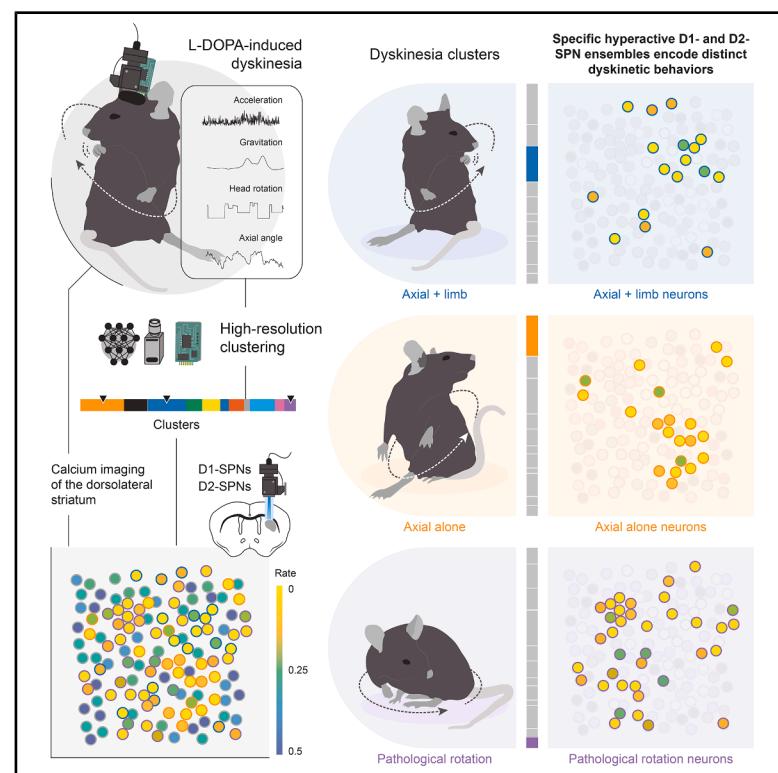


Abnormal hyperactivity of specific striatal ensembles encodes distinct dyskinetic behaviors revealed by high-resolution clustering

Graphical abstract



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In brief

Alcacer and Klaus et al. combine motion sensors and video data to automatically classify dyskinetic behaviors in a mouse model of L-DOPA-induced dyskinesia. Calcium imaging reveals that distinct dyskinesia-related behaviors are encoded by specific hyperactive D1- and D2-SPN ensembles, linking abnormal movements to structured striatal circuit activity.

Highlights

- Unsupervised clustering of IMU and video data classifies dyskinetic behaviors
- Automated classification identifies two dyskinesia types and pathological rotations
- Specific hyperactive D1/D2-SPN ensembles encode distinct dyskinetic behaviors
- Dyskinetic ensembles are active during normal movements with similar kinematics



Article

Abnormal hyperactivity of specific striatal ensembles encodes distinct dyskinetic behaviors revealed by high-resolution clustering

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SUMMARY

L-DOPA-induced dyskinesia (LID) is a debilitating complication of dopamine replacement therapy in Parkinson's disease and the most common hyperkinetic disorder of basal ganglia origin. Abnormal activity of striatal D1 and D2 spiny projection neurons (SPNs) is critical for LID, yet the link between SPN activity patterns and specific dyskinetic movements remains unknown. To explore this, we implemented a data-driven method for clustering movements based on high-resolution motion sensors and video recordings. In a mouse model of LID, this method identified two main dyskinesia types and pathological rotations, all absent during normal behavior. Using single-cell-resolution imaging, we found that specific sets of both D1- and D2-SPNs were abnormally active during these pathological movements. Under baseline conditions, these SPN sets were active during behaviors sharing physical features with LID movements. These findings indicate that ensembles of behavior-encoding D1- and D2-SPNs form new combinations of hyperactive neurons mediating specific dyskinetic features.

INTRODUCTION

In Parkinson's disease (PD), the degeneration of dopaminergic neurons that project to the striatum, causes poverty and slowness of movement. Dopamine (DA) replacement therapy with L-DOPA is still the most effective treatment, but it leads to a development of abnormal involuntary movements in the majority of patients within a few years.¹ Although the involuntary movements are collectively referred to as L-DOPA-induced dyskinesia (LID), they have variable clinical presentations, manifesting with different combinations of fast hyperkinetic motions and dystonic features (sustained twisting movements and abnormal postures) in different body parts.² In addition to being a medically important problem, LID provides a study paradigm to unveil patterns of striatal activity disrupting the control of movement sequences.^{3–6}

Movement control depends on the interplay of two main pathways originating from two populations of striatal neurons. Spiny projection neurons (SPNs) expressing DA D1 receptor

(D1-SPNs) project directly to the basal ganglia output nuclei forming the classically named “direct pathway,” while D2-receptor-expressing SPNs (D2-SPNs) influence basal ganglia output indirectly via intermediate nuclei.^{7,8} Canonical models postulate that direct and indirect pathways have opposite effects on movement: activation of D1-SPNs facilitates movement, whereas activation of D2-SPNs inhibits movement.^{9–11} However, accumulating evidence suggests that both pathways increase their activity at movement onset and that coordinated activation of D1- and D2-SPNs is required for proper action initiation.^{12–15} The role of SPNs in LID has been studied using parkinsonian rodent models treated with L-DOPA and developing axial, limb, and orofacial abnormal involuntary movements (AIMs). The recording of striatal activity during the expression of AIMs has revealed opposite changes in the average activity levels of D1- and D2-SPNs.^{5,6} Moreover, SPN-type-specific stimulations using chemogenetic⁹ or optogenetic methods^{4,6,16} concordantly show that LID is aggravated by increasing the activity of D1-SPNs and blunted by stimulating D2-SPNs. Taken together,



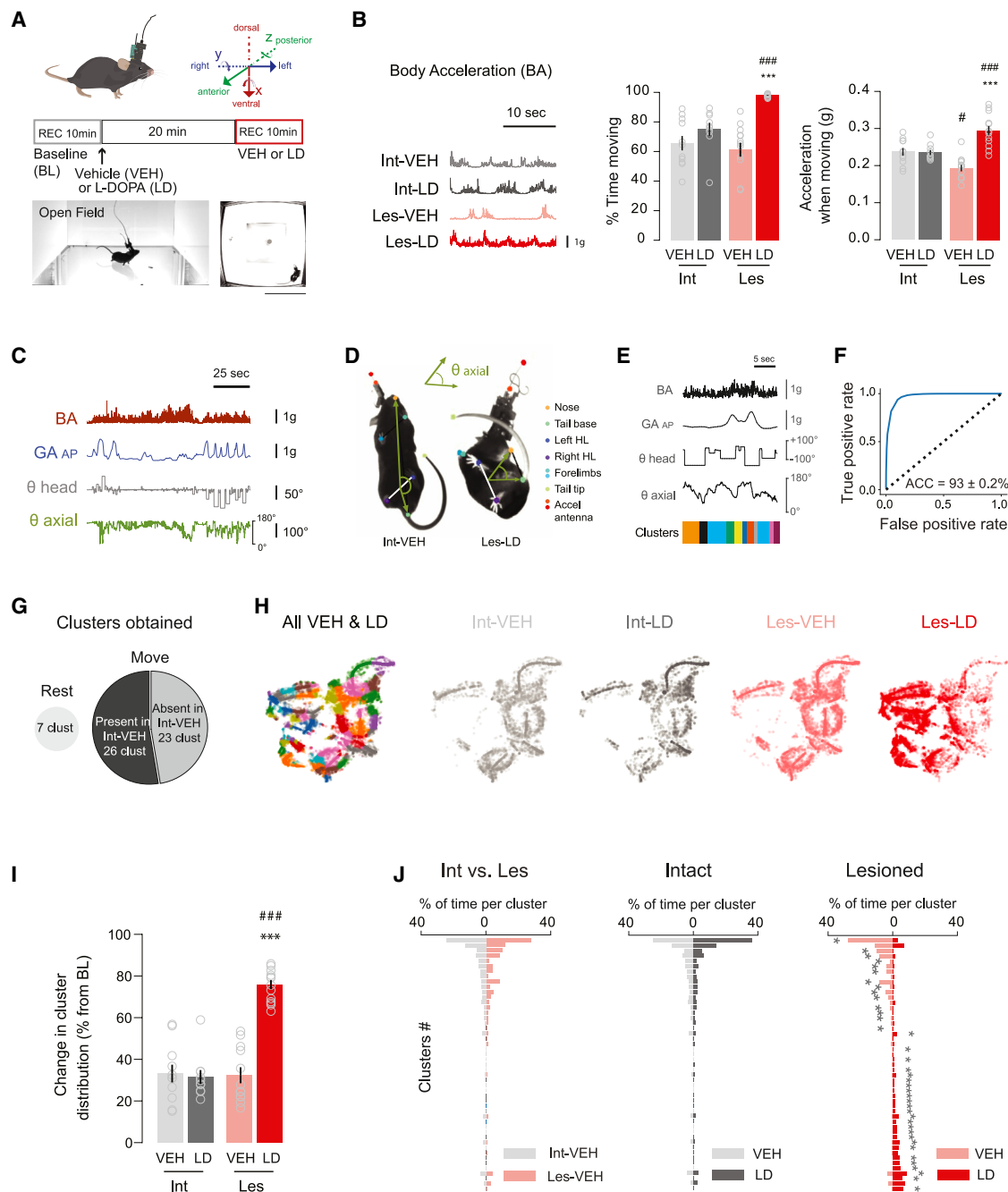


Figure 1. Behavioral changes in dyskinetic mice revealed by unsupervised behavioral clustering

(A) Schematic of the microendoscope and wireless IMU mounted on the mouse's head. Right: orientation of the IMU's six axes: three for acceleration (x, dorsoventral; y, right-left; z, posterior-anterior) and three for rotational velocity (gyroscope). Below: experimental timeline (STAR Methods) and side/bottom camera views of the open field (scale bar, 20 cm).

(B) Example body acceleration (BA) traces in the four conditions. Bar plots show mean time spent moving (% of session) on the left and average acceleration during movement (g) on the right, $\pm$ SEM ($n = 11-13$ mice). One-way ANOVA $p < 0.001$. Bonferroni: *** $p < 0.001$ vs. Les-VEH; ### $p < 0.001$ vs. Int-LD.

(C) Example traces of the four features used for the clustering: BA, GA_{AP} (gravitational acceleration along the antero-posterior axis), θ head and θ axial (axial bending angle; STAR Methods).

(D) Video frames from the bottom camera showing an Int-VEH (left) and a Les-LD (right) mouse with nine body points tracked using DeepLabCut. θ axial is computed from hindlimbs, tail base, and nose (STAR Methods; Video S1). The Int-VEH mouse shows a flat posture ($\sim 180^\circ$), while the Les-LD mouse exhibits pronounced bending ($\sim 40^\circ$).

(E) Top: time series of BA, GA_{AP} , θ head, and θ axial. Bottom: corresponding behavioral clusters obtained by affinity propagation on similarity of discretized time series (STAR Methods).

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these studies have established a causal link between LID and a disrupted interplay between direct and indirect pathway, with concomitant D1-SPN hyperactivity and D2-SPN underactivity. However, this level of explanation cannot account for the phenomenological diversity and temporal structure of LID. Recent studies have shown that specific ensembles of D1- and D2-SPNs encode specific behaviors.¹⁵ Consequently, different forms of LID may result from the specific activation of distinct neuronal groups including both SPN populations. To answer this question, there is a need to develop new methods enabling to monitor dyskinetic motions with high precision. In both patients with PD and animal models, the classification and quantification of dyskinetic movements is based on rating scales that assign dyskinesia severity scores to different body parts during monitoring periods of 1–2 min. Although these rating methods are well validated for translational research,^{17,18} they do not offer the temporal resolution that would be necessary to resolve neuronal events accounting for the different motor components that make up LID.

In this study, we introduce an automated approach to quantify dyskinetic movements with high temporal resolution in freely moving mice, enabling detailed investigation of the striatal pathophysiology associated with LID. We developed a semi-supervised approach using unsupervised behavioral clustering based on inertial measurement units (IMUs) and video, combined with a supervised higher-order clustering based on dyskinesia annotations. Using this approach, we were able to capture different types of dyskinesia as well as the presence of pathological rotational movements, ultimately enabling the classification of dyskinetic behavior with high accuracy. In order to investigate whether and how the activity patterns of D1- and D2-SPNs relate to specific dyskinesia types, we combined the usage of the IMUs with calcium imaging in freely behaving mice. We could therefore quantify the activity patterns of D1- and D2-SPN ensembles during each dyskinesia cluster and the pathological rotations. Our results show that specific patterns of AIMs are encoded by specific ensembles of D1-SPN and also D2-SPN showing abnormally high firing activity.

RESULTS

L-DOPA increases body acceleration during movement in dyskinetic mice

To induce hemiparkinsonism, mice received unilateral 6-hydroxydopamine (6-OHDA) injections into the medial forebrain bundle,⁹ resulting in near-complete loss of dopaminergic fibers in the ipsilateral striatum (Figure S1A). Following L-DOPA treat-

ment (6 mg/kg/day, 3–4 days), lesioned mice developed classical axial, limb, and orofacial AIMs, quantified using the classical dyskinesia severity scale^{19,20} (Figure S1B).

To study changes in motion patterns, we recorded high-resolution acceleration and angular velocity using head-mounted wireless IMUs and video (Figure 1A). Recordings were performed in a first cohort of intact (Int, $n = 12$) and lesioned (Les, $n = 13$) mice at baseline (BL) and after VEH or L-DOPA injection (Figure 1A; STAR Methods).

