Parkinsonism originates in a discrete secondary and dystonia in a primary motor cortical-basal ganglia subcircuit

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Abstract

Although manifesting contrasting phenotypes, Parkinson’s disease and dystonia originate from similar pathophysiology. Previously, we lesioned a discrete dorsal region in the globus pallidus (rodent equivalent to globus pallidus externa) in rats and produced parkinsonism, while lesioning a nearby ventral hotspot induced dystonia. Presently, we injected anterograde fluorescent-tagged anterograde multi-synaptic tracers into these pallidal hotspots. Viral injections in the Parkinson’s hotspot fluorescent labeled a circumscribed region in the secondary motor cortex, while injections in the dystonia hotspot labeled within the primary motor cortex. Custom probability mapping and N200 staining affirmed the segregation of the cortical territories for Parkinsonism and dystonia to the secondary and primary motor cortices. Intracortical microstimulation localized territories specifically to their respective rostral and caudal microexcitable zones. Parkinsonian features are thus explained by pathological signaling within a secondary motor subcircuit normally responsible for initiation and scaling of movement, while dystonia is explained by abnormal (and excessive) basal ganglia signaling directed at primary motor corticospinal transmission.

Introduction

Parkinson’s disease (PD) is characterized by a paucity, diminutive, and slow movements. In contrast, dystonia is heralded by excessive and sustained, ineffective movements. These dissimilar conditions can originate from similar pathophysiology and commonly co-occur in the same individual. For instance, motor features of PD result from degeneration of substantia nigra pars compacta dopamine (DA) neurons, while dopa-responsive dystonia (DRD) results from genetic defects in DA production. Further, parkinsonism is a frequent feature of DRD, while dystonia commonly occurs in PD. Similarly, strokes in the basal ganglia (BG) can produce isolated or combined symptoms of dystonia and parkinsonism. Additionally, the globus pallidus interna (GPI), the principal BG motor outflow nucleus, is commonly targeted via deep brain stimulation (DBS) to treat both disorders.

A deficiency of DA causes disinhibition of GABAergic DA D2 inhibitory striatal neurons, which, in turn, is expected to silence GP externa (GPe) neurons. Previously, to elucidate the behavioral effects of silencing GPe neurons, we injected the neurotoxin ibotenate focally into the posterolateral motor territory of GP (rodent equivalent of GPe) in rats. Consistent with the clinical observations in humans and as predicted by the classical basal ganglia model, large ibotenate lesions in the motor territory of GP induced both parkinsonism and dystonia. More highly restricted lesions, in turn, separately induced these two clinical features. A highly circumscribed dorsal hotspot lesion produced pure characteristic parkinsonian features, including severe immobility and contralateral forelimb flexion posturing, while a focal ventral hotspot lesion induced isolated, severe contralateral and truncal dystonic extension posturing, with characteristic EMG dystonic co-contraction activity. In ibotenate-lesioned dystonic rats, the downstream abnormal neuronal discharge rates and patterned activity in the entopeduncular nucleus (EP; rodent equivalent of Gpi) were indistinguishable from those recorded in jaundiced kernicterus dystonic
rats. The EP neuronal activity in parkinsonian lesioned rats was typical of that in humans with PD, including high discharge rates, prominent burst and 4–6 Hz oscillatory activity. The projections from GP to EP and GPe to GPi innervate topographically equivalent discrete regions of these nuclei. Therefore, to further address the translatability of these findings to humans, we scrutinized our blinded post-operative selection of effective GPi DBS contacts (4 metal contacts, 1.5 mm in length, with 0.5 mm separation) in PD and dystonia patients, typically placed with the lowest contact at the bottom and the 3rd contact near the top of GPi. As predicted from our rodent studies, the effective DBS contact for treating PD was located in the dorsal posterolateral motor territory of GPi and for dystonia was located more ventrally.

The BG have for a long time been considered to be organized into distinct reentrant motor, associative, and limbic BG thalamocortical (BGTC) anatomical circuits and subcircuits. Our previous findings provided compelling evidence that two contrasting movement conditions originate from equivalent pathophysiological disturbances originating along two different BGTC motor subcircuits. Based on the reported locations of GPi labeling from multi-synaptic retrograde herpes viral tracer injections in different motor cortical territories in primates, we hypothesized that the dorsal Parkinson's GPe and GPi territories contribute to a BG-supplementary motor area (SMA) subcircuit and the ventral dystonia territories to a BG-primary motor subcircuit. These proposed distinct cortical territorial contributions to PD and to dystonia are consistent with a majority of (though not all) functional imaging studies.

Further, we reasoned that resultant higher order motor signaling disturbances in the SMA could account for the characteristic poor initiation and abnormally scaled features of parkinsonism, while excessive, poorly regulated activation of primary motor cortex projections to the spinal cord could explain dystonic motor features.

Results

Multisynaptic tracer injections in the parkinsonian and dystonia hotspots in GP

To test our predictions about which cortical regions chiefly contribute to parkinsonism and dystonia, we injected fluorescent tagged recombinant vesicular stomatitis virus (rVSV) into the two previously defined corresponding GP hotspots in rats and traced the labeling to the level of the cortex. Replication competent VSV with its native G protein (VSV-G) has been shown to multi-synaptically spread, predominately via anterograde transport. A total of 36 Long Evans rats (22 females and 14 males, 8–40 (mean = 17.6 ± 10) weeks old, 200–700 (mean = 379.6 ± 160.4) g) were used for these studies. Using microelectrode extracellular recording guidance, rVSV was stereotactically administered unilaterally targeting either the previously defined dorsal parkinsonian (L3.6, P1.6, D4.0) or ventral dystonia (L3.5, P1.6, D2.9) GP hotspot (Fig. 1a). Refer to Table 1 for individual injection details.
Table 1
Details for individual rats injected with fluorescent labeled rVSV

<table>
<thead>
<tr>
<th>Rat ID</th>
<th>Sex</th>
<th>Age (wks)</th>
<th>Wgt (g)</th>
<th>GP injection site</th>
<th>Vol (ul)</th>
<th>Incubation period (days)</th>
<th>Remarks</th>
<th>Cortical projections</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>16</td>
<td>216</td>
<td>ventral</td>
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<td>1</td>
<td>short incubation</td>
<td>-</td>
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<tr>
<td>2</td>
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<td>16</td>
<td>213</td>
<td>ventral</td>
<td>1.0</td>
<td>2</td>
<td>short incubation</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>13</td>
<td>192</td>
<td>ventral</td>
<td>0.4</td>
<td>1</td>
<td>short incubation</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>9</td>
<td>224</td>
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<td>0.6</td>
<td>4</td>
<td>M1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F</td>
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<td>315</td>
<td>ventral</td>
<td>0.6</td>
<td>2</td>
<td>short incubation</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>11</td>
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<td>ventral</td>
<td>0.5</td>
<td>2</td>
<td>short incubation</td>
<td>-</td>
</tr>
<tr>
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<td>F</td>
<td>15</td>
<td>249</td>
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<td>0.5</td>
<td>4</td>
<td>lateral M1</td>
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<tr>
<td>8*</td>
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<td>19</td>
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<td>dorsal</td>
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<td>-</td>
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<tr>
<td>9</td>
<td>M</td>
<td>20</td>
<td>712</td>
<td>dorsal</td>
<td>0.8</td>
<td>4</td>
<td>severe parkinsonism M2</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>22</td>
<td>667</td>
<td>dorsal</td>
<td>0.6</td>
<td>4</td>
<td>processing issues</td>
<td>-</td>
</tr>
<tr>
<td>11^</td>
<td>M</td>
<td>23</td>
<td>671</td>
<td>ventral, light dorsal</td>
<td>0.8</td>
<td>4</td>
<td>severe dystonia M1, M2</td>
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</tr>
<tr>
<td>12</td>
<td>M</td>
<td>28</td>
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<td>-</td>
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<td>0.5</td>
<td>4</td>
<td>M2, M1</td>
<td></td>
</tr>
</tbody>
</table>

