Restoration of locomotor function following stimulation of the A13 region in Parkinson's mouse models.

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ABSTRACT

Parkinson's disease (PD) is characterized by extensive motor and non-motor dysfunction, including gait disturbance, which is difficult to treat effectively. This study explores the therapeutic potential of targeting the A13 region, a heterogeneous region of the medial zona incerta (mZI) containing dopaminergic, GABAergic, and glutamatergic neurons that has shown relative preservation in PD models. The A13 is identified to project to the mesencephalic locomotor region (MLR), with a subpopulation of cells displaying activity correlating to movement speed, suggesting its role in locomotion. We show that photoactivation of this A13 region can alleviate bradykinesia and akinetic features, while increasing turning in a mouse model of PD. These effects combine disease-specific rescue of function with a possible gain of function. We identified areas of preservation and plasticity within the mZI connectome using whole-brain imaging. Our findings suggest a global remodeling of afferent and efferent projections of the A13 region, highlighting the zona incerta's role as a crucial hub for the rapid selection of motor function. The study unveils the significant pro-locomotor effects of the A13 region and suggests its promising potential as a therapeutic target for PD-related gait dysfunction.

SIGNIFICANCE STATEMENT

This work examines the function of the A13 region in locomotion, an area with direct connectivity to locomotor regions in the brainstem. A13 stimulation can restore locomotor function and improve bradykinesia in a PD mouse model.

INTRODUCTION

Parkinson's disease (PD) is a complex condition affecting many facets of motor and non-motor functions, including visual, olfactory, memory and executive functions (Cenci and Björklund, 2020). Due to the widespread features of PD, focusing on changes within a single pathway cannot account for all symptoms. Gait disturbance is one of the hardest to treat; pharmacological, deep brain stimulation (DBS) and physical therapies lead to only partial improvements (Nonnekes et al., 2020, 2015). While the subthalamic nucleus (STN) and globus pallidus (GPi) are common DBS targets for PD, alternative targets such as pedunculopontine nucleus (PPN) and the zona incerta (ZI) have been proposed with mixed results in improving postural and/or gait dysfunctions (Caire et al., 2013; Ferraye et al., 2010; Gut and Winn, 2015; Hamani et al., 2011; Moro et al., 2010; Nonnekes et al., 2015; Okun and Foote, 2010; Ossowska, 2019; Stefani et al., 2007; Thevathasan et al., 2018). Part of the issue with targeting the ZI with DBS strategies is the relative lack of knowledge regarding its downstream anatomical and functional connectivity with motor centers. Recent work with photoactivation of subpopulations of PPN neurons in PD models shows promise for similar ZI-focused strategies (Masini and Kiehn, 2022).

The ZI is recognized as an integrative hub, with roles in regulating sensory inflow, arousal, motor function, and conveying motivational states (Chometton et al., 2017; Mitrofanis, 2005; Monosov et al., 2022; Sharma et al., 2024; Wang et al., 2020; Yang et al., 2022; Zhao et al., 2019). As such, it is well placed to be involved in PD and has seen increased clinical and preclinical research over the last two decades (Blomstedt et al., 2018; Ossowska, 2019; Plaha et al., 2008). However, little attention has been placed on the medial zona incerta (mZI), particularly the A13, the only dopamine-containing region of the rostromedial ZI (Bolton et al., 2015; Kim et al., 2017; Sharma et al., 2018). Recent research in primates and mice (Peoples et al., 2012; Roostalu et al., 2019; Shaw et al., 2010) indicates that the A13 is preserved in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-based PD models.

Recently, we discovered that the A13 located within the ZI projects to two areas of the mesencephalic locomotor region (MLR), the PPN and cuneiform nucleus (CUN) (Sharma et al., 2018), suggesting a role for A13 in locomotor function. Indeed, mini-endoscopic calcium recordings from calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) populations in the rostral ZI, which includes the A13 nucleus, show a subpopulation of cells whose activity correlates with movement speed (Z. Li et al., 2021). Since this region projects to the MLR, it is a potential parallel motor pathway to target for gait improvement. Photoactivation of glutamatergic MLR neurons alleviates motor deficits in mouse models that transiently blocked dopamine transmission or lesioned SNc with 6-OHDA (Fougère et al., 2021; Masini and Kiehn, 2022). Phenomena such as kinesia paradoxa (Glickstein and Stein, 1991) in PD patients support the existence of preserved parallel motor pathways that can be engaged in particular circumstances to produce normal movement.