We first quantified overall movement such as the percentage of time the animals moved per session and their body acceleration (BA) captured with the IMUs (Figure 1B). Open-field trajectories differed across conditions, with Les-LD mice showing confined movements (Figure S1C) yet with high BA (see example BA traces in Figure 1B). Lesion alone did not affect time moving but significantly reduced BA while moving (Figure 1B, $p < 0.05$, Les-VEH vs. Int-VEH). L-DOPA significantly increased both time moving and BA when moving in lesioned mice ($p < 0.001$, Les-LD vs. Int-LD and Les-VEH) but not in intact mice. Together, these findings confirm that L-DOPA reverses bradykinesia by enhancing movement duration and acceleration in lesioned mice. However, these metrics did not capture the details of movement characteristics.

Unsupervised clustering of motor features reveals new behavioral clusters in dyskinetic mice

To capture fine-grained behavior, we used unsupervised behavioral clustering of the IMUs and video data.^{15,21} We extracted specific features capturing head and body posture as well as three-dimensional motion. In particular, we used the following four movement features (Figure 1C): (1) total BA, which distinguishes movement versus rest; (2) gravitational acceleration along the antero-posterior axis (GA_{AP}), which detects postural changes; (3) head rotational movements along the dorsoventral axis obtained with the gyroscope (θ head, or angular velocity); and (4) axial bending angle extracted from the video (θ axial), which is inversely related to the degree of upper body torsion and correlates with the axial component of dyskinesia²² (Figure 1D, STAR Methods; Video S1).

Using a first cohort of mice ($n = 12$ Int, $n = 13$ Les; 81 sessions), we created a library of the standard repertoire of the animal behavior in our paradigm using the unsupervised clustering algorithm (Figure S2A; for details of the clustering see Figures S2A–S2D and STAR Methods). With the above-mentioned features, we identified a total of 56 behavioral clusters (Figures 1E and 1G), which were well separated as shown by a ROC analysis (Figure 1F); seven classified as rest (i.e., periods where mice

(F) Clusters show strong separability, with 93% accuracy in ROC analysis using a single EMD threshold. True positive are two segments belonging to the same cluster; false positive are two clusters belonging to different clusters (STAR Methods).

(G) Clustering yielded 56 total clusters: seven corresponding to rest and 49 to movement. “Move” clusters were further divided into those present in Int-VEH (26 clusters) and those absent in Int-VEH (23 clusters).

(H) t-SNE visualization of behavioral clusters. Each dot represents a behavioral segment in 2D t-SNE space. Left: all VEH and LD sessions, with each color indicating a distinct cluster. Right: segments from each condition (Int/VEH, Int/LD, Les/VEH, Les/LD). Note the similar distribution in Int-VEH, Int-LD, and Les-VEH, contrasting with the distinct pattern in Les-LD.

(I) Change in the cluster distributions vs. baseline (BL, means $\pm$ SEM). One-way ANOVA, $p < 0.001$. Bonferroni: *** $p < 0.001$ vs. Les-VEH; ### $p < 0.001$ vs. Int-LD.

(J) Percentage of time spent per cluster ($n = 19$ –20 mice). Left: Int-VEH and Les-VEH (Mann-Whitney U, Benjamini-Yekutieli (BY)-corrected $p > 0.05$). Middle: intact, VEH vs. LD (Wilcoxon, BY $p > 0.05$). Right: lesioned, VEH vs. LD (Wilcoxon, BY, $p < 0.05$ in 39/49 clusters).

did not move) and 49 as move clusters (Figure 1G, STAR Methods). From the 49 moving clusters, 26 were present in Int-VEH, considered as normal behavior. The remaining 23 were absent in Int-VEH, labeled as pathological behaviors. Since Les-LD mice did not have periods of rest (see Figure 1B), we included only the moving clusters for further analyses to avoid the increase in time moving as a confounding factor. A t-distributed stochastic neighbor embedding (t-SNE) projection of the moving clusters showed a similar spatial distribution in Int-VEH, Int-LD, and Les-VEH mice, with behavioral segments shifted to the right side of the map. In contrast, Les-LD mice displayed a clear shift, with clusters localized to the left (Figure 1H).

We then quantified changes in cluster distribution in each group relative to their BL (Figure 1I; note that no group differences were found at BL; Figure S2E). Clusters associated with the intact condition (Int-VEH vs. Int-LD) showed a similar change vs. BL, indicating no effect of L-DOPA treatment on the moving behavior of intact mice, with similar results obtained from lesioned mice treated with vehicle (Figure 1I). In contrast, Les-LD animals showed a dramatic change in cluster distribution (Figure 1I, $p < 0.001$ vs. the other three groups). While these analyses indicate that clusters absent in Int-VEH (see Figure 1G) emerge specifically in Les-LD mice, they do not reveal the changes of individual behavioral clusters in the dyskinetic condition. Therefore, we next compared the percentage of time spent per cluster in intact and lesioned mice after VEH and L-DOPA. To this end, we added an additional cohort of $n = 7$ Int and $n = 7$ Les mice by unsupervised clustering of individual recording sessions and by matching the resulting clusters to the library (Figure S2B; STAR Methods). In Les-VEH, time spent in each cluster remained largely unchanged from Int-VEH, even though some individual clusters seemed to be more strongly expressed in the lesioned cohort (Figure 1J, left panel). In the intact condition, L-DOPA did not change the percentage of time spent in each cluster (Figure 1J, middle panel). In contrast, Les-LD mice exhibited both gain and loss of specific clusters, including newly emerging ones (Figure 1J, right panel). In summary, our unsupervised clustering approach revealed a profound reorganization of movement structure in lesioned mice treated with L-DOPA, marked by the emergence of new behavioral clusters associated with dyskinesia.

Behavioral clustering captures specific dyskinesias and pathological rotations

To assess how behavioral clusters relate to classical dyskinesia subtypes, we annotated two well-defined AIMs^{19,20} from video: axial (trunk twisting contralateral to lesion) and limb (fluttering forelimb movements) (Figure 2A, STAR Methods; Video S2). Frame-by-frame annotation yielded three combinations: axial-alone, limb-alone, and axial + limb dyskinesias.

We then correlated each behavioral cluster with these annotated dyskinetic events and grouped the clusters accordingly. We found clusters that were correlated specifically with one type of dyskinesia annotation (Figure 2B) or more than one (Figure S3A). From the 49 moving clusters, 21 were significantly correlated with the dyskinesia annotations: nine with axial + limb; 11 with axial alone, and only one with limb alone (Figure 2C). Time spent in these clusters strongly correlated to the time spent

in axial + limb or axial-alone annotations but not with limb alone (Figure S3B). The remaining clusters included 20 present in Int-VEH mice, labeled “other N” for normal behavior, and eight absent in Int-VEH that featured prominent contralateral head rotations, designated as pathological rotations (path rot, Figure 2C).

Each cluster group had composite behavioral signatures such that a single movement feature was not sufficient to differentiate them (Figures 2D, S3C, and S3D). The axial + limb cluster group was the most static (low BA values), with the strongest axial torsion (θ axial $\sim 70^\circ$) but moderate head rotation, comparable to other-N cluster group (Figure 2D). Axial alone was more dynamic (higher BA) and combined strong head rotations (low θ head values) with a higher degree of upper body torsion (θ axial $\sim 90^\circ$). The limb-alone cluster group had low BA and gravitational acceleration (GA), the lowest head rotation, and low axial torsion (θ axial $\sim 120^\circ$). Path-rot cluster group was the most dynamic of all, while also showing the strongest head rotations, occurring while the body was in a relatively straight position, as indicated by higher θ axial values compared to both axial alone and axial + limb ($p < 0.001$ for both comparisons, Figure 2D; Table S2 for statistics).

To verify the specific association of each cluster group with the presence of dyskinesia, we examined the percentage of time per cluster group between the four experimental conditions (Figure 2E). Intact (VEH and LD) and Les-VEH mice spent the majority of the time in the other-N cluster group (60%–70% of time during moving), whereas Les-LD mice showed a shift toward dyskinesia and path-rot behaviors (Figure 2E). We observed that the limb-alone cluster group was highly expressed in all other cohorts but the Les-LD. This was confirmed with a group comparison of percentage of time per cluster, revealing that axial + limb and axial-alone clusters were highly specific to the Les-LD cohort but not limb alone (Figure 2F). We hypothesized that limb AIMs could share kinematic features with certain phases of a grooming sequence (described by Berridge and Whishaw²³). Indeed, a linear support vector classifier showed that the limb-alone cluster could predict grooming in intact mice with an accuracy of 78% (Figure S4A). Given its overlap with grooming and low prevalence in Les-LD mice (3.1%), the limb-alone cluster was excluded from further analyses.

The emergence of distinct dyskinesia clusters led us to hypothesize that these could be used to predict specific dyskinesia subtypes. To test this, we applied a behavioral classifier using a leave-one-out cross-validation approach, which achieved 92% accuracy for axial + limb and 67% for axial-alone dyskinesias (Figure 2G; see STAR Methods). Next, we examined the relationship between dyskinesia clusters and classical AIM scores by correlating the time spent in each behavioral cluster with the total axial and limb AIMs during the 10-min recording. Axial + limb and axial-alone clusters were positively correlated with axial AIMs, with a subset of clusters showing significant correlations (Figure 2H, red points, left panel). As expected, axial + limb clusters were also positively correlated with limb AIMs, whereas axial-alone clusters showed overall negative correlations (Figure 2H, right panel). In contrast, correlations for limb-alone, path-rot, and other-N clusters were more variable, with significant associations limited to negative correlations (Figure S4B). Taken together,

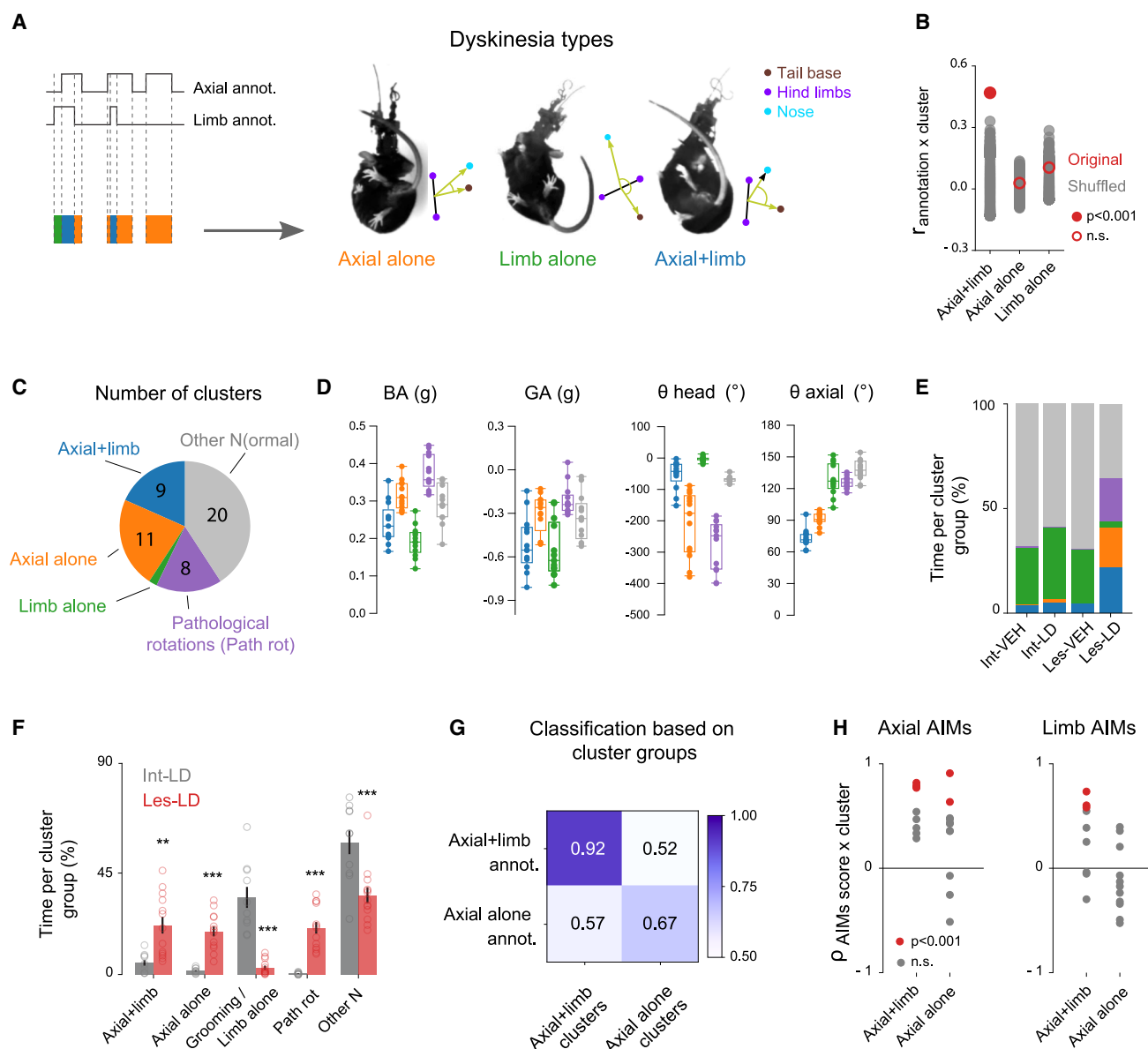


Figure 2. Behavioral clustering captures specific dyskinesias and other pathological behaviors

(A) Schematic representation of frame-by-frame annotation of three dyskinesia types: axial alone (orange), limb alone (green) and axial + limb (blue) (see Video S1).
(B) Example cluster for one mouse that significantly correlated with the axial + limb annotation but not with axial alone or limb alone. Bootstrap significance: closed red circle = $p < 0.001$.