* Parkinsonian rat shown in Fig. 1B1

^ Dystonic rat shown in Fig. 1B2

+ In animals with prolonged incubations beyond 4 days, the cortical fluorescent labeling consistently advanced extensively beyond the primary cortical projection sites.

d 1/ 5th, d1 1/10th viral dilution
<table>
<thead>
<tr>
<th>Rat ID</th>
<th>Sex</th>
<th>Age (wks)</th>
<th>Wgt (g)</th>
<th>GP injection site</th>
<th>Vol (ul)</th>
<th>Incubation period (days)</th>
<th>Remarks</th>
<th>Cortical projections</th>
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<td>4</td>
<td></td>
<td>M1</td>
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<td>4</td>
<td></td>
<td>M2, M1</td>
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<tr>
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<td>4</td>
<td></td>
<td>M2</td>
</tr>
<tr>
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<td>M</td>
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<td>4</td>
<td></td>
<td>M2</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>11</td>
<td>436</td>
<td>dorsal and ventral</td>
<td>0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4</td>
<td></td>
<td>M1, M2</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>12</td>
<td>420</td>
<td>ventral</td>
<td>0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2</td>
<td>short incubation</td>
<td></td>
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<tr>
<td>22</td>
<td>F</td>
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<td>415</td>
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<td>short incubation</td>
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<tr>
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<td>3</td>
<td>short incubation</td>
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<tr>
<td>25</td>
<td>F</td>
<td>8</td>
<td>265</td>
<td>NA</td>
<td>0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>Surgical complications, no perfusion</td>
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<tr>
<td>26</td>
<td>F</td>
<td>9</td>
<td>269</td>
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<td>extensive labeling+ M2, M1</td>
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<tr>
<td>27</td>
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<td>284</td>
<td>dorsal and ventral</td>
<td>0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6</td>
<td>extensive labeling M1, M2</td>
<td></td>
</tr>
</tbody>
</table>

* Parkinsonian rat shown in Fig. 1B1

^ Dystonic rat shown in Fig. 1B2

+ In animals with prolonged incubations beyond 4 days, the cortical fluorescent labeling consistently advanced extensively beyond the primary cortical projection sites.

<sup>d</sup> 1/5th, <sup>d1</sup> 1/10th viral dilution
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<th>Rat ID</th>
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<th>Vol (ul)</th>
<th>Incubation period (days)</th>
<th>Remarks</th>
<th>Cortical projections</th>
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</thead>
<tbody>
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<td>10</td>
<td>264</td>
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<td>12</td>
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<td>7</td>
<td>extensive labeling</td>
<td>M1, M2</td>
</tr>
<tr>
<td>31</td>
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<td>21</td>
<td>712</td>
<td>ventral</td>
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<td>4</td>
<td>processing issues</td>
<td>none</td>
</tr>
<tr>
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<td>F</td>
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<td></td>
<td>M2</td>
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<tr>
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<td>M</td>
<td>23</td>
<td>620</td>
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<td>0.25</td>
<td>4</td>
<td>Excessive leakage</td>
<td>M2</td>
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<td>M1</td>
</tr>
<tr>
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<td>4</td>
<td>caudal M2, M1</td>
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<tr>
<td>36</td>
<td>M</td>
<td>19</td>
<td>318</td>
<td>dorsal</td>
<td>0.4</td>
<td>4</td>
<td>M2</td>
<td></td>
</tr>
</tbody>
</table>

Grey shaded brains used for GP-cortical correlation

* Parkinsonian rat shown in Fig. 1B1

^ Dystonic rat shown in Fig. 1B2

+ In animals with prolonged incubations beyond 4 days, the cortical fluorescent labeling consistently advanced extensively beyond the primary cortical projection sites.

\[ d \] 1/ 5th, \[ d1 \] 1/10th viral dilution

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Select VSV-induced motor features affirm accurate delivery of viral constructs to the targeted motor subcircuit

After several days, VSV begins to be lethal to infected cells \(^{28,29}\). Thus, after a delay, we expected to observe behavioral changes coincident with VSV-induced cytotoxicity (silencing) of GP hotspot neurons at the injection site. Among rats receiving a larger volume of viral construct (0.75–0.9 µl), moderate to severe parkinsonism (rats #8, 9, 23) or dystonia (rats #11, 14) developed when sufficient survival times were permitted. The classical movement disorder features were preceded on Day 2–3 by the onset of a turning tendency ipsilateral to the injection side. The movement disorder features developed rapidly on Day 4 (Fig. 1b1 (rat #8) and b2 (rat #11)) and were indistinguishable from that induced previously with ibotenate \(^{17}\) (Fig. 1c1 and c2) and closely resembled the human conditions (Fig. 1d1 and d2). Dorsal hotspot rVSV injections induced parkinsonism characterized by a paucity of spontaneous generalized...
movement, with a highly suppressed response to audio and tactile stimuli. The contralateral forelimb was held in a flexed posture and infrequently utilized. Ventral hotspot injections induced prominent neck and truncal twisting, and contralateral forelimb and hindlimb extensions. These animals showed normal spontaneous activity, though exhibited regular falls due to the dystonic posturing. See Fig. 1e1 and e2 for scoring of parkinsonian and dystonia features. For additional details, refer to our previously published videos for comparable illustrative behavioral features. The onset of the parkinsonian or dystonic features coincided with the timing (on Day 4) of delayed local viral-induced GP neuronal degeneration, as seen on silver-stained sections (not shown). The reproducibility of the isolated clinical syndromes confirmed both the overall accuracy and discreteness of the rVSV injections. The largely successful, discrete targeting of the intended GP hotspots was additionally supported by chiefly isolated fluorescent transsynaptic cortical labeling (Fig. 2).

Subsequently, to limit the severity of the motor features and to better restrict the local spread of the viral labeling, the viral dosage was limited to 0.2–0.4 ul and/or diluted (1/5th or 1/10th dilution using Dulbecco's PBS) in the last 13 animals. At these lower viral dosages, the animals self-maintained their body weights and when permitted to survive for 4 days (rats # 26–36) consistently displayed similar but milder parkinsonian or dystonic motor features. At the lower dosages, the consistent induction of mild behavioral features were sufficient to affirm the accuracy of each injection. Although the lower dosages permitted us to keep animals for extended periods, we quickly realized that maintaining animals beyond 4 days led to confounding local cortical spread of the virus beyond the primary targeted cortical regions. As such, we restricted the subsequent reconstruction of the primary cortical projections to 4-days post-injection (DPI) rats (perfused on day 4). Fortuitously, on day 4, the virus consistently reaches the cortex from GP at approximately the same time that it induces prominent degeneration of GP neurons and the resultant induction of the behavioral phenotype. The 4 DPI timing of transsynaptic cortical labeling was consistent with reports of VSV transit of one synapse per day.