Further evidence supporting the importance of parallel motor pathways in PD includes those reporting functional alterations in A13 (Hoffman et al., 1997; Périer et al., 2000). Nigrostriatal lesions affect A13 cellular function and lead to anatomical remodeling in monoaminergic brain regions including widespread alterations in dopaminergic, noradrenergic, cholinergic, serotoninergic, and other monoaminergic neuronal populations (Braak et al., 2003; Kish et al., 2008; Lim et al., 2009; Perez-Lloret and Barrantes, 2016; Roostalu et al., 2019; Scatton et al., 1983; Zweig et al., 1989). There is additional evidence showing parallel motor pathways in the A13. For example, the A13 connectome encompasses the cerebral cortex

(Mitrofanis and Mikuletic, 1999), central nucleus of the amygdala (Eaton et al., 1994), thalamic paraventricular nucleus (Li et al., 2014), thalamic reuniens (Sita et al., 2007; Venkataraman et al., 2021), CUN and PPN (Sharma et al., 2018), superior colliculus (SC) (Bolton et al., 2015), and dorsolateral periaqueductal grey (PAG) (Messanvi et al., 2013; Sita et al., 2007), making the A13 an potential hub for goal-directed locomotion (Choi and McNally, 2017; Eaton et al., 1994; Messanvi et al., 2013; Mok and Mogenson, 1986; Moriya et al., 2020; Ogundele et al., 2017; Manjit K. Sanghera et al., 1991; M. K. Sanghera et al., 1991; Sita et al., 2007; Venkataraman et al., 2021).

Considering the role of the A13 region in gait control, particularly its potential as a therapeutic target to enhance gait in Parkinson's Disease (PD), we focused on exploring the benefits of photoactivating this anatomically defined area. This targeted region predominantly encompasses the A13, along with a portion of the medial zona incerta (mZI), collectively referred to as the A13 region throughout this study. We identified areas of preservation and plasticity within the mZI connectome using whole-brain imaging techniques. While endogenous compensatory mechanisms from the remaining and remodelled A13 region connectome were inadequate in overcoming locomotor deficits, photoactivation of the A13 region rescued bradykinetic and akinetic symptoms in a mouse model of 6-hydroxydopamine (6-OHDA) mediated unilateral nigrostriatal degeneration. These findings indicate that the A13 region produces powerful pro-locomotor effects in normal and PD mouse models. Moreover, PD-related bradykinesia is ameliorated with A13 region photoactivation in the presence of remodelling of the A13 region connectome. Because we found the zona incerta is a hub for the rapid selection of motor function (Wang et al., 2020), we then mapped the input and output patterns finding evidence of global remodeling of afferent and efferent projections of the A13 region. Some of these data have been published in abstract form (L. Kim et al., 2021).

RESULTS

Unilateral 6-OHDA mouse model has robust motor deficits

The overall experimental design is illustrated in Figure 1A, along with a schematic in Figure 1B showing injections of 6-OHDA in the medial forebrain bundle (mFB) and AAVDJ-CaMKIIα-ChR2 virus into the medial zona incerta (mZI). We confirmed substantia nigra pars compacta (SNc) degeneration in a well-validated unilateral 6-OHDA-mediated Parkinsonian mouse model (Thiele et al., 2012). The percentage of tyrosine hydroxylase (TH^+) cell loss normalized to the intra-animal contralesional side was quantified. 6-OHDA produced a significant lesion that decreased TH^+ neuronal SNc populations. As previously reported (Boix et al., 2015), the SNc ipilesional to the 6-OHDA injection (n = 10) showed major ablation of the TH^+ neurons compared to sham animals (Figure 1C and D: n = 12).