(C) Left: summary of the behavioral cluster groups obtained after correlating the behavioral cluster to the dyskinesia annotations across $n = 13$ mice (STAR Methods).

(D) Feature characteristics of the five cluster groups. Box-and-whiskers diagrams show the means $\pm$ SEM ($n = 13$ mice) of each of the four features (BA, GA, θ head, and θ axial) per cluster group. One-way RM ANOVA: all $p < 0.001$ (Table S2).

(E) Time spent per cluster group. In Int-VEH, Int-LD, and Les-VEH, mice spent $>50\%$ of the time in the other-N and $\sim 30\%$ in the limb-alone (grooming) cluster. Les-LD mice showed more variable distribution across all five cluster groups.

(F) Time spent in each cluster group (means $\pm$ SEM, Int-LD vs. Les-LD, $n = 10-13$). Two-way RM ANOVA: effect of group $\times$ cluster, $p < 0.001$. Bonferroni: $**p < 0.01$, $***p < 0.001$.

(G) Support vector machine (SVM) classifier accuracy for predicting axial + limb and axial-alone dyskinesia types. Accuracy significantly above shuffle (paired t test: both $**p < 0.001$).

(H) Correlation between axial and limb AIMs scores and the fraction of time spent in each behavioral cluster. Each point represents a cluster from the respective cluster group, averaged over $n = 13$ Les-LD mice. Red points = significant Spearman correlation ($p < 0.001$).

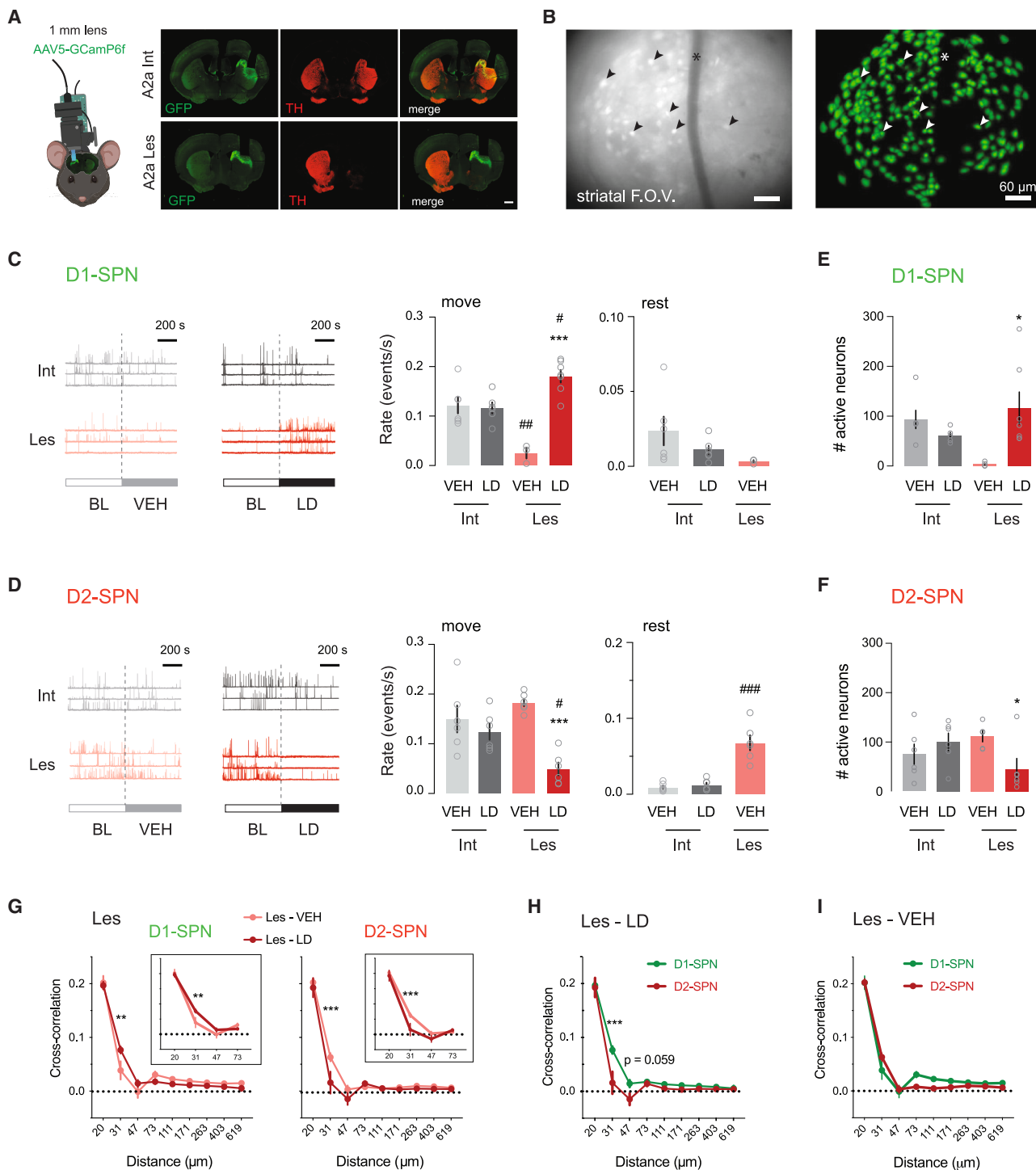


Figure 3. L-DOPA oppositely modulates average SPN activity in 6-OHDA lesioned mice

(A) Schematic of the implanted microendoscope and wireless IMU mounted above a 1-mm GRIN lens. Right: striatal coronal sections from A2a-Cre Int (top) and Les mice (bottom) show GCaMP6f expression (green) and tyrosine hydroxylase (TH) loss (red) in lesioned striatum. Scale bar, 1 mm.

(B) Striatal field of view (FOV) from a D1-Cre lesioned mouse after LD. Left: maximum projection (3,000 frames). Arrows indicate active neurons; asterisk a blood vessel. Right: ROIs ($n = 283$ neurons) detected by CNMF-E across baseline and LD sessions. Scale, 60 μ m.

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these results indicate that axial + limb and axial-alone clusters are highly predictive of the occurrence of axial and limb dyskinesias as rated using a well-validated method, supporting the use of clustering as an objective readout.

Comparison of D1- and D2-SPN activity across experimental conditions

To examine how dyskinetic behaviors are encoded in the striatum, we performed one-photon calcium imaging in D1- and D2-SPNs using GCaMP6f expressed via Cre-dependent adeno-associated viruses (AAVs) in D1-Cre or A2a-Cre mice. Following 6-OHDA lesion, virus injection, and gradient index (GRIN) lens implantation in the dorsolateral striatum (Figure 3A), we recorded neural activity during 10-min open-field sessions before and after VEH or L-DOPA treatment (STAR Methods). Up to 280 neurons per session were recorded (Figure 3B) and aligned to behavioral clusters (Videos S3 and S4). Calcium events were extracted using a constrained non-negative matrix factorization for microendoscopic data (CNMF-E),^{15,24,25} and spike events were detected using the MLspike algorithm²⁶ (STAR Methods).

We first assessed how L-DOPA modulated mean calcium activity and the number of active neurons (i.e., mean activity ≥ 0.5 events/s; see also Figure S5D). In D1-SPNs, calcium event rates during movement were significantly reduced in Les-VEH mice vs. Int-VEH ($p < 0.01$), but significantly increased in Les-LD mice (Figure 3C). Resting activity was comparable across Int and Les-VEH groups (Les-LD excluded due to near-continuous movement; cf. Figure 1B). The number of active D1-SPNs also peaked in Les-LD mice ($p < 0.05$ vs. Les-VEH; Figures 3E and S5D). In contrast, D2-SPNs showed a decrease in activity after L-DOPA in lesioned mice but no change in intact mice (Figure 3D, see ratio D1/D2-SPN activity in Figure S5A, summary table in Figure S5B and the relative change in event rate from BL in Figure S5C). Resting activity was significantly higher in Les-VEH compared to intact controls ($p < 0.001$ vs. Int-VEH), while the number of active D2-SPNs dropped significantly in Les-LD vs. Les-VEH (Figures 3F and S5D, and ratio D1/D2-SPN active neurons in Figure S5E). Confirming previous reports,^{5,6} our data show that L-DOPA oppositely modulates striatal output pathways in the DA-denervated striatum, increasing both calcium event rate and the number of active neurons in D1-SPNs while reducing these parameters in D2-SPNs during movement. The effect of L-DOPA on the number of active neurons is in line

with the notion that DA biases striatal activity toward the direct or indirect pathway by changing the overall number of SPNs recruited during behavior.²⁷

We then quantified the spatial cross-correlation of calcium events between pairs of active neurons as in Klaus et al.¹⁵ In all experimental groups, the cross-correlation declined sharply between 20 and 47 μm of intercellular distance (Figures 3G–3I and S5F–S5H). Lesion alone did not alter spatial coactivity (data not shown), but L-DOPA significantly increased D1-SPN coactivity and reduced D2-SPN coactivity at ~ 30 μm only in lesioned mice (Figure 3G; see data from intact mice in Figure S5F). In Les-LD, D1-SPN pairs showed a ~ 5 -fold higher correlation than D2-SPN pairs at that same critical distance (Figure 3H), a difference not observed in VEH-treated or intact animals (Figures 3I, S5G, and S5H). These findings suggest that the spatiotemporal activation pattern of nearby D1- vs. D2-SPN ensembles changed substantially during LID only at intercellular distances less than 40 μm .

Specific SPN ensembles encode for dyskinesia types and pathological rotations

While previous studies have shown opposing effects of L-DOPA on D1- and D2-SPNs in lesioned mice,^{4–6} the encoding of specific dyskinetic behaviors by these pathways remains unclear. We therefore set out to determine how D1- and D2-SPNs encoded the behavioral clusters of dyskinetic mice.

We first quantified average calcium event rates for each SPN type across behavioral clusters (i.e., axial + limb, axial alone, path rot, and other N). No significant differences in average event rate were found between cluster groups for either D1- or D2-SPNs (Figure 4A). In all behavioral clusters, the D1/D2 event ratio in Les-LD was 1.5- to 3.5-fold larger than in Int-LD mice (hatched line in Figure 4A), particularly in axial-alone and path-rot clusters (Figure 4A right panel). These data suggest that overall increases and decreases in D1- and D2-SPN activities are not driven by specific behavioral cluster groups.

Although average event rates of D1- and D2-SPNs did not differ across behavioral clusters, we investigated whether dyskinesia or pathological rotations could be decoded from SPN ensemble activity. We therefore trained a linear classifier (STAR Methods) to decode dyskinesia (axial + limb and axial alone) and path-rot behaviors from deconvolved SPN ensemble activity. D1-SPN ensembles predicted dyskinesia with $\sim 70\%$ accuracy, while D2-SPNs achieved $\sim 60\%$ (Figure 4B). Decoding

(C) D1-SPN event rates during movement ($n = 3$ –7 mice; 6–18 sessions per group). Example traces from three neurons in Int (top) and Les mice (bottom). Dashed line separates BL and treatment periods. Scale bar, 200 s. Bar graph represents the mean $\pm$ SEM of event rates per mouse and session when moving (move) and at rest (see Video S3). One-way ANOVA, $p < 0.001$. Bonferroni: $^{##}p < 0.005$ Les-VEH vs. Int-VEH; $^{***}p < 0.001$ Les-LD vs. Les-VEH and $^{#}p < 0.05$ vs. Int-LD. No rest activity difference across groups.

(D) D2-SPNs event rates, as in (C) (means $\pm$ SEM, 6 mice per group and 8–14 sessions per group). One-way ANOVA, $p < 0.001$. Bonferroni: $^{***}p < 0.001$ vs. Les-VEH; $^{#}p < 0.05$ vs. Int-LD. Rest activity: one-way ANOVA, $p < 0.001$. Bonferroni: $^{###}p < 0.001$ vs. Int-VEH (see Video S4).

(E) Number of active D1-SPNs when mice were moving. Values are number of active neurons $\pm$ SEM ($n = 3$ –7 mice; 6–18 sessions per group). One-way ANOVA, $p < 0.05$. Bonferroni: $^{*}p < 0.05$ vs. Les-VEH.

(F) Number of active D2-SPNs as in (E) ($n = 6$ mice; 8–14 sessions per group). One-way ANOVA, $p < 0.05$. Bonferroni: $^{*}p < 0.05$ vs. Les-VEH.

(G) Spatiotemporal cross-correlation (means $\pm$ SEM, Les mice, VEH vs. LD). Two-way RM ANOVA: effect of distance, $p < 0.001$. D1-SPNs showed increased coactivity. Bonferroni: $^{**}p < 0.01$; D2-SPNs decreased coactivity $^{***}p < 0.001$, both at ~ 31 μm .

(H) Spatiotemporal cross-correlation as in (G) (Les mice, D1- vs. D2-SPNs). Two-way RM ANOVA: effect of distance, $p < 0.001$. D1-SPNs showed increased coactivity vs. D2-SPNs. Bonferroni: $^{***}p < 0.001$.

(I) No difference in D1- vs. D2-SPN coactivity in Les-VEH.