**Focal GP hotspot injections label distinct cortical motor territories**

Post-mortem sections were imaged using a Keyence digital microscope and aligned to Paxinos & Watson rat brain atlas sections (Supplemental Fig. 1b). The coordinates of the cortical projections were then digitally registered (Supplemental Fig. 1c). Of 22 4-DPI rats, 17 (n = 10 predominant dorsal, n = 6 ventral, n = 1 dorsal and ventral GP hotspot injections) were used for the labeling reconstructions (Table 1). The additional five 4-DPI rats were excluded due to missed target, poor perfusion, processing issues or excessive leakage. Among the 17 reconstructed injections, all injections leaked to varying extents into the nearby unintended hotspot with one injection evenly encompassing both hotspots. The injections also all showed varying degrees of leakage into the traversed striatum, reticular thalamus, and substantia innominata. Most injections produced dense cortical neuronal labeling, with 3 injections (rat # 20, 27, 32) producing more modest labeling due to limited transfection of GP neurons. The dorsal (parkinsonian) GP injections were observed to project predominately to secondary motor cortex (M2; Fig. 2b1a and b1b). In
contrast, ventral (dystonia) injections predominately projected to primary motor cortex (M1; Fig. 2b2a and b2b).

**Probability mapping of the parkinsonian and dystonia cortical territories**

The 17 reconstructed injections were next scrutinized in detail and subjected to statistical verification of their GP-cortical relationship. First, tetrachoric correlation was used to grossly support the anatomical relations between the cortical labeling and the two principal injection sites (step 1). Upon supporting the relations, a custom probability algorithm was implemented to delineate cortical voxels which have strong and consistent associations with either of the two GP hotspots (step 2). Next, after filtering out poorly correlated voxels, spatial autocorrelation (global Moran I) and Getis-Ord GI hotspot analysis were used to establish whether the GP-cortical projections are both significantly clustered (step 3). Finally, independent t-test on the filtered cortical voxels was used to confirm that the dorsal versus ventral GP hotspots project to two non-overlapping cortical regions (step 4). Details of the results for each step are as follows:

1. **Tetrachoric matrix supports anatomical relations between the cortical labeling and the two principal injection sites**

   Upon deriving the impression that the cortical labeling from the dorsal GP hotspot injections was projecting predominately to M2 and that from ventral GP injections to M1, a tetrachoric correlation was calculated to objectively assess the strength of the correlations between the binary injection locations (dorsal and ventral GP) and the primary projection locations (M2 and M1). Table 2 indicates the tetrachoric matrix correlation values with percentage of M1-M2 digitized projections paired with each GP injection sites (details in the methods section). The correlation coefficient of the matrix was −0.818, indicating a strong relation between the individual GP injection sites and the specific M1 versus M2 cortical labeling. The negative value indicates a correlation of the ventral GP site with M1 and the dorsal site with M2.

<table>
<thead>
<tr>
<th>Dorsal GP injection</th>
<th>Ventral GP injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 labeling</td>
<td>11.17%</td>
</tr>
<tr>
<td>M2 labeling</td>
<td>68.13%</td>
</tr>
<tr>
<td>M1 labeling</td>
<td>88.83%</td>
</tr>
<tr>
<td>M2 labeling</td>
<td>31.87%</td>
</tr>
</tbody>
</table>

2. **Probability mapping to delineate cortical voxels with strong and consistent associations with either of the two GP hotspots**
Next, towards defining the separate cortical parkinsonian and dystonia territories, we derived a novel 3D spatial custom GP-cortical correlation algorithm. Refer to online Methods, Projection probability mapping, for methodological details. Briefly, the distribution of VSV within and surrounding GP was established based on visual inspection of fluorescent labeling and/or silver-staining (FD NeuroSilver kit II) of damaged neurons and scored (from 0 = none to 10 = complete labeling) for the dorsal and ventral GP hotspots. The cortical region of interest (ROI) was then defined to encompass M1 and M2 and surrounding regions and segmented into a 0.1 x 0.1 x 0.1 mm$^3$ voxel grid. From 3D cortical labeling plots, the algorithm generated individual voxel scores (0–10) for extent of labeling, accounting for immediate and/or surrounding voxel labeling. For each voxel, median scores were generated across 17 animals. Cortical voxels were then defined as being related or not related to the dorsal or ventral hotspots based on the following criteria: i. a high median score ($\geq$ threshold Th1, defined below), ii. low variability (standard deviation $\leq$ Th2), and ii. repeat labeling ($\geq$ Th3). Using stringent criteria, thresholds were set as follows: Th1 = 6, Th2 = 1, Th3 = 3 rats to accommodate 70% active (non-zero) data points in the region of interest. The distribution of these probability maps related to dorsal and ventral GP hotspots were then compared. To additionally establish a relaxed probability map, more moderate criteria were set as follows: Th1 = 5, Th2 = 2, Th3 = 3. For both the strict and relaxed criteria, voxels that satisfy the set criteria were then plotted in a final 3D realization representing cortical probability maps (2D realization are presented in Figs. 3 and 4, left column).

3. Spatial autocorrelation and hotspot analysis establish that both GP-cortical projections are significantly clustered

Global Moran’s I (GMI) function values for the M1 (GMI = +0.453) and M2 (GMI = +0.585) territories derived from the probability mapping indicate significant spatial autocorrelations, both $p < 0.05$ (i.e., less than a 5% probability that the distinct clusters resulted randomly). Getis-Ord Gi* statistics (z-scores) modified for 3D data were calculated providing a statistical hotspot analysis using the local pattern of spatial association to identify local spatial clusters with high median scores (Figs. 3 and 4, middle columns). For statistically significant positive z-values (Gi*; z-score $> 1.96$ ($p < 0.05$)), the larger the z-values, the more intense is the clustering of the cortical labeling. The Getis-Ord distribution maps reveal the progressive inwards confidence for the defined parkinsonian M2 and dystonia M1 central hotspot regions. Subsequently, the probability distribution maps generated in step 2 (Figs. 3 and 4, left column) were filtered to include only significant z-scores (Gi*). The resultant final distribution maps define the statistically significant cortical projects (Figs. 3 and 4, right column). The final maps define two distinct cortical territories, one contributing to a dorsal BG-rostral cortical (M2) parkinsonian subcircuit (center of mass (COM) for the stringent protocol: L2.07, A3.08 D3; Fig. 3 right column and Fig. 6a) and one to a ventral BG-caudal cortical (M1) dystonia subcircuit (COM: L2.12, A-0.14, D1.90; Fig. 4, right column, and Fig. 6a).