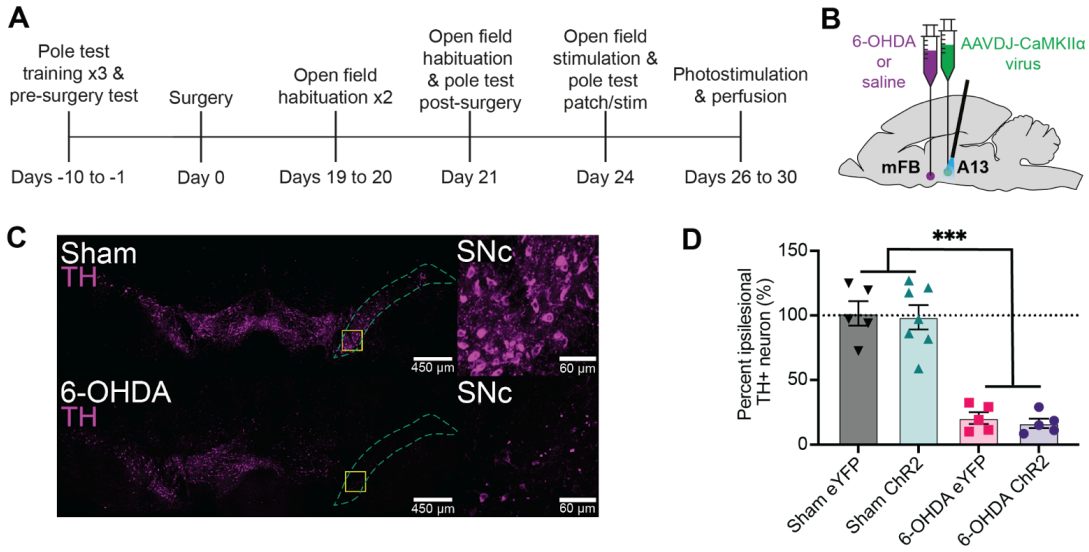

Figure 1. Experimental design and confirmation of unilateral TH$^+$ depletion in the SNc via 6-OHDA lesion. (**A**) Illustration of experimental timeline. (**B**) Dual ipsilateral stereotaxic injection into the mFB and A13 region. (**C**) TH$^+$ cells in SNc of sham (top) compared to 6-OHDA injected mouse (bottom). Magnified areas outlined by yellow squares are shown on the right. (**D**) Unilateral injection of 6-OHDA (6-OHDA ChR2: $n = 5$, 6-OHDA eYFP: $n = 5$) into the MFB resulted in greater percentage of TH$^+$ loss compared to sham in the SNc (sham ChR2: $n = 7$, sham eYFP: $n = 5$, 2-way ANOVA), regardless of virus type ($F_{1,18} = 104.4$, $p < 0.001$). ***$p < 0.001$. Error bars indicate SEMs.

A13 region photoactivation generates pro-locomotor behaviors in the open field

6-OHDA lesions produce bradykinetic and akinetic phenotypes in the open field (L.-X. Li et al., 2021; Magno et al., 2019; Sanders and Jaeger, 2016). To understand the impact of A13 region photoactivation on locomotion in sham and PD model mice, on-target localization of the optical fiber above the A13 region, centered on the mZI, along with YFP reporter expression, was confirmed (Figure 2; Figure S1) in mice given sham or 6-OHDA injections. Corroborating the *post hoc* targeting, we found evidence for c-Fos in neurons within the A13 region in photostimulated ChR2 mice (Figure 2D). We observed an increase in c-Fos fluorescence intensity in the A13 region of photostimulated 6-OHDA ChR2 mice compared to the 6-OHDA eYFP mice. There was also an increase in the number of ChR2 cells with c-Fos labeling in 6-OHDA ChR2 mice compared to the 6-OHDA eYFP mice. However, there was no net increase in TH$^+$ cells labeled with ChR2 and c-Fos suggesting a heterogeneous population of activated cells. Before *post hoc* analysis, mice were monitored in the open field test (OFT), where the effects of the 6-OHDA lesion were apparent, with 6-OHDA lesioned animals demonstrating far less movement, fewer bouts of locomotion, and less time engaging in locomotion (Figure 3A-E). Using instantaneous animal movement speeds that exceeded 2 cm/s as per Masini & Kiehn (2022), we plotted instantaneous speed (Figure 3B-E) and analyzed one-minute bins (Figure 3H). As was expected, 6-OHDA lesioned animals had lower movement speeds than sham control animals ($p < 0.001$). One animal from the 6-OHDA eYFP group was excluded because it did not meet the speed threshold during recording. Notably, photoactivation of the A13 region often generated dramatic effects, with mice showing a distinct increase in locomotor behavior (Figure 3A, Movie S1 & Movie S2). Both sham and 6-OHDA ChR2 mice showed a significant increase in locomotor distance travelled during periods of photoactivation (Figure 3F, $p < 0.01$). One sham animal showed grooming behavior on stimulation and was excluded from the analysis.