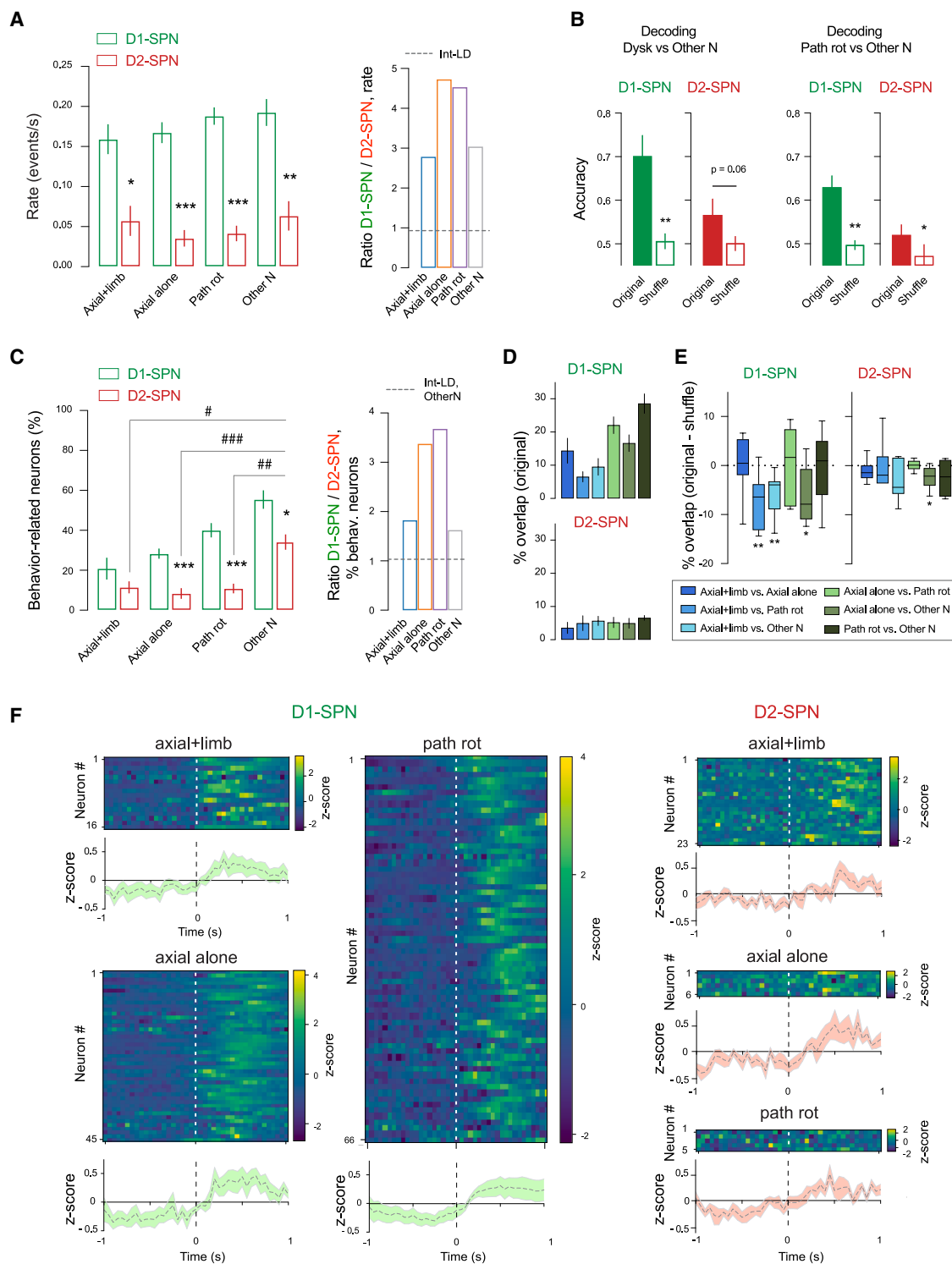


Figure 4. Specific D1-SPN and D2-SPN ensembles are associated with dyskinesia and pathological rotations

(A) Average event rate of D1- and D2-SPNs during each behavioral cluster group in Les-LD mice ($n = 6-7$, 2-3 sessions/mouse). Values are means $\pm$ SEM. Two-way RM ANOVA: SPN type $p < 0.001$. Bonferroni: $*p < 0.05$ (axial + limb), $***p < 0.001$ (axial alone, path rot), $**p < 0.01$ (other N). Right: D1/D2 event rate ratio vs. Int-LD baseline. Right: D1/D2 event rate ratio vs. Int-LD baseline. Dashed line is the ratio in Int-LD during move.

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of path rot reached ~60% and ~50% for D1- and D2-SPNs, respectively. Performance dropped to chance when neuron identities were shuffled, confirming that decoding relies on the consistent activity of specific neurons within the ensemble. These findings show that dyskinesia and pathological rotations are encoded by specific SPN ensembles rather than by overall activity levels, with D1-SPNs carrying more predictive information than D2-SPNs.

Further analysis revealed distinct subsets of “behavior-related neurons” (specific SPNs with significantly increased activity during particular behavioral clusters; Figure 4C). Among 164 D1- and 176 D2-SPNs per field of view (all SPNs detected during both BL and LD sessions), 21%–55% of D1-SPNs and 8%–34% of D2-SPNs were behavior related, with D1-SPNs more broadly recruited across all cluster groups. Notably, D2-SPNs were more often associated with the other-N group than with any dyskinesia or path-rot cluster, pointing to a larger recruitment of D2-SPNs during the expression of relatively normal motions despite the ongoing effect of L-DOPA. The D1/D2 ratio of behavior-related neurons was biased toward D1-SPNs, particularly in axial-alone and path-rot clusters, with a >2-fold increase compared to Int-LD during other N (Figure 4C, right).

Axial + limb and axial-alone dyskinesia share some behavioral characteristics (i.e., the axial component) that could be reflected in an overlap between the neuronal ensembles related to them. To examine whether neuronal ensembles were shared across behaviors, we computed the overlap between SPNs associated with different clusters (Figures 4D and 4E). The overlap between any of the behavior-related D1- and D2-SPNs during LD sessions remained low (6%–27% for D1-SPNs, 4%–7% for D2-SPNs; Figure 4D). By correcting for random overlap (via shuffle control), we observed that the overlap was not more than the one expected by chance (i.e., no significant positive increase from 0; 0% overlap corresponding to no shared neurons and 100% to a complete overlap between the neuronal groups). These data indicate that the behavior-related neurons are specific to their cluster group.

Finally, we analyzed the temporal dynamics of SPN recruitment around the onset of behavioral events. Heatmaps of single-neuron activity aligned to behavior start times showed a significant increase in SPN activity following the onset of axial + limb, axial-alone, and path-rot events (Figure 4F). These dynamics further support the existence of behavior-specific, temporally precise recruitment of SPN ensembles during dyskinesia.

Activity of dyskinesia- and pathological rotation-tuned SPN sets across behavioral clusters

We next examined how the behavior-related SPNs in Les-LD mice modulate their activity across behavioral clusters (Figure 5A). D1-SPNs tuned to axial + limb and axial-alone clusters showed the highest event rates, exceeding 2-fold the average D1-SPN firing in Les-LD (~0.4 Hz) during their associated axial + limb and axial-alone clusters. D2-SPNs also exhibited increased firing in their associated clusters, sometimes surpassing the levels observed in intact mice, but still firing at lower rates (~0.18 Hz) than the coactive D1-SPNs. These data indicate that behavior-related SPNs are broadly active but reach peak firing during their associated clusters.

Previous studies have identified dyskinesia-specific D1-SPNs that are exclusively activated during LID.⁴ To explore how these ensembles emerge with L-DOPA, we analyzed their recruitment during the baseline condition, taking advantage of our experimental paradigm that allowed for extracting the activity of dyskinesia-specific SPNs also during the preceding off-drug behavior. Under BL conditions, about 60% of SPNs were associated with clusters of motor features emerging during normal movement (data not shown), in line with previous findings from intact mice.¹⁵ A large proportion of SPNs tuned to dyskinesia and path rot were also active during other-N clusters at BL (referred to as BL-other N): 68% for D1-SPNs and 61% for D2-SPNs (Figure 5B). Their event rates were comparable to those seen in intact mice at baseline (Figure 5C), suggesting that these dyskinesia-encoding neurons are already engaged during non-dyskinetic movements.

The finding that the dyskinesia and path-rot neurons were indeed active during some of the BL-other-N clusters raised the question of whether such clusters were kinematically similar to dyskinesia or path-rot movements. To address this question, we identified the dyskinesia/path-rot clusters to which each SPN group was tuned in Les-LD and compared their earth mover’s distance (EMD) similarity to the following BL-other-N clusters: (1) BL-other-N clusters to which the same neurons were positively modulated (i.e., behavior-related; see STAR Methods), and (2) to the BL-other-N clusters to which the neurons were not positively modulated (i.e., “other”) (Figure 5D). We observed that the EMD similarity between the axial + limb clusters and the behavior-related BL-other-N clusters was significantly higher for both SPNs than the one between the axial + limb clusters and all the other clusters in the BL-other-N category (Figure 5D, left). A similar trend emerged in the EMD similarity analysis of axial alone and path rot vs. BL-other N, particularly for the D1-SPN group

(B) Behavioral decoding using SPN ensemble activity. Values are means $\pm$ SEM. SVM classifier accuracy for dyskinesia (left) or path rot (right) vs. other N (see STAR Methods). Accuracy significantly higher than shuffled controls. Paired t test: dyskinesia/D1-SPN $**p < 0.01$; D2-SPN $p = 0.06$; path rot/D1-SPN $**p < 0.01$; D2-SPN $*p < 0.001$. Note that the shuffle data were permutations of the neuron labels, thus preserving the average activity information.

(C) Percentage of behavior-related D1- and D2-SPNs in Les-LD mice. Values are means $\pm$ SEM. Two-way RM ANOVA: SPN type $p < 0.001$. Bonferroni: $***p < 0.001$ (D1 vs. D2, axial alone and path rot); $*p < 0.05$ (other N); $#p < 0.05$, $##p < 0.01$, $###p < 0.001$ for intra-D2-SPN group comparisons. Right: D1/D2 ratio of behavior-related neurons per cluster. Dashed line is the ratio in Int-LD during other-N cluster groups.

(D) Percentage of overlap between behavior-related D1- and D2-SPNs neuronal groups (original data, STAR Methods). Values are means $\pm$ SEM ($n = 6$ –7 mice, 2–3 sessions/mouse).

(E) Percentage of shuffle-corrected overlap between behavior-related D1- and D2-SPNs neuronal groups. Values are means $\pm$ SEM. Shuffle-corrected overlap is the overlap minus the shuffle overlap. Unpaired t tests vs. 0: $**p < 0.01$ (axial + limb vs. path rot/other N); $*p < 0.05$ (path rot vs. other N).

(F) Heatmaps of D1- and D2-SPN activity aligned to behavioral onset (0 s). Averaged Z scored $\Delta F/F$ traces show significant activation after onset: D1-SPNs, $*p < 0.05$; D2-SPNs, $**p < 0.01$ (t test -1 to -0.1 s vs. 0.1 to 1 s).

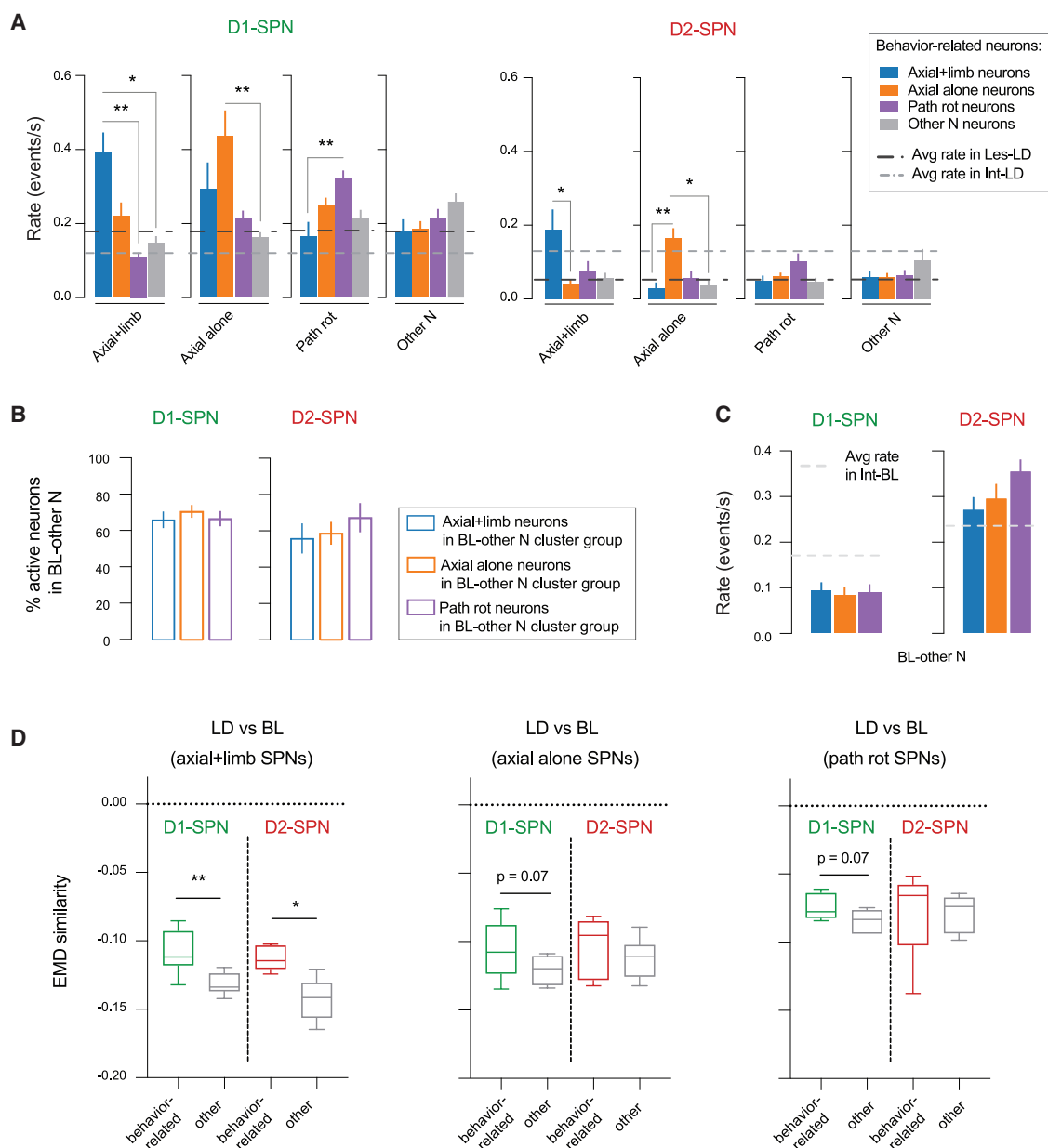


Figure 5. Activity features in SPN ensembles tuned to specific dyskinesia types

(A) Average event rates of behavior-related D1- and D2-SPNs during each behavioral cluster group ($\pm$ SEM $n = 6-7$ mice, 2–3 sessions/mouse). Black and gray dashed lines indicate mean rates in Les-LD and Int-LD during movement, respectively. D1-SPNs showed highest activity during their matching clusters (e.g., axial + limb, axial alone, path rot). Kruskal-Wallis: D1-SPNs, axial + limb ($**p < 0.01$), axial-alone ($**p < 0.01$), and path-rot ($*p < 0.05$) cluster groups, but not for other N; D2-SPNs, axial-alone cluster group ($**p < 0.01$), but not for axial + limb ($p = 0.05$, not significant), path rot or other N.