4. Independent t-test on the filtered cortical voxels confirms that the dorsal and ventral GP hotspots project to two non-overlapping cortical regions
Finally, to verify that the two hotspots are spatially significantly different from each other, the distributions (distance from bregma) of the two hotspot coordinates were compared using independent sample test. The test rejected the null hypothesis that the two hotspot coordinate distributions are statistically at the same location ($p < 0.005$).

**N200 staining and ICMS testing affirm that the parkinsonian subcircuit is confined to the microexcitable M2 motor territory.**

Additionally, to better define the borders of M2 and thereby assure that the dorsal GP hotspot subcircuit does not encroach into the cingulate cortex rostrally or into M1 posteriorly, we stained representative brains ($n = 2$) with N200 $^{32,33}$ (see Methods for details). The N200 staining affirmed that the cortical fluorescent labeling from the dorsal GP hotspot injections was confined to M2 (Fig. 5a). Although the existence of an SMA equivalent in rodents remains controversial, the motor responses evoked by electrical stimulation in the microexcitable zone in M2 $^{34}$ and the direct physiological influences of the M2 microexcitable area on M1 $^{34}$ closely resemble that of SMA. Thus, to better define the M2 parkinsonian territory and its potential relation to the human SMA, as well as to better define the M1 dystonia cortical hotspot, we also performed intracortical micro-stimulation (ICMS) in additional rats ($n = 6$). These studies indeed revealed the parkinsonian cortical territory to lie chiefly within the M2 microexcitable zone. More specifically, it was found to be localized to the (proximal and distal) forelimb area, encroaching on the more lateral jaw area (Fig. 5b). Surprisingly, as for other groups, hindlimb responsive neurons were not identified in M2 with ICMS $^{34}$. The dystonia cortical hotspot encompassed the M1 microexcitable forelimb area, as well as potentially the hindlimb area.

**Discussion**

In the current study, we focally injected multi-synaptic anterograde tracers into the rodent equivalent of the GPe and delineated the associated cortical territories of two of the most common disorders, parkinsonism and dystonia. We demonstrated that parkinsonism originates along a circumscribed dorsal BG-secondary motor cortex subcircuit, while dystonia originates along a ventral BG-primary motor cortex subcircuit (Fig. 6b). Further, we showed that parkinsonian hypokinetic and, opposing, dystonic hyperkinetic features originate from equivalent disturbances, with their differences accounted for by their involvement of two distinct BGTC motor subcircuits. In the case of dystonia, the pathological motor subcircuit directly involves (abnormally activating) the primary motor subcircuit, while in parkinsonism, the hypokinetic motor features are accounted for by interrupting normal signaling in a higher order secondary motor subcircuit.

By demonstrating that two highly different movement conditions originate from disturbances in specific motor subcircuits, our present findings underscore the distinct contributions of the brain's motor subdivisions to normal and, as investigated here, to pathological motor behavior. Our findings of a dorsal
posterolateral GP (parkinsonian)-M2 circuit and a ventral posterolateral GP (dystonia)-M1 circuit in rodents are anatomically consistent with findings from tracer and electrophysiology investigations in normal primates. Further, evidence for the contribution of segregated BGTC circuits to functionally distinct normal and pathological behaviors are widely reported. Deficits in specific motor, associative, and limbic loops have, for example, been shown to differentially induce dyskinesia, attention deficits and stereotypy, respectively. Moreover, subdivisions of the motor circuit have been reported to differentially contribute to distinct motor actions, like licking and turning. More directly relevant to our study findings, upon demonstrating anatomically segregated BGTC motor subcircuits, Hoover and Strick suggested that these subcircuits are likely to contribute differentially to the various motor manifestations of PD, including rigidity, tremor, and akinesia. In support of this hypothesis, our current studies parceled out a dorsal BG-M2 motor subcircuit accounting for akinesia and flexed posturing (classical features of PD) and a ventral BG-M1 subcircuit accounting for dystonic motor features (a common feature in PD).

Our observations of a segregated akinetic parkinsonian BG subcircuit involving M2 and a dystonia subcircuit involving M1 are largely supported by prior studies. By injecting herpes simplex retrograde multi-synaptic tracers into distinct arm regions of the frontal cortex in primates, Hoover and Strick demonstrated the arm representation of SMA, primary motor, and ventral premotor areas discretely connected to dorsal, middle, and most ventral posterolateral territories of GPI, respectively. Our findings in experimental rats of posterolateral dorsal BG-M2 (parkinsonian) and centro-ventral BG-M1 (dystonia) circuits are anatomically consistent with these findings. Our previously reported observations that the effective DBS contact for treating PD was located in the dorsal posterolateral motor territory of GPI and for dystonia was located more ventrally further supports the contribution of this dorsal-ventral segregated circuitry contributions to parkinsonism and dystonia and supports the conservation of this arrangement across species. Single neuron thalamocortical projection electron microscope reconstructions by Kaneko and colleagues reveal an analogous maintenance of segregated pallidal-receiving subcircuits in the rostro-medial VA-VL thalamic complex, with dorsal pallidal-receiving VL neurons projecting prominently to M2 and ventral pallidal-receiving VL neurons projecting predominately to M1. The authors further demonstrated that pallidal-receiving neurons in rostral MV project predominately project to M2, while pallidal-receiving neurons in the caudal VM project predominately to M1.

Our analyses revealed that focal injections in the basal ganglia produced highly clustered and, for the most part, confined cortical projection labeling. Our anatomical studies supported the presence of predominately segregated BGTC motor subcircuits with minimal divergence of the transmitted BG signals on route to their primary cortical targets. Although the final cortical hotspots in M1 and M2 were found to be largely non-overlapping, a small percentage of projections were observed to project more diffusely across regions of premotor and primary motor cortex. Although we suggest this represents true relatively minor overlap in the projections, our methodology cannot confidently exclude the presence of modest cortical-cortical tracer spread. We also cannot conclude that our analysis methodology was able to fully
account for potential overlap in the targeted hotspot injections. In support of there being incomplete segregation of the motor subcircuits, Kaneko et al. reported mild divergence of M1 and M2 predominate thalamocortical projections in their VL-VM single neuronal projection reconstruction studies. Although the combined evidence supports that motor information processing is delegated to largely distinct BGTC motor subcircuits, there therefore is likely to be a small level of crosstalk between across these parallel subcircuits. In depth electrophysiological investigations are required to better understand the contribution of divergent (albeit, relatively minor) signaling pathways. Further, using neural tracing and immunostaining, Karube and colleagues observed that M1 provides direct cortical innervation predominately to dorsal arkypallidal GP-STR projecting neurons, while M2 predominately targets ventral arkypallidal GP neurons. While requiring replication, this study suggests a means for the otherwise predominately segregated motor networks to more strongly influence, conceivably in a feedback manner, other subcircuits at the level of the basal ganglia. This potentially important finding however requires replication in rodents, as well as in primates.