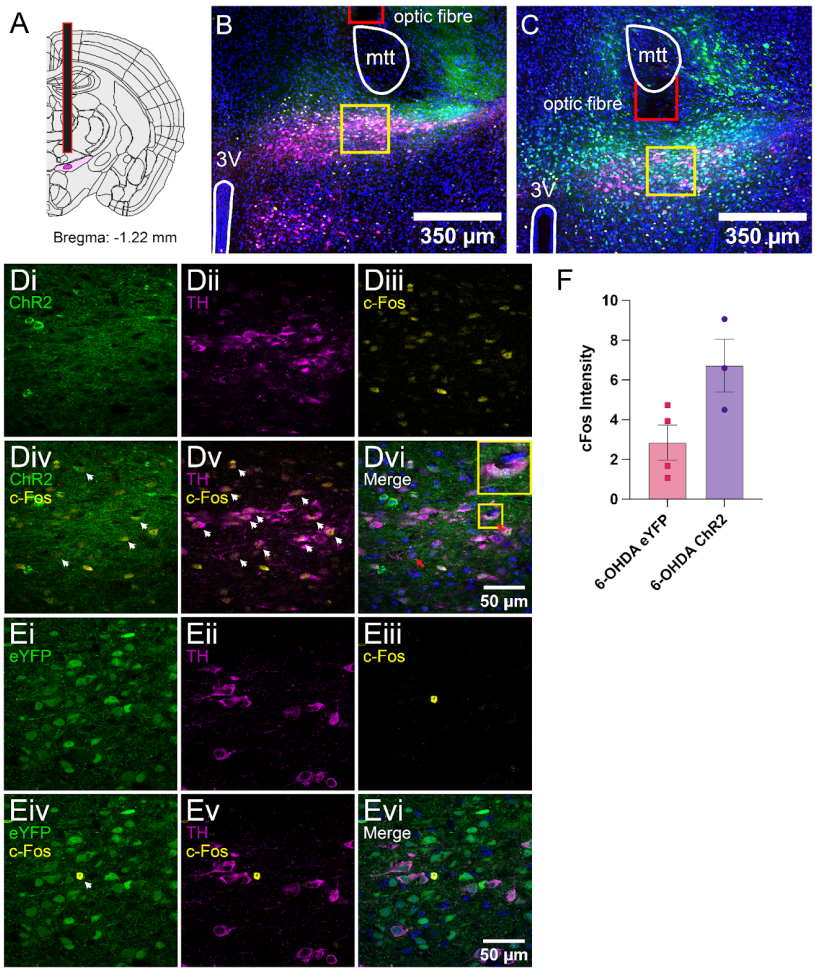

Figure 2. *Post hoc* c-Fos expression and targeting of the mZI and A13. (A) Diagram showing the A13 DAergic nucleus in dark magenta encapsulated by the ZI in light magenta. The fiber optic tip is outlined in red. Atlas image adapted from the Allen Brain Atlas (Goldowitz, 2010). **(B)** Tissue images were obtained from a 6-OHDA ChR2 animal and **(C)** a 6-OHDA eYFP animal. Images show the distribution of DAPI (blue), eYFP (green), c-Fos (yellow), and TH (magenta). Landmarks are outlined in white (3V: third ventricle; A13 and mZI as shown in A), and the optic cannula tip is shown in red. Higher magnification images of the A13 DAergic nucleus are outlined by the yellow boxes in a 6-OHDA ChR2 animal **(Di-Dvi)** and a 6-OHDA eYFP animal **(Ei-Evi)**. Images show isolated channels in the top rows of the respective groups: eYFP **(i)**, TH **(ii)**, and c-Fos **(iii)**. Merged channels for eYFP and c-Fos **(iv)**, TH and c-Fos **(v)**, and a merge of all four channels **(vi)** are presented in the bottom rows of their respective groups. White arrowheads in the merged images highlight overlap in merged markers. Red arrows show triple colocalization of eYFP, c-Fos and TH. **(Dvi)** contains a magnified example of triple-labelled neurons, as highlighted in the yellow box. **(F)** Graph shows increase in the intensity of c-Fos fluorescence after photoactivation in 6-OHDA ChR2 mice ($p = 0.05$).

We tested whether photoactivation led to a single bout of locomotion or if there was an overall increase in bouts, signifying that animals could repeatedly initiate locomotion following photoactivation. Mice in the ChR2 groups demonstrated an increase in the number of locomotion bouts with photoactivation, indicating a greater ability to start locomotion from rest, and that photoactivation was not eliciting a single prolonged bout (Figure 3G, $p < 0.01$). Photoactivation increased the total duration of locomotor bouts (Figure 3I, $p < 0.001$). We did note a refractory decrease in the distance travelled by the sham ChR2 animal group. To control for this, we compared the pre-stimulation timepoints to the baseline one-minute averages to ensure that the animal locomotion distance travelled returned to a stable state before stimulation was reapplied (Figure S2, $p = 0.78$).