(B) Percentage of the dyskinesia/path-rot SPNs associated with at least one other-N behavioral cluster during baseline (named BL-other N) (mean $\pm$ SEM; $n = 6-7$ mice, 2–3 sessions/mouse).

(C) Behavior-related D1- and D2-SPNs event rate during other N during BL (average event rate $\pm$ SEM, $n = 6-7$ mice, 2–3 sessions/mouse). Dashed line is the average rate in Int-BL.

(D) EMD behavioral-similarity analysis comparing dyskinesia (Les-LD) versus baseline (Les-BL) for behavioral clusters that are associated with neurons in the following groups: axial + limb, axial-alone, and path-rot SPNs. Specifically, for each SPN, we identified the significant dyskinesia clusters (Les-LD) and compared their EMD similarity to BL-other-N clusters (Les-BL) for which the neuron was positively modulated (i.e., behavior-related, see STAR Methods) vs. the remaining other-N clusters (“other”). Note that 0 is identical behavior, and more negative values are more dissimilar behaviors. Values are means $\pm$ SEM. Paired t tests showed higher similarity in axial + limb neurons than unrelated clusters in both D1 ($**p < 0.001$) and D2 ($*p < 0.05$); no differences in axial-alone or path-rot groups ($n = 7$ mice for D1-SPNs and $n = 6$ mice for D2-SPNs).

(Figure 5D, middle and right). These findings suggest that SPNs engaged during dyskinesia and pathological rotations are already active at baseline during normal movements with shared kinematic features.

DISCUSSION

We here present an automated approach to detect dyskinetic movements with sub-second resolution in freely moving mice. We developed a semi-supervised approach using unsupervised behavioral clustering based on IMUs and video combined with a supervised group clustering based on dyskinesia annotations. Using this approach, we were able to capture different types of dyskinesia and other behaviors absent in intact mice classified as pathological rotations. To investigate the interplay between D1- and D2-SPNs during specific dyskinesia types, we combined this behavioral analysis with SPN calcium imaging in freely behaving mice. We could therefore quantify the activity patterns of D1- and D2-SPN ensembles during each dyskinesia cluster. Our results show that the two dyskinesia types and the pathological rotations were encoded by highly specific sets of D1- and D2-SPNs. These sets of hyperactive SPNs emerge from combinations of D1- and D2-SPNs that encode normal behaviors under baseline conditions.

The present study introduces the first application of a non-invasive miniaturized wireless IMU version for mice to quantify pathological movements. The unsupervised behavioral clustering method was based on previous work developed by Klaus and collaborators,¹⁵ where body movements of healthy mice in an open-field arena were monitored and clustered using wired IMUs and video data. As in Klaus and colleagues, we used total BA and GA in the antero-posterior axis; however, diverging from their approach, the head rotation parameter was taken from the IMUs and in particular from the gyroscope component, rather than from the video. The addition of the fourth feature, the axial bending angle, was key to capture the axial component of dyskinesia.²² Using the combination of these four features, the unsupervised behavioral clustering detected significant changes in the cluster distribution of dyskinetic mice, shown by the emergence of a new behavioral space represented in Figure 1H. Our approach enabled us to prove that LID is not simply an acceleration or exaggeration of normal movements but involves the emergence of abnormal motor motifs that are not found in non-dyskinetic animals, whether intact or parkinsonian.

To establish the nature of the obtained behavioral clusters, we used the dyskinesia annotations to group the clusters into higher-order groups. As a result, we obtained cluster groups corresponding to three specific dyskinesia types: axial combined with limb, axial alone, and limb alone. The latter was discarded from further analyses due to a large degree of overlap with the kinematic properties of limb movements during grooming in intact animals. Based on the combined use of four relevant primary features, the behavioral signatures identified by the cluster groups represent with high fidelity the posture-motion dynamics of dyskinetic mice exhibiting axial and limb AIMs. For example, as dyskinetic mice develop limb AIMs, they stop locomoting and execute characteristic fluttering movements with the forelimb contralateral to the lesion. In the axial + limb cluster group,

this was reflected by a decrease in the BA and rotations, along with a position of the head closer to the floor (lower GA). When dyskinetic mice develop axial AIMs without limb involvement, we would expect an increase in BA and head rotation combined with a twisted, dystonic body posture (low axial angle) and with the head raised toward a higher GA level. These features were indeed observed in the axial-alone clusters. In addition, the cluster expression of axial limb and axial alone was significantly correlated with the corresponding AIMs scores. These results show that our behavioral-clustering method can detect and quantify dyskinetic motor patterns with very high kinematic precision.

Interestingly, our clustering analysis detected an abnormal behavior that we defined as pathological rotations (path rot). The path-rot cluster group included behavioral motifs that were not correlated to classical axial and/or limb AIMs yet pathological because they were totally absent in intact and Les-VEH mice. This cluster group was characterized by pronounced head rotation (large head angle) occurring simultaneously with high BA. The axial bending angle was, however, comparable to that measured in the other-N cluster group. Path-rot behavior most likely corresponds to an initial phase of axial dyskinesia, which typically starts with a tight contralateral twisting of the head preceding the torsion of the body and the stopping of forward body motions. Contralateral rotations are frequently measured in unilateral models of PD-LID using automated video-tracking or rotary sensors with photobeam detectors. Measured in this way, contralateral rotations correlate poorly with LID ratings,²⁸ and they are indeed induced to a much larger degree by non-dyskinesiogenic treatment with DA agonists compared to L-DOPA.^{28,29} In many circumstances, contralateral rotations have been found to reflect therapeutic-like effects of antiparkinsonian or antidyskinetic treatments (discussed in Cenci and Crossman¹⁷). Importantly, the high kinematic precision offered by our method enabled us to isolate pathological components of the animal's rotational behavior, where an abnormally tight turning of the head occurs while the mouse is moving forward (high BA).

In the second part of our study, we aimed at revealing the neural activity patterns of D1- and D2-SPNs during dyskinesia. As previously reported,^{5,6} dyskinetic mice exhibited an increased average D1-SPN activity and a reduced D2-SPN activity during periods of movement. We also investigated whether SPNs would exhibit altered spatiotemporal activation patterns, as indicated by Parker and colleagues.⁵ Overall, cross-correlations between pairs of active SPNs exhibited the same spatiotemporal configuration in all experimental groups, with a sharp decline between 20 and 47 μm and hardly any cross-correlation at intercellular distances larger than 100 μm . This suggests that small groups of contiguous SPNs fire together also under parkinsonian and dyskinetic conditions, presumably driven by the same excitatory input,³⁰ resembling the situation reported in intact mice during movement.^{15,31} At variance with the results of Parker et al.,⁵ our data therefore indicate that the gross spatial organization of SPN coactivity is maintained during LID, although the number of active neurons, their event rates, and the relative D1-SPN/D2-SPN recruitment are profoundly abnormal. Importantly, however, a marked difference in coactivation was found between

SPN types at intercellular distances below 40 μm , with a larger coactivation of D1-SPNs compared to D2-SPNs. This short intercellular distance corresponds to the microdomain with largest probability of SPN-to-SPN synaptic contact formation via axon collaterals.³² It is also the microdomain where largest coactivity of SPNs is found under physiological movement.^{15,31} Given that cross-correlation ratios of D1- and D2-SPNs were basically identical under normal conditions (see [Figures S5G](#) and [S5H](#)), the highly significant divergence between SPN types at this critical intercellular distance represents a substantial change. The data indicate that dyskinetic movements are associated with a high level of D1-SPN coactivation concurring with a deficient co-recruitment of D2-SPNs specifically in the distance range where D2 SPNs normally exert maximal collateral inhibition onto contiguous D1-SPNs.³² The reduced coactivation of D2 SPNs at this short intercellular distance therefore suggests weaker lateral inhibition onto nearby D1 SPNs, contributing to direct pathway overactivity during the expression of LID.

This study establishes a direct link between specific dyskinetic movements to the neural activity of striatal ensembles with single-cell resolution. At first, we saw that average calcium event rates of D1- and D2-SPNs did not differ significantly between behavioral clusters. These data indicated that the average increase in D1-SPN and decrease in D2-SPN activity was not driven by a specific behavior but also that the average SPN activity per se could not explain the emergence of different types of dyskinesia. Next, taking advantage of the single-cell resolution, we could identify specific groups of D1- and D2-SPNs whose activity was correlated to each behavioral cluster group. Although these specific sets of neurons fired also under other conditions, their activity was maximal during their associated behaviors. The sets of D1-SPNs linked with the two dyskinesia cluster groups had doubled their activity compared to the average activity during the moving periods. Although the entire D2-SPN population showed very low calcium event rates in Les-LD, the D2-SPN ensembles associated with dyskinesia or pathological rotation exhibited markedly higher activity than the rest of the D2 population during the expression of their corresponding behavioral clusters. This was particularly clear for D2-SPNs tuned to the axial + limb and axial-alone clusters, which showed a 3-fold increase in event rate compared to the average value found in Les-LD (and also higher than the Int-LD average rate) during the expression of those specific behaviors. However, despite showing a relatively high activity, these D2-SPN groups fired at an approximately 2-fold lower rate than the D1-SPNs tuned to the same behavioral cluster. The marked hyperactivity of certain groups of D1-SPNs over the average of that population is likely to depend on a stronger excitatory drive as shown in Ryan et al.³⁰ Importantly, we found that the majority of the SPNs associated with the dyskinesia and pathological rotation clusters were also active during normal behaviors at baseline, suggesting that their marked activity levels during LID might trigger a shift from normal to abnormal motion patterns. Under baseline conditions, neurons associated with axial and limb AIMs appear to encode for behaviors that share physical similarity with the specific dyskinesia types to which they are tuned, suggesting that the predetermined phenotype of hyperactive neurons determines which pattern of dyskinesia will emerge on L-DOPA.

In summary, using an advanced quantitative approach to quantify dyskinetic movements, our study unveils the underlying changes in D1- and D2-SPN activity with unprecedented granularity. Using *in vivo* recordings with single-cell resolution, we not only confirm the existence of specific sets of D1-SPNs associated with dyskinetic motions but also describe the presence of a small group of D2-SPNs that are disproportionately active in each dyskinesia cluster. The large disparities in event rates and cross-correlation found between D1- and D2-SPNs suggests that, despite their relatively high activity levels, dyskinesia-specific D2-SPNs fail to brake nearby hyperactive D1-SPNs via inhibitory axon collaterals. While confirming that an imbalance between D1- vs. D2-SPN activity in favor of the former is a generic signature of LID, our results indicate that specific combinations of abnormally active SPNs dictate the moment-to-moment expression of particular LID features. Interestingly, SPN ensembles tuned to specific dyskinetic movements also encode similar normal behaviors at baseline, suggesting that L-DOPA may repurpose existing motor circuits to generate abnormal outputs rather than recruiting entirely new pathways.

Limitations of the study

While this study provides high-resolution mapping of dyskinesia-related activity in striatal SPN ensembles, several limitations remain. A key constraint is that D1- and D2-SPNs were imaged in separate animals, preventing simultaneous assessment of their dynamic interactions during specific dyskinetic events. Although our behavioral clustering captured axial and limb dyskinesias with high precision, we were unable to analyze orofacial AIMs due to camera-angle and resolution limitations, potentially omitting important components of LID. In addition, while ensemble activity was strongly correlated with specific dyskinetic behaviors, causality was not established; future studies using cell-type-specific manipulations of identified ensembles will be needed to confirm their functional contributions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and data supporting the findings of this study should be directed to and will be fulfilled by the lead contact, Cristina Alcacer (cristina.alcacer81@gmail.com).

Material availability

This study did not generate new materials.