Our demonstration that N200 only sparsely labeled layers 3–5 neurons in the delineated parkinsonian cortical territory affirmed its localization to M2, while our ICMS mapping revealed this territory to be more specifically chiefly localized to the M2 forelimb area. Although an SMA territory has not been previously defined in rats, the dorsal postrolateral localization of the parkinsonian territory in GP and its localization to the rostral forearm microexcitable zone strongly implicate the identified parkinsonian M2 territory to represent the rodent equivalent to SMA. Based on anatomical trace and cortical stimulation investigations in primates, the BGTC SMA forearm reentrant subcircuit localizes to mid-depth regions of the posterolateral GPi, located between the dorsal primary motor and the ventral premotor forearm territories. This defined mid-depth posterolateral localization of the SMA subcircuit corresponds to the localization of the immediate upstream hotspot in GP in which we previously induced parkinsonism in rats via ibotenate lesions and induced here via viral-induced neuronal toxicity. Also, as we have discussed, the mid-depth localization of the hotspot in the posterolateral GP for inducing parkinsonism corresponds to the equivalent localized anatomical site in GPi for reversing parkinsonism in PD patients via DBS. In additional support of our contention that the defined M2 parkinsonian cortical territory represents the primate SMA, the SMA, in difference from other premotor areas, provides substantial direct projections to the spinal cord and, as such, represents a rostral electrically microexcitable cortical motor area (as observed in our rats). Furthermore, in primates, lateral premotor areas have been shown to largely control distal forelimb movements. In contrast, SMA neurons, like primary motor area neurons, strongly signal both proximal and distal movements. Not only were the fluorescent labeled M2 parkinsonian and M1 dystonia territories in our rats localized to their respective forelimb microexcitable zones, but they both similarly induced both proximal and distal forelimb movements to relatively low threshold layer 5 directed electrical stimulation.

For our anatomical tracing studies, we chose to use a multi-synaptic viral construct from the Rhabdoviridae family developed by Beirer et al. The direction of the viral propagation can be controlled by the selection of a specific glycoprotein. As chosen for our studies, VSV with native G protein
(VSV-G) reliably spreads preferentially in the anterograde direction. Even with meticulous stereotactic tracer delivery, a degree of unavoidable leakage and spread of the tracer constructs occurs outside of the targeted regions and complicates the assessment of the labeled projection circuitry. Unfortunately, most published anatomical studies neither report nor account for this critical leakage and spread. In the present study, the two injection targets were relatively small, circumscribed regions that are vertically separated by only ~ 0.3 mm. Thus, it was especially important here to incorporate methodology that would reliably allow us to accurately delineate the cortical labeling originating specifically from the targeted parkinsonian and dystonia-related GP sites. Because we were unable to adequately prior methodology to account for unavoidable tracer leakage and imprecisions in targeting, we developed our own probability modeling algorithm. To complement the dependability of our algorithms, we also varied the trajectorial approach in different experiments. This served to avoid regular leaking into the same regions and thereby, enhanced our ability to delineate contributions of unintended tracer injections into both transversed sites and extension beyond the intended injection sites. We are confident that the algorithm output accurately delineated cortical voxels that correspond to the corresponding subcircuitry involvement of the targeted dorsal versus ventral GPe hotspot. Further, by defining high z-scores for the clustered cortical projections, we are confident that the Getis-id clustering hotspot analysis effectively masked any cortical labeling originating from leakage or spread. Although, we believe that our algorithm was robust enough to yield reliable results, future electrophysiological and neuromodulation investigations will be important to confirm these findings.

We propose that in people with PD, pathological disinhibition of striatal neurons from deficient DAergic input within the SMA-BG subcircuitry induces silencing of dorsal SMA circuitry GPe neurons, which ultimately causes pathological SMA-corticospinal and SMA-primary motor-cortical spinal signaling. Because the SMA normally contributes to the executive control of movement \cite{50,51}, pathological BG-induced SMA executive motor signaling leads to the classical poor initiation and abnormally scaled movement features in PD. In contrast, per our modeling comparable pathophysiological BG signaling along the M1 subcircuit produces excessive and poorly regulated thalamocortical activation of M1, leading to the characteristic excessive dystonic co-contractions with spread of muscle activations to unintended joints. Consistent with our present findings, functional cortical imaging studies in humans with PD have demonstrated abnormal cortical activation principally in the SMA \cite{23,24,52,53}, while investigations in dystonic subjects have, in contrast, suggested principal disturbances in primary motor cortex, though also variably implicated prefrontal motor regions \cite{54–56}. In further support of the translational implications of our findings to humans, Magno and colleagues \cite{48} reported that optogenetic stimulation of ICMS defined M2 pyramidal neurons ameliorated parkinsonian features in DA depleted mice.

In summary, we have provided experimental evidence that parkinsonism and dystonia, two of the most common movement disorders originate similarly, but along two distinct motor subcircuits. These two opposing conditions, one heralded by a paucity of movement and the other by motor hyperactivity, differ due to their involvement of distinct motor subcircuits. This is in major conflict with the longstanding
The classical basal ganglia model which suggested that these conditions originate due to opposing signaling along the same pathways. Although investigators and clinicians in the movement disorders field have for a long time recognized a need to rectify the model due to its many shortcomings, the critical contributions of the separate motor subcircuits to specific movement disorder conditions have not been previously justly recognized. The present findings and proposed novel modeling have not only major implications for PD and dystonia, but also, more generally, for understanding a wide variety of other movement disorders. Precise definition of the relevant pathological motor subcircuits could lead to novel superficial, and potentially non-invasive, targeted stimulation therapeutic approaches to the multitudes of disabling movement disorders.

**Methods**

**Animals and scientific rigor**

All experiments were approved and monitored by the Institutional Animal Care and Use Committee of the Hunter Holmes McGuire Veterans Affairs Medical Center and performed in accordance with regulatory guidelines. Long Evans wild-type rats were used for this study. Animals were initially obtained from Charles River, MA, USA and maintained as an in-house breeding colony in the McGuire Research Institute's animal facility. The rats were housed on 12-h light/ 12-h dark cycle with food and water ad libitum. Animals were housed in groups of 2 or 3 per cage before procedures and were single-housed post-surgery.

All experiments were conducted in strict accordance with the ARRIVE guidelines. To increase scientific rigor, rats were randomly chosen for the experiments, irrespective of the strain, sex, age, and weight. A total of 22 females and 14 males were used, age range: 8–48 weeks old and weights: 200–720 g at the time of stereotactic injection of the tracers. See Table 1 for details of the rats and stereotactic injections. All data were reviewed by multiple team members to ensure its validity and to minimize operator biases.

**Viral vector**

All anterograde transsynaptic tracing experiments utilized vesicular stomatitis virus (VSV) native glycoprotein (G) VSV-Venus [VSV-G VSV-Venus] Replication Competent vector (7.56E + 10 TU/ml), obtained from the Salk Institute Viral Vector Core and aliquots (5 µl) were stored at -80°C. Center of Disease Control guidelines for viral handling were strictly followed.

**Surgery and target localization**

The stereotactic injection surgeries were carried out under 2–4% isoflurane anesthesia (with 1 L/min oxygen) using sterile techniques. Adequate depth of sedation was assured by regularly assessing for responses to toe pinch. Ophthalmic ointment was applied to protect the eyes during surgery. The rat's body temperature was monitored and maintained via rectal temperature probe and a feedback-controlled heating pad. Respiration and heart rate were monitored at regular intervals. The fur on the rat's head was clipped. Then the position of the rat's head was secured with a bite bar, non-rupturing ear bars, and the
stereotactic device (KOPF). After disinfection with betadine, an incision was made on the top of the head and the overlying tissue was scraped to expose the skull. To ensure proper targeting, the pitch of the rat’s head was measured using a dial indicator and adjusted to make the head level. A 2–5 mm burr hole was made with a drill bit in the skull centered above the targeted GP (L3.3mm, AP -1.6mm referenced to bregma). Prior to viral tracer injection, the location of the GP dystonia or parkinsonian hotspot target was confirmed via stereotaxic microelectrode recording (MER), typically over 2–3 penetrations, utilizing ultrafine 100µm heptodes (Thomas Recording, GmbH) mounted in a 7-heptode capacity Eckhorn manipulator (Thomas Recording) attached to the KOPF stereotaxic system. Refer to our prior publication 57 for additional details of the mapping procedures.

**Tracer injection**

Prior to initiating MER, a glass micropipette (80 µm I.D., Thomas Recording) was secured in one of the heptode positions in the Eckhorn manipulator and connected via Tygon flexible tubing (0.25 mm I.D.) to a Hamilton syringe mounted in a syringe pump. To assure the administration of the viral load, 2–4 µL of Dulbecco’s phosphate buffered saline (dPBS) was drawn first into the micropipette with a small air bubble (~ 2 mm). dPBS was used, rather than water or regular PBS, to minimize the risk of hydrocephalus. Also, some of the utilized tracer agents are prepared in Dulbecco’s PBS from the producer. Next, fluorescent tagged VSV tracer solution (0.2–1 µL) was drawn into the micropipette. After confirming the precise location of the intended target using MER, the tracer was injected at a rate of 100–200 nL/min while monitoring the movement of the air bubble. To minimize leakage of tracer on withdrawal of the micropipette, the system was left in place for an additional 10 min prior to retracting.

After completion of a tracer injection, the burr hole was sealed with bone wax and the incision was sutured closed. Bupivacaine was injected into the skin around the incision and buprenorphine (0.25–1.6 mg/kg, i.p.) was administered prior to discontinuing the isoflurane. Subsequently, rats were placed in a clean cage and allowed 24 hours for recovery.

**Behavioral assessment**

Successful discrete targeting of injections into either the dorsal or ventral GP hotspots was affirmed by induction of selective motor conditions, parkinsonism or dystonia, respectively, upon delayed local viral induced neuronal degeneration. The health status and motor behavior of rats were assessed at least twice daily following injections. Health status monitoring included weights, pain and stress indicators, including vocalization, posture, grooming, porphyrin staining, and suture condition. Motor behavior assessments included recording of the level and direction of any turning tendency. Parkinsonian and dystonic symptoms were quantified based on previously reported rating scales [Appendix B of 17]. Briefly, parkinsonian features were scored from 0–4 (normal to extreme) for (1) generalized spontaneous and (2) stimulation-induced movement and for (3) contralateral hypokinesia-flexion posturing. Dystonic features were scored for (1) contralateral fore- and hindlimb extension, (2) midline posturing, and (3) dystonia-related falls (producing total parkinsonian and dystonia scores each of 0–12). For features presenting midway in severity between one category and the next higher one, an additional 0.5 was added.
Histology and imaging

After 1–7 days of viral incubation, the rats were sacrificed with an overdose of pentobarbital and transcardiac perfusion via the left ventricle, with saline (200mL), followed by 4% formaldehyde solution (300mL). The brains were extracted and initially placed in 4% formaldehyde overnight for post-fixation. The brains then were placed in 30% sucrose in PBS solution until the brains sank (~ 2 days) for cryopreservation. The cerebrum was vertically bisected along the interhemispheric fissure. The injected side of the cerebral hemispheres were then molded in Tissue-Tek OCT (Sakura). Using a Leica CM1850 cryostat, the brains were then sliced into sagittal sections (50 µm), typically saving every third section from approximately lat. 0.5 to 4.0 mm for a total of 24 sections and floated in PBS. The sections were next mounted on slides (Fisherbrand Superfrost Plus) and stained with fluorescent nuclear stain 4′,6-diamidino-2-phenylindole (DAPI).

In the earliest experiments, prior to the acquisition of a microscopy capable of directly imaging yellow fluorescent proteins (YFP), fluorescence was enhanced using standard immuno-fluorescent techniques. Briefly, after mounting on slides, tissue were placed in acetone for antigen retrieval, blocked with normal goat serum, then treated with rabbit/ anti-GFP primary antibody. After incubation, rinsing, and additional blocking, the tissue were treated with goat anti-rabbit secondary antibody conjugated with Alexa 488 fluorescent dye for visualization with standard GFP filter sets.

In most cases, the brain sections were imaged with a Keyence BZ-X800 digital microscope. The slides were scanned with 4x objective lens (Nikon PlanFluor DL 4x 0.13/16.50mm PhL) in two fluorescence channels: EYFP; DAPI (CHROMA 49003-UF1-ET-EYFP, 49000-UF1-ET-DAPI), and in phase contrast visible light. For each channel, each slide was scanned as a 231-image mosaic composed of 960x720 pixel 8-bit monochrome images; 21 columns x 11 rows with approximately 15% overlap. The Keyence image stitching feature was then employed to produce a single large 14405 x 5768-pixel image for each channel (Supplemental Fig. 1c).

Also, to investigate the extent and timing of delayed VSV-induced neuronal damage in the injected hotspot areas, we additionally stained sections from select VSV injected rats with silver stain. For silver-staining methodological details refer to our prior publication.

N200 labeling

N200 histology was performed using a standard primary/ secondary antibody immunofluorescence process for floating tissue. Briefly, formalin fixed brains were immersed in 30% sucrose/ PBS solution until the tissue sank. 50 µm thick tissue sections were sliced in the sagittal plane on a cryostat and placed in PBS in individual wells of a 24-well plate. Tissue sections were first immersed in 3% hydrogen peroxide for 1 hour. Sections were rinsed 3 times with PBS/ Triton X-100 solution and then blocked in a solution of PBS / Triton X-100 / normal donkey serum for 1 hour. Each section was then treated with 250µl of primary antibody solution consisting of PBS, Triton X-100, donkey serum, and rabbit anti-neurofilament 200 (Sigma) diluted 1:400. The tissue was incubated overnight at 4°C, and then rinsed 3
times and blocked again for 1 hour. Secondary antibody solution, 250 µl/ well, similar to the primary solution but with Alexa Fluor 568 donkey anti rabbit (Invitrogen) diluted 1:500 was then applied and incubated at room temperature for 2 hours. The tissue was again rinsed 3 times before mounting on slides. After drying for 10–30 minutes, the slides were cover-slipped with Prolong Glass antifade mounting media (Invitrogen). The mounting media was allowed to cure overnight before microscope imaging.