Data and code availability

- Tabular datasets and behavioral videos are available on Zenodo: [10.5281/zenodo.15620107](https://doi.org/10.5281/zenodo.15620107) (tables) and [10.5281/zenodo.15619965](https://doi.org/10.5281/zenodo.15619965) (videos).
- All resources used are archived at [10.5281/zenodo.15621612](https://doi.org/10.5281/zenodo.15621612).
- Protocols are provided in the [STAR Methods](#) and at <https://www.protocols.io/>.
- Custom behavioral clustering code and other custom made codes are available upon publication; see [key resources table](#) for DOI, and [10.5281/zenodo.15621612](https://doi.org/10.5281/zenodo.15621612).
- Further information is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

C.A. and M.A.C. conceived the project. C.A. and R.M.C. designed the experiments. C.A., A.K., and R.M.C. conceptualized the analyses. A.K., S.F.A., and C.A. configured the features and thresholds for the unsupervised behavioral clustering algorithm. C.A. and A.K. performed the behavioral clustering. C.A. performed the surgeries, one-photon calcium imaging experiments, dyskinesia scoring and annotations, and histological analysis. A.K. and C.A. performed the DeepLapCut analysis. M.M. helped organize the data and performed the analyses of average calcium imaging. A.K. and C.A. performed all other analyses. C.A., M.A.C., A.K., and R.M.C. wrote the paper. R.M.C. and M.A.C. supervised and guided all aspects of the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
- **METHOD DETAILS**
 - 6-Hydroxydopamine lesion and virus injection
 - Chronic lens implantation and inertial sensor holder placement
 - Drug treatment
 - Open-field experiments
 - Wireless inertial measurement unit (IMU)
 - One-photon calcium imaging
 - Measurement and quantification of body acceleration with the IMU
 - Abnormal involuntary movements (AIMs)
 - Manual annotations of dyskinesia
 - Video tracking and axial bending angle extraction
 - Unsupervised behavioral clustering
 - Library of mouse behavior and matching procedure
 - Behavioral cluster groups
 - Visualization of behavioral similarity in two dimensions
 - Classifier of *axial+limb* and *axial alone* dyskinesia and of grooming
 - Calcium imaging processing and analysis
 - Decoding dyskinesia and pathological rotations based on SPN ensemble activity

- Calculation of neuronal overlap
- Analysis of SPN activity during non-dyskinetic periods
- Analysis of grooming-related neurons
- Tissue preparation and immunohistochemistry
- Quantification of striatal dopamine depletion

● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
6-OHDA hydrochloride	Sigma-Aldrich	H4381
L-ascorbic acid, 99%	Sigma-Aldrich	A92902
3,4-Dihydroxy-L-phenylalanine	Sigma-Aldrich	D9628
Benserazide hydrochloride	Sigma-Aldrich	B7283
Antibodies		
Rabbit anti-TH	Peel Freez Biological	Cat # P40101-150; RRID: AB_2617184
Rabbit anti-GFP Alexa Fluor 488 conjugate	Invitrogen (Molecular Probes)	Cat# A-21311; RRID: AB_221477
Alexa Fluor 594 goat anti-rabbit	Jackson ImmunoResearch Labs	Cat#115-585-045; RRID: AB_2338062
Bacterial and Virus Strains		
AAV5.CAG.Flex.GCaMP6f.WPRE.SV40	University of Pennsylvania Vector Core	Cat#100835-AAV5, #AV-5-PV2816
Experimental Models: Organisms/Strains		
D1-Cre, Tg(Drd1a-cre) FK150Gsat/Mmucd	MMRRC	#029178-UCD
A2a-Cre, B6.FVB(Cg)-Tg(Adora2acre) KG139Gsat/Mmucd	MMRRC	#036158-UCD
Softwares and Algorithms		
Unsupervised behavioral clustering algorithm	This paper; Klaus et al. ¹⁵ ; Frey and Dueck ³³	https://zenodo.org/records/13990593
CNMF-E	Klaus et al. ¹⁵ ; Pnevmatikakis et al. ²⁴ ; Friedrich et al. ³⁴ ; Zhou et al. ²⁵	https://github.com/zhoup/CNMF_E
Bonsai 2.4.	Lopes et al. ³⁵	https://bonsai-rx.org/ ; RRID:scr_017218
DeepLabCut	Mathis et al. ³⁶	https://github.com/DeepLabCut/DeepLabCut ; RRID: SCR_0021391
Python Video Annotator	Champalimaud Foundation	https://github.com/video-annotator/pythonvideoannotator
Spyder 5.2	Spyder/Python	https://www.spyder-ide.org/ ; RRID:SCR_017585
GraphPad Prism 9	GraphPad Software	https://www.graphpad.com/ ; RRID: SCR_002798
Inscopix Data Processing Software	Inscopix Inc.	https://www.inscopix.com/software-analysis-miniscope-imaging
ImageJ	NIH	https://imagej.nih.gov/ij/index.html ; RRID: SCR_003070
MATLAB	MathWorks	https://www.mathworks.com/products.html ; RRID: SCR_001622
Adobe Illustrator CS5	Adobe	https://www.adobe.com/products/illustrator.html ; RRID: SCR_014198

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The study was performed in bacterial artificial chromosome (BAC) transgenic mice expressing Cre recombinase under the control of the dopamine D1 receptor (D1-Cre, Tg(Drd1a-cre) FK150Gsat/Mmucd; MMRRC #029178-UCD) for targeting of direct-pathway SPNs, and adenosine A2a receptor (A2a-Cre, B6.FVB(Cg)-Tg(Adora2acre) KG139Gsat/Mmucd; MMRRC #036158-UCD) for targeting indirect-pathway SPNs. All lines have been backcrossed onto C57Bl6/J mice for at least 8 generations. Experimental mice were 3 to 5-month-old males housed on a 12-h light/dark cycle with *ad libitum* access to food and water. Experimental groups were

balanced within litters. All animal procedures were reviewed and performed in accordance with the Champalimaud Center for the Unknown Ethics committee guidelines and approved by the Portuguese Veterinary General Board (Direção Geral de Veterinária, Ref. No. 0421/000/000/2014). Sample size is detailed in the Results or figure legends.

METHOD DETAILS

6-Hydroxydopamine lesion and virus injection

6-hydroxydopamine (6-OHDA) lesion and virus injection were performed in the same surgery. Surgeries were performed under isoflurane (1%–3%, plus oxygen at 1–1.5 L/min) anesthesia on a stereotactic frame (David Kopf Instruments, Model 962LS), with a mouse adaptor (David Kopf Instruments, Model 923-B Mouse Gas Anesthesia Head Holder). Throughout each surgery, mouse body temperature was maintained at 37°C using an animal temperature controller (ATC1000, World Precision Instruments) and afterward, each mouse was allowed to recover from the anesthesia on a heating pad. After shaving and disinfecting the surgical area of the mouse head with 70% ethanol and iodine, a small incision was made on the skin to allow for alignment of the skull and drilling of the injection holes for 6-OHDA and virus injections. Chronic striatal DA denervation was produced through unilateral injection of 6-OHDA in the medial forebrain bundle (MFB). The toxin 6-OHDA hydrochloride (Sigma-Aldrich, Portugal) was dissolved in 0.02% ice-cold L-ascorbic acid/saline (3.2 µg free-base 6-OHDA/µL), and 1 µL was injected into the MFB (coordinates: AP = − 0.7, ML = − 1.2, DV = − 4.7), using a capillary attached to a Nanojet II Injector (Drummond Scientific, USA) at a rate of 4.6 nL per pulse every 5 s. The capillary was left in place for 2 min before and 10 min after the injection. After injecting the toxin, the capillary was replaced with a new one to inject the virus, which was injected into the striatum ipsilateral to the lesion. Each animal was then unilaterally injected with 600 nL (volume injected based on⁹ to cover a broad area of the striatum) of AAV5.CAG.Flex.GCaMP6f.WPRE.SV40 (titer: 3.82×10¹³; University of Pennsylvania Vector Core) into the right dorsal striatum (AP = + 0.5, ML = − 2.3, DV = 2.3). After the surgery, the wound was closed with tissue glue (Vetbond tissue adhesive, 3M, USA) and the animal received a subcutaneous injection of the analgesic Carprofen (5 mg/kg s.c; 10 µL/10 g body weight). To prevent dehydration, mice received a subcutaneous injection of sterile glucose-ringer acetate (0.6 mL) immediately after the surgery. During the first 2 to 3 weeks post-surgery, mice received daily subcutaneous injections of sterile glucose-ringer acetate solution (0.1 mL/10 g body weight) and dietary supplementations, as necessary. In addition, mice were kept in a warming cabinet at 27°C to keep a constant body temperature avoiding hypothermia.⁹ Lesions were also verified at the end of the study using tyrosine hydroxylase (TH) immunohistochemistry,^{9,20} and only mice with more than 90% TH depletion were included in the study (Figure S1A).

Chronic lens implantation and inertial sensor holder placement

Following the same surgical procedures, 3 weeks after 6-OHDA and viral injections, a gradient index (GRIN) lens (diameter: 1 mm, length: 4 mm; Inscopix) was implanted in the right dorsolateral striatum directly above the viral injection site (AP = + 0.5, ML = − 2.3, DV = 2.3), after carefully aspirating 1.8–2 mm of the overlying cortical tissue with a 30-gauge blunt needle. Care was taken to minimize bleeding before inserting the lens. Once in place, the lens was secured to the skull using superglue and a self-curing adhesive resin cement (Super-Bond C&B Kit, SUN MEDICAL CO., LTD., Japan). A small screw was placed on the surface of the skull and a layer of resin surrounded the lens and covered the screw to increase the lens bond to the skull and to minimize motion artifacts during imaging. To the self-curing adhesive resin cement, we added the mixture of black Ortho-Jet powder and liquid acrylic resin (Lang Dental, USA) on top, and finally a tape was placed to protect the lens surface. One week after the GRIN lens implantation, the microendoscope baseplate (nVistaHD, Inscopix) was attached to the microendoscope, and placed at the best focal plane to observe neuronal structures and blood vessels when present. Once the best focus was found, the baseplate was secured with a first layer of self-curing adhesive resin cement and a second layer of black cement to permanently secure the baseplate to the head cap prior to removing the microscope and attaching a baseplate cover (Inscopix) to the baseplate. Once the baseplate was placed, a small connector or holder for the wireless IMU was cemented on the back of the baseplate. The imaging field of view was inspected and allowed to clear for several days prior to imaging and behavioral experiments. Mice were excluded prior to the collection of experimental data based on imaging quality due to bad focal plane, movement artifacts or a lack of cells.

Drug treatment

L-DOPA methyl ester (L-3,4-dihydroxyphenylalanine also called L-DOPA or LD; 6 mg/kg; from Sigma Aldrich, Portugal) and the peripheral DOPA decarboxylase inhibitor benserazide-HCl (12 mg/kg; from Sigma Aldrich, Portugal) were dissolved together in physiological saline (9 g/L NaCl). L-DOPA was injected intraperitoneally with a volume of 10 mL/kg body weight. Vehicle (VEH) solution corresponding to physiological saline (9 g/L NaCl) was used as control. VEH was given for 2 consecutive days during which mice were recorded for imaging and behavior, and the following day, L-DOPA was administered for 3–4 consecutive days while recorded as well for imaging and behavior.

Open-field experiments

Experiments were conducted in a white open-field arena (40 cm × 40 cm) with a transparent acrylic on the bottom and on the side, placed inside a sound-attenuating chamber. A video camera (Flea3, Point Gray Research) was placed on the bottom of the arena to record mouse behavior from the bottom at 30–40 frames per second (fps). Before starting the experiments, mice were habituated

for 2–3 days (1 h per mouse per day) to the head-mounted equipment by using a replica of the microendoscope with the same weight (~ 2 g) and the actual wireless inertial sensor (~ 1.8 g). On the day of the experiment, mice were lightly anesthetized with isoflurane to facilitate mounting (and removal) of the microendoscope and the inertial sensor. Fifteen minutes after initial recovery from anesthesia, mice were video-recorded and striatal activity and acceleration were simultaneously recorded for 10 min, this period being referred to as baseline (BL). After BL, mice were taken out of the arena, injected with the L-DOPA or VEH and placed in a cage close to the arena. After a period of 20 min, at the peak of the L-DOPA,²⁰ the animals were placed back inside the open field arena and recorded/imaged for another 10 min (see *Timeline* Figure 1A). Note that the microendoscope and the inertial sensor were left in place along the whole session, from the BL till the end of the L-DOPA/VEH recording. Acceleration was recorded using head-mounted wireless inertial sensors with a sampling rate of 200 Hz (for inertial sensor's details see next section). The inertial sensor was secured to a connector or holder placed on the back of the microendoscope, as described above, with a consistent alignment of its axes (see Figure 1A). We note that a subset of mice ($n = 14$, 7 Int and 7 Les), were used for the behavioral clustering only and not for the calcium imaging part. These mice were recorded in a slightly different experimental setup, with a smaller open field arena (20 cm $\times$ 20 cm) and with 4 mirrors surrounding the open field box. This subset of mice had a different alignment and axes were corrected in a pre-processing step before subsequent data analyses.