Registration and reconstruction

Single-channel Keyence images of entire sagittal sections were imported into GIMP image software [version 2.10.12] as individual layers and overlaid. This allowed for each channel to be selectively switched on and off for visual analysis of the fluorescent labeling within the context of the whole brain slice (Supplemental Fig. 1). Registration of the sagittal slices was then performed by cropping and rotation of the individual brain slice images before aligning them with respect to each other as separate layers in GIMP. After alignment, slice images were exported in a format to be imported into ImageJ for further analysis. This facilitated 3D reconstruction of each brain hemisphere. Supplemental Fig. 1 shows representative images from each step. The cortical projected neuronal labelling was then digitally registered for each rat using custom MATLAB script employing image processing toolbox [MATLAB 2019A], which also converts imageJ coordinates into atlas coordinates. The location and the spread of injections in GP was similarly registered.

Connectivity modeling

(i) Correlating cortical labeling with the injection

Tetrachoric correlation was used to assess for significant correlations between the dorsal (parkinsonian) and ventral (dystonia) GP injections and the cortical labeling. For the dorsal GP injected cases, for example, the digitized projection coordinates in M1 and M2 were assigned into categories ‘dorsal GP–M1’ and ‘dorsal GP-M2’, respectively. Similar assignments were performed for ventral GP projections in M1 and M2. Sum of all the labelled coordinates for each of the four paired combinations were calculated. Percentage of each category with respect to total M1 and M2 coordinates were then arranged in a correlation matrix and the tetrachoric correlation was calculated based on following formula:

\[
Tetrachoric\text{correlation} = \cos \left( \frac{\pi}{2} \sqrt{\frac{ad - bc}{\frac{1}{a} + \frac{1}{c}}} \right)
\]

(ii) Projection probability modeling

A novel 3D spatial algorithm was developed to define statistically significant cortical regions anatomically connected to the two GP hotspots related to parkinsonism and dystonia. This was
accomplished by correlating the location and spread of viral infection in GP injection to the distribution of the neuronal projection labeling in the cortex.

1. First, the distribution of VSV spread in GP and beyond was established based on visual inspection of fluorescent labelling and/or silver-staining of damaged neurons. Scores (from 0 = none to 10 = complete labeling) were then assigned to following: 1) dorsal GP hotspot (Ds) and 2) ventral GP hotspot (Vs).

2. The cortical region of interest (ROI) was defined to encompass M1, M2, cingulate cortex, and surrounding regions (L 0–5 mm, AP +5 to -5 mm, and D 0–4 mm). The ROI was divided into a 50 x 100 x 40-point grid, totaling 200,000 voxels, each of 0.1 x 0.1 x 0.1 mm

3. From the generated 3D cortical labeling plots (Methods, Registration and reconstruction (Supplemental Fig. 1)), all voxels which are fluorescently labeled or have labeling in their immediate surrounding voxels are scored (0–10) each for correlations with Ds and Vs. For example, for Ds, the cortical voxel scores are calculated as follows:

\[
D_{\text{Dorsal Score for Voxel, } DV_{x,y,z}^{\text{Rn}}} = \begin{cases} 
Ds \times 1 & \text{if } D_i < 0.2 \\
Ds \times 0.5, \text{ if } 0.2 \leq D_i \leq 0.3 \\
0, \text{ if } D_i > 0.3
\end{cases}
\]

Where, \(DV_i\) is the voxel score for \(i^{th}\) voxel in rat \#n, with center coordinates described as \(x,y,z\).

Ds is the dorsal spread score estimated in point 1 above.

Di is the mean distance between the center of voxel \(V^i (x,y,z)\) and the nearest VSV label.

4. Arrays of DV and ventral hotspot (VV) were generated for each of the voxels from the cortical ROI for each rat. For each rat, three scores were separately assigned to each voxel that lies within the volume of lesion spread. Therefore, each voxel was assigned 19 different scores, equal to the number of lesioned animals.

5. Final individual voxel score for dorsal and ventral GP hotspot. Initially, NaN (not a number) score was assigned to all the voxels in the ROI. For each voxel, median and standard deviation (SD) of the assigned scores was calculated. Voxels deemed sparsely sampled (< 3 non-NaN values) or with coefficient of variation (CV) > 1 were assigned NaN values, thereby excluding these data from further analysis.

\[
\text{Final score, } f (x) = \begin{cases} 
\text{medianscore, if } \text{medianscore} > Th1; \\
STD < Th2; \\
\text{and number of non-zero scores } > \text{Th3}## \\
\text{NaN, otherwise}
\end{cases}
\]
Where, thresholds, $\text{Th}_1 = 6$, $\text{Th}_2 = 1$, and $\text{Th}_3 = 3$.

The thresholds were $\text{Th}_1 = 5$, $\text{Th}_2 = 2$, and $\text{Th}_3 = 3$ for relaxed hotspot region.

6. Voxels with score 1 are drawn in the 3D realization and the values were compared.

(iii) Hotspot analysis

The final GP and cortical hotspots defined from the above steps were tested for spatial autocorrelations and significant associations. Global Moran's I function (GMI) $^{58,59}$, modified for 3D data $^{17}$, was used on the filtered voxelated data to assess spatial autocorrelation. GMI classifies the overall spatial distribution as clustered (GMI $\sim +1$), dispersed (GMI $\sim -1$), or random (GMI $\approx 0$). Next, Getis-Ord $G_I^*$ statistics $^{31}$, modified for 3D data $^{17}$, was calculated providing a statistical hotspot analysis using the local pattern of spatial association to identify local spatial clusters with high or low efficacy values in the ROI. For statistically significant positive z-values ($G_I^*; z\text{-score} > 1.96 \ (p < 0.05)$), the larger the z-values, the more intense the clustering of hotspots. The probability distribution maps generated in above section (Figs. 3 and 4, middle column) were subsequently filtered to include only significant z-scores ($G_I^*$) and to generate final maps revealing the statistically significant hotspots.

(iv) Independent hotspot test

To ensure that the two hotspots are spatially significantly different from each other, the distribution of the two hotspot coordinates (distance from bregma) were compared using independent sample test.

**Intracortical microstimulation**

The rats were placed under anesthesia using i.p. ketamine (100 mg/ kg) and xylazine (16.67 mg/ kg) and secured to the stereotaxic device with ear bars. A burr hole was made in the skull exposing the M1 and M2 regions. Ketamine (30 mg/ kg) was subsequently injected every 70 min for the duration of the experiment. A stainless-steel EEG-head screw electrode (Invivo1) was secured in the skull contralateral to the burr hole for electrical stimulation return. Next, a tungsten monopolar electrode was inserted through the exposed dura in precise locations within M1 or M2 at a depth of 1.6–2.4 mm from the surface. Using an AM systems stimulus generator and isolator, biphasic pulse trains (pulse width 200–500 us, inter-pulse frequency 100–500 Hz, train length = 5–20 pulses, train frequency = 0.5-5 Hz, amplitude = up to 500 uA) were delivered and corresponding contralateral body part movements (front paw, hind limb, trunk, neck, whiskers, shoulder, jaw, tail) were recorded. The rats were perfused immediately after the experiment and the brain was processed to confirm the anatomy.

**Statistical analysis**

Group comparisons were made using nonparametric tests as indicated in the text, with significance defined as $p < 0.05$. All statistical analyses were conducted using standard MATLAB (MathWorks) functions.
Declarations

DATA AVAILABILITY

The raw histological images, digitized data, and the MATLAB custom scripts for probability modeling will be provided upon request. Correspondence and requests for materials should be addressed to M.S.B.

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Author Contributions M.S.B. conceived and secured funds for the project. M.S.B. and D.K. designed the experiments and analysis techniques. D.K. and G.W. implemented the experiments and collected the data. G.W., S.G., and Z.L.H. performed histological processing, imaging, and image registration. D.K. developed the hotspot algorithm and other programs for data analysis and registration. D.K. and M.S.B. interpreted the data and wrote the manuscript. All authors have approved the final version.

Declaration of interests.

The authors declare no competing interests.

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References


**Figures**
Figure 1

Induction of pure parkinsonism or dystonia affirms the successful injection of VSV in the respective GP motor territory hotspot. a. Under MER guidance, VSV was stereotactically injected into either the dorsal parkinsonian (green) or ventral dystonia (magenta) hotspot in the posteroventral motor territory of GP (rodent equivalent of GPe). b1/2. VSV-induced cytotoxic neuronal degeneration at 4 days post-injection produced movement disorder features indistinguishable from that (c1/c2) induced previously with the
cytotoxic agent ibotenate\textsuperscript{17}. \textbf{b1.} Side and bottom views of a rat (#8) illustrate the movement disorder features induced by a VSV injection in the dorsal parkinsonian GP hotspot. Over the illustrative 25 sec interval, the akinetic rat holds the contralateral (left) paw motionless in a characteristic flexed posture, while also never moving the contralateral hindlimb. During this interval, the rat even barely adjusts the ipsilateral limbs. \textbf{b2.} Illustrative movement disorder features induced by a VSV injection in the ventral dystonia GP hotspot (rat #11). The 16 sec interval demonstrates the animal to be constantly moving, but with dystonic, contortion posturing of the trunk and extension posturing of the contralateral (left) limbs. For comparison, \textbf{c1.} shows a photograph of a parkinsonian rat and \textbf{c2.} a dystonic rat induced by ibotenate lesions in the dorsal and ventral GP hotspots, respectively, with features which closely mimic the human conditions (\textbf{d1} and \textbf{d2}). \textbf{e.} As supported by the motor scores generated from more severely affected rats (n=4), VSV hotspot injections induced isolated classical features of parkinsonism or dystonia, thus validating the select and accurate targeting of the viral injections. \textbf{e1.} As evident from the scoring, rats with dorsal hotspot injections displayed a paucity of spontaneous generalized movement, with minimal response to audio and tactile stimuli, and prominent contralateral (forelimb and hindlimb) hypokinesia. \textbf{e2.} In contrast, rats with ventral hotspot injections showed relatively normal amounts of spontaneous and stimulation-induced movements, while displaying contralateral dystonic limb extension, neck and truncal twisting, and dystonic posturing related falls.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure2}
\caption{Focal VSV multi-synaptic anterograde tracer injections in the parkinsonian and dystonia GP hotspots label distinct secondary and primary cortical motor territories. Irrespective of the surrounding spread, stereotactic injections of multi-synaptic anterograde VSV tracer (\textbf{a}) into the previously defined dorsal parkinsonian GP hotspot (green) produced fluorescent labeling predominately confined to a circumscribed region of the secondary motor cortex (M2) (\textbf{b1a}), while ventral GP dystonia hotspot injections (magenta) predominately labeled primary motor cortex (M1) (\textbf{b2a}). The scale bar for \textbf{b1a,b} represents 1 mm. High}
\end{figure}
power magnifications of the illustrated cortical hotspots demonstrate the intense viral labeling of layer 4 and 5 (b1b) M2 and (b2b) M1 neurons. The scale bar represents 100 µm.

Figure 3

**Probability modeling and hotspot analysis for parkinsonian projections.** To account for viral leakage and extension of the injections beyond the hotspots, a custom spatial hotspot algorithm was employed across injections (n=17) grouped by dorsal vs (Fig 4) ventral GP injections. The algorithm defined cortical regions with fluorescent neuronal labeling that correlated specifically with neuronal labeling or cellular damage in the dorsal GP hotspots. The left column shows the cortical labeling (red) defined by the algorithm for the dorsal hotspot injections in four sagittal planes. The middle column indicates Getis-Ord G*I* statistics maps shows the z score values (color bar) for the efficacy distribution plots. Finally, the right column indicates the final masked projection coordinates (red asterisk) superimposed on the M1 (yellow) and M2 (green) territories as defined by the Paxinos and Watson 6th edition atlas. The dorsal posterolateral GP parkinsonian hotspot projections can be seen to project primarily to M2 (c1).
Figure 4

Probability modeling and hotspot analysis for dystonia projections. The ventral posteralateral GP dystonia hotspot projections can be seen to project chiefly to M1. Refer to Fig. 3 for specific details.
The parkinsonian subcircuit encompasses the M2 cortical microexcitable zone, while dystonia is localized to the M1 microexcitable zone. **a.** To investigate the suggested possibility that the labeled parkinsonian cortical hotspot encroached on the cingulate cortex rostromedially, we first stained select brains (n=3) with N200. Because N200 labels layer 3-5 neurons in cingulate cortex and M1, while only sparsely labeling L3-5 neurons in M2, N200 effectively discriminates M2\(^2\). As illustrated, N200 staining revealed the parkinsonian cortical hotspot (green outlined circle) to be confined specifically to M2. The white arrows indicate i) rostrally, the margins of cingulate cortex area 1 (cg1) and M2 and ii) caudally, the margins of M2 and M1. **b.** Next, we used microstimulation to define the topographical relations of the two cortical hotspots to the M2 (rostral) and M1 (caudal) microexcitable zones, which denote the two most predominant direct cortical projections to the spinal cord\(^3\). The parkinsonian (green) cortical hotspot and
the relaxed hotspot (dashed green) can be seen to be primarily confined to the rostral forelimb area (RFA, blue oval) of M2, encroaching on the lateral jaw (cyan diamond) area. The dystonia cortical hotspot was chiefly confined to the caudal forelimb area (CFA, purple oval) of M1, encroaching caudally on the hindlimb (orange oval) area.

![Cortical Hotspots of M1(blue) and M2(red)](image)

**Figure 6**

**Cortical hotspots and segregated subcircuitry.** *a.* shows the defined parkinsonian (green) and dystonia (magenta) cortical territories together in 3D to illustrate their positions and separation from each other. The study results strongly implicate that parkinsonism and dystonia originate along distinct secondary and primary motor subcircuits. *b.* Novel working model of segregated movement disorder subcircuitry. Illustrated is our new working model of segregated BGTC motor subcircuits specifically for parkinsonism and dystonia, two of the most common movement disorders. As discussed in the main text, the confinement of the parkinsonian cortical hotspot to the rostral microexcitable zone supports that the parkinsonian subcircuit is likely to represent the SMA subcircuit. The M2 (purported SMA) parkinsonian cortical territory provides high level (executive) motor-related signaling to the spinal cord and to the M1 (dystonia) territory (not illustrated), with the latter, providing direct corticospinal programming of the motor action. Disturbances in motor signaling isolated to these two subcircuits can readily explain the characteristic clinical features of Parkinson’s disease and dystonia.

**Supplementary Files**

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- [SupplementalFig.1.tif](image)