Wireless inertial measurement unit (IMU)

The wireless, custom-made inertial measurement units (IMU) (Champalimaud Hardware Platform; WEAR motion sensor system - <https://www.cf-hw.org/harp/wear>) delivers a self-centered 9-axis inertial sensor containing 3-axes for accelerometer, gyroscope and magnetometer (the latest was not used in this study). The wireless IMUs are small and light (~ 1.8 g) and can sample data up to 200 Hz, with a battery allowing to record up to 4 h of data. The IMUs are connected to the computer through a base station (HARP design, designed by the Champalimaud Hardware Platform), which provides hardware-based synchronization. We used Bonsai visual reactive programming (Bonsai v2.4, <https://bonsai-rx.org/>), which is compatible with the WEAR motion devices, to integrate and synchronize all the data coming from different sources such as the IMUs, the camera recordings and the calcium imaging recordings. Time stamps from the IMU, the video cameras and the microendoscope were then aligned using custom MATLAB scripts.

One-photon calcium imaging

One-photon imaging of intracellular calcium activity was acquired at 20 fps using an nVista microendoscope [lens: 1 mm diameter, 4 mm length, 0.5 numerical aperture; excitation: blue light-emitting diode (LED); excitation filter: 475/10 nm, 0.24–0.6 mW/mm²; emission filter: 535/50 nm; Inscopix, Palo Alto, CA] and with LED power set at 20%–40%, and gain level 4. Resulting calcium movies, video and IMU data were analyzed as described below.

Measurement and quantification of body acceleration with the IMU

The percentage of time moving per session was calculated using the body acceleration (BA) component of the IMU, which accurately tracks animal movement and correlates with pixel change in video measurements.^{15,21} As shown by Alves da Silva and colleagues, the average distribution of the logarithm of total BA was bimodal, with a very low acceleration distribution corresponding to immobility periods and a high acceleration distribution corresponding to periods of mobility. As done previously in the mentioned studies, to separate between rest and moving periods, we put a threshold at the value that separates the two peaks in the BA distribution. Using this threshold, which in our case corresponded to 0.05 g ($\ln(0.05) = -3$ a.u., Figure S2C), we calculated the percentage of time that the mice moved per session and the acceleration in the periods where the mice were strictly moving (Figure 1B).

Abnormal involuntary movements (AIMs)

Three topographic subtypes of abnormal involuntary movements (AIMs; axial, limb and orofacial) were rated for 1 min every 5 min for a total of 10 min following L-DOPA injection.^{9,19,20} Axial AIMs are twisting movements or dystonic postures of neck and upper body towards the side contralateral to the lesion; limb AIMs are circular or fluttering movements of the contralateral forelimb; orofacial AIMs include twitching of facial muscles, jaw movements, and contralateral tongue protrusion. During the test, every mouse was scored on a well-characterized severity scale that is based on the duration and persistence of each dyskinetic behavior (0 = no dyskinesia; 1 = occasional signs of dyskinesia; 2 = frequent signs of dyskinesia, present for more than 50% of the observation time; 3 = dyskinesia present during the entire observation time, but interruptible by mild external stimuli; 4 = continuous dyskinesia, not interrupted by mild external stimuli). L-DOPA, at a dose of 6 mg/kg, was administered for 3–4 days and AIM scores were rated each day/session at 20, 25 and 30 min post injection.

Manual annotations of dyskinesia

Manual annotations of dyskinetic movements induced by L-DOPA were made frame by frame using video data recorded with a bottom camera. The annotations were performed with the software *Python video annotator* (<https://github.com/video-annotator/pythonvideoannotator>), for a total of 10 min per mouse, a total of 13 mice and 1 or 2 days per mouse (depicted in Figure 2A and Video S2). Only two types of AIMs were annotated: axial and limb. The orofacial dyskinesia, although scored *live* (as described above), was not possible to annotate due to not enough resolution (30–40Hz) and angle view of the bottom camera. The criteria to

detect the beginning and end of specific axial AIMS was the following. Beginning of the annotation: i) torsion of the neck/upper trunk by almost 90° (bipedal position) and ii) contralateral (to the lesion) forelimb off the floor and ipsilateral forepaw extended close to the floor; end: i) 4 paws on the floor, quadrupedal position and ii) small deviation of the neck/upper trunk of less than 60°. For the limb AIMS, it was as follows: the digits of the contralateral forelimb flexed and held in a fist and close to the snout was considered the beginning of the annotation; the end of the annotation corresponded to the digits of the contralateral forelimb extended and far from the snout. The minimal duration of an annotation was 3 s.

Along the whole recording period, we obtained frames where axial or limb were present and frames where axial and limb appeared at the same time. Therefore, the following three combinations of annotations were considered for the analysis: *axial alone*, *limb alone* and *axial+limb*. We observed that *limb alone* did not occur very often, when present, limb was generally combined with axial (Pearson correlation between limb and axial AIMS shows a positive correlation, data not shown).

Video tracking and axial bending angle extraction

We used the video recordings of the bottom camera and DeepLabCut software (<https://github.com/DeepLabCut/DeepLabCut>) to track 9 regions of interest: nose, tail base and tail tip, left and right hindlimbs, left and right forelimbs, and, in order to extract additional head posture details, the base and tip of the accelerometer antenna (depicted in Figure 1D and Video S1). The data set for the training of the network consisted of a total of 2840 manually labeled frames selected from 81 sessions ($n = 12$ Int, $n = 13$ Les mice; including VEH and LD together with their corresponding BL sessions). We used a training and test fraction of 0.95 and 0.05, respectively. The trained network was used to extract the positions of all regions of interest during the entire video session resulting in a set of time series for each region (i.e., x- and y-positions and the likelihood, which is an indicator of the “quality” of the extracted position). In a post-processing step, for each region of interest, we linearly interpolated the x- and y-time series if the likelihood was less than 0.6.

Once we obtained the correct tracking of all regions of interest in all mice/all sessions, we proceeded to the extraction of the axial bending angle (θ axial) as described in²². In short, the θ axial was defined as the angle generated from the intersection between 2 vectors emerging from the mid-point between the hindlimbs (mid_HL) to the nose and to the tail base (Figure 1D).

Based on the X and Y coordinates for the nose ($Nose(x_{Nose}, y_{Nose})$), hindlimbs $L_HL(x_{L_HL}, y_{L_HL})$, $R_HL(x_{R_HL}, y_{R_HL})$ and base $Tail(x_{Tail}, y_{Tail})$, and the mid_HL , we calculated the v_Nose and v_Tail vectors as follow:

$$mid_HL = ((x_{R_HL} + x_{L_HL}) / 2, (y_{R_HL} + y_{L_HL}) / 2)$$

$$v_Nose = (x_{Nose} - x_{mid_HL}, y_{Nose} - y_{mid_HL})$$

$$v_Tail = (x_{Tail} - x_{mid_HL}, y_{Tail} - y_{mid_HL})$$

Then, to get the axial angle, θ axial, we calculated the cosine of the angle between the “nose” and “tail” vectors:

$$\cos(\theta) = (v_Nose \cdot v_Tail) / (|v_Nose| |v_Tail|)$$

where $v_Nose \cdot v_Tail$ is the dot product of the two vectors and $|v_Nose|$ and $|v_Tail|$ are their Euclidean lengths.

Using the trained DLC network, the axial bending angle was then calculated for this cohort of mice ($n = 12$ intact, $n = 13$ lesioned) for all $n = 142$ sessions and the entire 10 min recordings (in total 16.9 h).

For another group of mice that were used for calcium imaging (D1-Cre: lesioned $n = 3$ and intact $n = 4$; A2a-Cre: lesioned $n = 4$ and intact $n = 3$), we only had video-recordings from a camera placed on top of the open field that did not allow us to use the tracking method described above. We therefore developed a custom Python script to track semi-automatically the 4 body parts needed to get θ axial, i.e., the nose, tail base, left HL and right HL. We annotated these body parts every 5 to 20 frames for a total of 10 min recordings per mouse and session ($n = 7$ lesioned mice with 2 days per mouse, including VEH and LD; $n = 7$ intact mice with 2 days per mouse, LD). The calculation of the θ axial in this group of mice was done as described above.

Unsupervised behavioral clustering

Behavior was quantified based on the time series of acceleration and gyroscope data obtained from the IMU and the bending angle obtained with video. In short, we extracted features that capture head and body posture and motion in three dimensions. In particular, we used the following features given by the sensors: (i) total body acceleration (BA) to distinguish movement versus rest, (ii) gravitational acceleration along the anterior-posterior axis (GA_{AP}) to extract postural changes such as those observed during rearing¹⁵; (iii) gyroscope (or angular velocity) along the dorsoventral axis that infers the head rotational behavior (θ head) and (iv) axial bending angle (θ axial), to get a measure of the axial component of dyskinesia. Example traces of each feature are shown in Figures 1C and S2A.

Total body acceleration was defined as the square root of the sum of the squares of the BA of each axis ($BA = \sqrt{BA_{AP}^2 + BA_{ML}^2 + BA_{DV}^2}$), where $BA_{AP/ML/DV}$ denote the body acceleration in the anteroposterior, mediolateral, and dorsoventral axis, respectively, with respect to the animal’s head. The individual BA components were calculated by median-filtering the raw acceleration and gyroscope time series with a 7th-order one-dimensional median filter and by a subsequent Gaussian filter (type *fspecial*), to remove noise peaks. Gravitational acceleration, GA , was obtained for each axis by median and subsequent low-pass filtering (0.5 Hz cutoff,

first-order Butterworth filter and a *filtfilt* filter). We used GA_{AP} to quantify postural changes (i.e., vertical head position) in the open field during resting, locomotion and rearing (as in¹⁵).

Head angular velocity of the animal was obtained from the gyroscope component of the IMU data (processed as mentioned above). To capture rotational behavior, we used the dorsoventral axis and measured the cumulative head angle (θ head) from the beginning until the end of a head rotation for all rotations with a minimum of ± 10 deg/sec. The value for each rotation was set to the estimated total rotation angle (Figure S2A).

The signal distribution of the above-mentioned features, BA, GA_{AP} , θ head and θ axial of all mice and sessions, was used to define the thresholds for binning the feature's time series (histogram of distributions and thresholds shown in Figure S2C). The thresholds of all features were defined as follows. The threshold for BA was set to separate moving and resting as described in *Measurement and quantification of body acceleration with the IMU*. For GA_{AP} , two thresholds were defined (GA_{AP} thresholds (au) = -0.4 and 0) to capture vertical head movements including head down, head forward and head up (including rearing). For the θ axial feature, one single threshold of 90 deg was imposed, which corresponded to the 90 deg angle of the upper torso of the mice. Finally, for the θ head feature we used a threshold of ± 25 deg to detect smaller rotations or head deviations and a higher threshold of ± 165 deg to capture stronger (i.e., pathological) rotations (see Figure S3). Here, positive and negative values indicate right/ipsilateral and left/contralateral rotations, respectively. Note that the strong pathological ipsilateral rotations never happened in this study.

With the time series of the features binned using the thresholds described above, we did sliding windows of 250 ms with 80% overlap and compared them. For this, we used a similarity measure based on the so-called earth mover's distance (EMD).^{15,37} EMD calculates the pairwise similarity between consecutive sliding windows to get the change-points. Change-points are defined as significant changes in acceleration data corresponding to peaks detected above a threshold that was set to the average change-point value (0.005). The signal between two change-points, termed behavioral blocks or behavioral segments, had an average duration of 1.04 s (minimum duration: 100 ms, median duration: 550 ms). The obtained similarity measures were clustered using affinity propagation^{15,33} on the EMD similarity of the behavioral blocks. Combined, for all the 600 s long recordings, this resulted in a $45,571 \times 45,571$ similarity matrix. After matching with the entire data set (see next section *Library of mouse behavior and matching procedure*), the clustering revealed a total of 56 clusters, whose signatures are shown in the *Cluster exemplars* matrix in Figure S2D. To measure the accuracy of the clustering, we did a ROC (receiver operating characteristic) analysis, which determines whether two behavioral segments belong to the same or to different behavioral clusters based on a single threshold (Figure 1F). "True positive" corresponds to behavioral segments that were correctly classified as belonging to the same cluster whereas "false positive" corresponds to the behavioral segments from the same cluster that were misclassified as belonging to different clusters ($n = 25$ mice). Based on the probability of BA being below the "moving" threshold (see Figures S2A, S2C, and S2D), we defined 49 out of the 56 clusters as "moving" clusters (i.e., probability of resting < 0.5) and the remaining 7 clusters as "resting" (i.e., probability of resting > 0.5).

Library of mouse behavior and matching procedure

Due to the computational complexity of the clustering algorithm, we created a library of mouse behavior by running the clustering for a subset of 81 sessions ($n = 25$ mice in total; $n = 12$ Int, $n = 13$ Les; ~ 13 h of data) out of a total of 142 sessions. These included the four conditions, i.e., Int and Les, each treated with VEH and LD plus the BL sessions and one to three days per animal and treatment. We defined clusters present in a condition if they occurred a minimum of 3 s per session (corresponding to 0.5% of the session duration). Using this definition, we found 26 clusters present in Int-VEH and 23 clusters absent in Int-VEH (Figure 1G). As mentioned above, our entire data set consisted of a total number of 142 sessions, which corresponded to 16.9 h of acceleration and video data from $n = 39$ mice. These additional sessions were clustered on a session-by-session basis and matched to the library a posteriori using a matching procedure based on the closest EMD. We confirmed the accuracy of this approach by comparing the EMD similarity between original (library) and matched data with that of shuffled data (Figure S2B, right panel).

Behavioral cluster groups

For each of the 49 moving clusters, we calculated the Pearson correlation for each mouse between the occurrence of the cluster and each of the 3 combinations of dyskinesia annotations (*axial+limb*, *axial alone* and *limb alone*) and considered a correlation to be significant if the *pp*-value was smaller than 0.05 (Figures S3A and S3B). We found clusters that were positively correlated with specifically one type of dyskinesia annotation, such as cluster #20, which correlated to *axial alone* in 10 out of 13 mice (Figure S3A), and others that were positively correlated with more than one type of annotation, such as cluster #7, which was correlated to *axial+limb* and *axial alone* annotations. We also found clusters that were negatively correlated to annotations (data not shown). Next, we grouped behavioral clusters at the cohort level into the following dyskinesia cluster groups: *axial+limb*, *axial alone* and *limb alone*. Due to the variability of some of the dyskinetic behavior across mice, we used the following criteria: a cluster must be significantly positively correlated to a combination of dyskinesia annotation in at least two mice. If significant negative correlations were present in some mice, the number of mice with significant positive correlations should be at least two more than the number of mice with significant negative correlations. This resulted in 9 *axial+limb* clusters, 11 *axial alone* clusters and one *limb alone* cluster (Figure 2C). In the remaining moving clusters, we found 20 clusters that were present in Int-VEH and referred to them as *other N* (*N* for "normal"). The remaining clusters appeared only in Les-LD and were largely characterized by strong pathological rotations (*path rot*). We note that behavioral clusters of the *other N* group also appeared in dyskinetic mice (Figure 2E, Les-LD). Although the behavioral signature

of the clusters in *other N* Int-VEH and Les-LD was very similar, we observed differences in the probability of occurrence as shown in Figure S4B.

Visualization of behavioral similarity in two dimensions

For the visualization of the behavioral segments in the two-dimensional space as done previously,¹⁵ we used the non-linear dimensionality reduction technique t-SNE (*t-distributed stochastic neighbor embedding*)³⁸ (Figure 1H). In Figure 1H left, we show the space representation of the VEH and LD sessions of the library ('All VEH & LD'), where each dot represents a behavioral segment and each color corresponds to a different cluster. On the right is the behavioral representation of each of the four conditions Int/VEH or LD and Les/VEH or LD. We used the matrix of pairwise EMD for all behavioral histograms (i.e., 300 ms time bins) as the input for the algorithm. We verified that the Euclidean distance in the two-dimensional projection appropriately reflected the true EMD (data not shown).

Classifier of axial+limb and axial alone dyskinesia and of grooming

To evaluate the predictive power of the dyskinesia cluster groups (Figure 2G), we trained a linear support vector classifier that predicts the type of dyskinesia (i.e., annotation) based on the *axial+limb* and *axial alone* cluster groups. Specifically, we used a leave-one mouse-out cross-validation approach and a fraction of 0.8 and 0.2 for training and testing, respectively. Importantly, the no-dyskinesia data was sampled from both Les-VEH as well as from Int-VEH/LD to account for false-positive classifications. Data for training and testing was balanced by subsampling the data repeatedly and calculating the average accuracy ($n = 5000$). The chance level accuracy for shuffled data was calculated by random permutation of the classification labels. For the classification of grooming vs. no grooming (Figure S4A), we used the same approach as above using the annotations of grooming behavior in $n = 6$ mice.

Calcium imaging processing and analysis

Calcium recordings were pre-processed in batches using Mosaic and the *Inscopix Data Processing Software library* with a custom MATLAB script for spatial binning and motion correction. Background and neuropil contamination in the one-photon microendoscopic data were corrected for using a constrained non-negative matrix factorization for microendoscopic data (CNMF-E;^{15,24,25}) and single-neuron footprints and their corresponding activities, C_{raw} , were extracted from up to 318 neurons (see Table S3 for number of neurons per mouse and experimental condition). In order to compare neuronal activities between BL and treatment (i.e., VEH or LD), we matched neuronal footprints within a session (i.e., when the microendoscope was kept in place) using a combination of correlation matching of the spatial footprints and manual curation.

To account for the decay dynamics of the genetically encoded calcium sensor (GCaMP6f), we quantified the event rate for each neuron by deconvolving the C_{raw} activity traces using spike deconvolution. Since small fluctuations in the baseline fluorescence can lead to spuriously detected spikes, we used the MLspike algorithm which explicitly models a time-varying baseline.²⁶ We used the following parameters for the spike detection: $t_{decay} = 400$ ms, offset correction to obtain strictly positive C_{raw} values, multiplicative drift estimation with drift parameter 0.2, drift baseline start 0 and drift mean and standard deviation estimated for each trace by fitting a normal distribution to the distribution of offset-corrected C_{raw} .

For the quantification of the event rate (events/sec) during the resting periods, we used the following criteria for immobility: the mouse has to have a BA lower than the threshold 0.05 g, for at least 500 ms. In the Les-LD condition, the criteria of immobility was not attained, there were only a few periods in a few mice where the mice spent from 500 to 700 ms with a BA below 0.05 g.

For the calculation of active neurons in Figure 3, it was necessary to define 'active' during the specific treatment (i.e., VEH or LD) because, as explained above, the neuronal footprints were determined based on the BL plus the treatment recordings. We quantified the number of active neurons as a function of the activity threshold (Figure S5D) and chose a threshold of 0.05 events/s for the main analysis in Figure 3.

For the calculation of the spatiotemporal correlations, we used the same approach as in¹⁵ based on the C_{raw} time series. In short, the Pearson correlation coefficient of pairwise correlations together with the corresponding inter-neuronal distances of the spatial footprints was calculated for all neuronal pairs within an imaging session. The correlation values were binned into nine bins of logarithmically scaled spatial distances (15–750 μ m) and averaged over all neurons within an imaging session. Spatiotemporal correlation plots represent the averages $\pm$ SEM across the mice.

For the analyses of behavior-related neurons (Figures 4 and 5), we analyzed the neuronal activity in relation to individual behavioral clusters. In order to identify an SPN as "axial+limb" or "axial alone", we determined whether the neuron was related to at least one *axial+limb* or *axial alone* behavioral cluster, respectively. "Behavior-related" was defined as any neuron whose activity is positively modulated above the shuffled average activity for that neuron (see¹⁵).

Decoding dyskinesia and pathological rotations based on SPN ensemble activity

To evaluate the predictive power of the deconvolved D1- and D2-SPN ensemble activities for dyskinesia and *path rot* behaviors, we trained a linear support vector classifier for the following binary classifications based on neuronal activity: (1) dyskinesia (*axial+limb* and *axial alone*) versus *other N*, and (2) *path rot* versus *other N*. Specifically, we considered each behavioral segment as a sample and averaged the deconvolved activity of each SPN during that time period (i.e., an experimental session with n neurons results in an n -dimensional vector representing the ensemble SPN activity during the behavioral segment, which serves as the input to the decoder). We balanced the number of samples for dyskinesia (or *path rot*) and *other N* using a subsampling approach and calculated

the average accuracy over $n = 5000$ repetitions. For training and testing, we used a fraction of 0.8 and 0.2 of the data, respectively. The chance level accuracy for shuffled data was calculated by random permutation of the neuron labels, therefore preserving the average activity information. We note that the goal of this analysis was not to maximize the predictive power of the decoder for specific behavioral clusters, which has been shown to depend on the behavior similarities between behavior clusters.¹⁴ Rather, we wanted to test the hypothesis that SPN ensemble activities carry dyskinesia- and *path rot*-specific information. Due to the sparse activity of the deconvolved SPN signals (i.e., many time periods with zero activity), we did not normalize the activity across the neurons for each segment. However, we performed an additional control measure (data not shown), i.e., normalization of activity to unity for each behavior segment using C_{raw} . This approach gave similar results to the decoding based on deconvolved ensemble activity described above, demonstrating that indeed the ensemble activity and not just the average activity contains dyskinesia and *path rot* specific information.

Calculation of neuronal overlap

To quantify the neuronal overlap between different groups of behavior-related neurons (e.g., axial+limb vs. other N (LD), Figure 4D), we calculated the overlap as:

$$\text{Overlap} = 100 * (\# \text{ of neurons related to both behavioral cluster groups}) / (\# \text{ of neurons related to either behavioral cluster group})$$

Thus, in the case where all neurons are only related to one of the behavior cluster groups but not the other, the overlap is 0%. In the case where all neurons are related to both behavioral cluster groups, the overlap is 100%. Shuffled-corrected measures were obtained by subtracting the average overlap values from 5000 random shuffles where the ensemble pattern was randomized for each of the individual behavior clusters (thus preserving the average activity for each behavior cluster across neurons).

Analysis of SPN activity during non-dyskinetic periods

To assess striatal activity during non-dyskinetic behavior, we quantified SPN dynamics in intact and dyskinetic mice during periods classified as *other N* clusters. Specifically, we compared D1- and D2-SPN activity between intact mice treated with L-DOPA (Int-LD) and lesioned mice treated with L-DOPA (Les-LD). We first quantified the average calcium event rate of all detected D1- and D2-SPNs within the *other N* cluster group for both Int-LD and Les-LD cohorts (Figure S6A). We then determined the proportion of behavior-related neurons within each SPN population (Figure S6B). Finally, we calculated the event rates of these behavior-related neurons separately for D1- and D2-SPNs (Figure S6C). Comparisons revealed that D1-SPNs in Les-LD mice displayed significantly higher event rates than in Int-LD mice, while D2-SPNs exhibited reduced activity in the Les-LD group. The proportion of D1-SPNs modulated during *other N* clusters was similar between groups, but the proportion of modulated D2-SPNs was significantly reduced in Les-LD mice. Among the behavior-related neurons, D1-SPNs in Int-LD showed lower event rates than in Les-LD, whereas the event rate of D2-SPNs did not differ significantly between groups.

Analysis of grooming-related neurons

To evaluate SPN activity associated with grooming, we focused on calcium imaging data recorded during grooming-related behavioral clusters in intact mice treated with L-DOPA (Int-LD). For each mouse, we calculated the average event rate of all recorded D1- and D2-SPNs during the grooming cluster group (Figure S6D). We also determined the proportion of SPNs significantly modulated during grooming episodes - defined as grooming-related neurons - and measured their event rate. Both D1- and D2-SPNs showed similar average activity (~ 0.1 Hz) during grooming. A small proportion of neurons (9–12%) were classified as grooming-related, exhibiting higher activity levels (~ 0.35 Hz) compared to the population average.

Tissue preparation and immunohistochemistry

Twenty minutes after an intraperitoneal injection of VEH or LD (6 mg/kg), mice were rapidly anesthetized with pentobarbital (500 mg/kg, i.p.; Sanofi-Aventis) and perfused transcardially with 4% (w/v) paraformaldehyde in saline phosphate buffer (PBS), pH 7.4. Brains were postfixed overnight in the same solution and stored at 4°C. Thirty micron-thick sections were cut with a Vibratome (Leica vibratome VT1000) and stored at -20°C in a solution containing 30% (v/v) ethylene glycol, 30% (v/v) glycerol, and 0.1 M sodium phosphate buffer, until they were processed for immunohistochemistry. The extent of dopamine denervation was verified in each animal by immunohistochemical staining for TH, and the transfection efficiency of the GCaMP6f-GFP viral constructs was verified using an antibody that recognizes the fluorescent reporter protein GFP, according to the following protocol for double immunofluorescence. **Day 1.** Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.10 M Tris and 0.14 M NaCl, pH 7.4), incubated for 5 min in TBS containing 3% H_2O_2 and 10% methanol, and then rinsed again. After 15 min incubation in TBS containing 0.2% Triton X-100, sections were blocked in serum solution, and then incubated overnight at 4°C in a mixture containing of a rabbit polyclonal α GFP primary antibody (1:1000, GFP Polyclonal Antibody, Alexa Fluor 488, Invitrogen (Molecular Probes) Cat#A-21311), and a rabbit polyclonal α TH antibody (1:1000, Rabbit anti-TH: Peel Freez Biological, Product code: P40101-150). **Day 2.** Sections were rinsed in TBS and incubated for 45 min with the secondary antibody goat Alexa 594-coupled (1:400; Alexa Fluor 594 goat anti-rabbit, Jackson ImmunoResearch Labs Cat#115-585-045). After final rinsing steps, sections were mounted in a polyvinyl alcohol mounting medium

(PVA-DABCO, Sigma Aldrich). Both placement of lens and viral transduction were confirmed using a Zeiss Axio Imager M2 fluorescence microscope.

Quantification of striatal dopamine depletion

Densitometry analysis of TH immunostaining on regions of interest (ROIs) in the dorsal striatum were performed using the free software *NIH ImageJ 1.43*. Images were digitized using a Zeiss Axio Imager M2 microscope connected to a digital camera (Nikon DM1200F). Staining intensities were calibrated on optical density (O.D.) standards provided by the software and the average O.D. in the ROI was calculated after background subtraction. Measurements were carried out in 6 rostro-caudal sections per animal throughout the striatum. Values from the lesion side were expressed as a percentage of the mean of the values from the intact contralateral side. Animals were excluded a posteriori if the striatal TH depletion was lower than 90%.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis and statistics were performed on GraphPad Prism software, MATLAB (MathWorks) and Python. The statistical tests used and the sample size of each analysis is stated in the figure legends. The analysis of variance was performed using ANOVA (one-way or two-ways ANOVA, factorial or by repeated measures, as appropriate) followed by Bonferroni's multiple-comparison test. For two-group comparisons, we used two-tailed Student's t test (paired or unpaired as appropriate) or non-parametric (Mann-Whitney U or Wilcoxon signed-rank test), with Benjamini–Yekutieli procedure for multiple tests *pp*-value correction. We used a bootstrap analysis, where indicated, for the case where we compared our data to shuffled data. Correlations were analyzed using Pearson correlation test, or the Spearman rank-order correlation test. Statistical details of experiments can be found in the results section and figure legends, and in [Table S4](#).