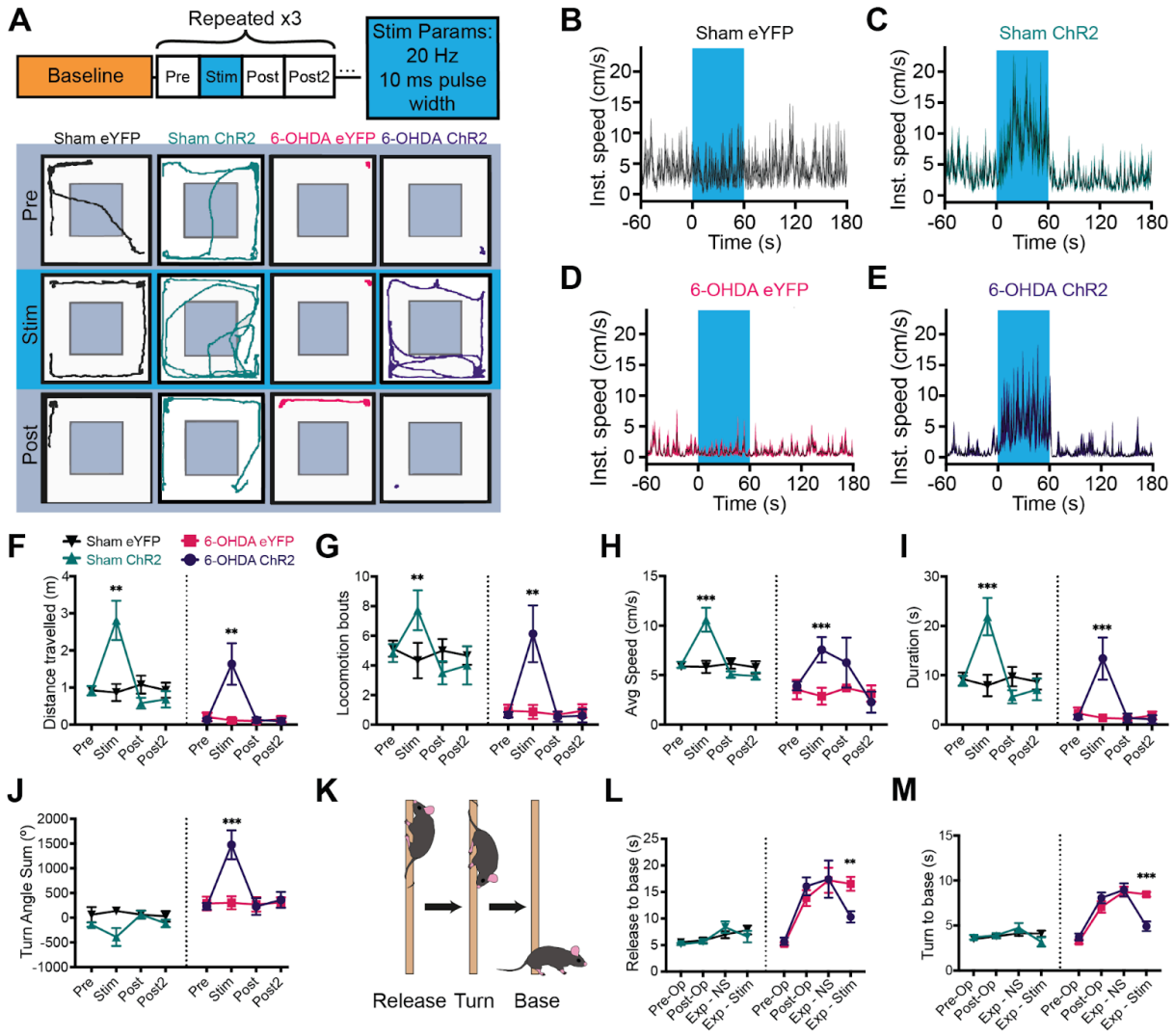

Figure 3. Ispilesional photoactivation of the A13 region in a unilateral 6-OHDA mouse model rescues motor deficits.

(**A**) Schematic of open field experiment design and example traces for open field testing (each testing box is 1 min - total test is 4 min) with unilateral photoactivation of the A13 region. (**B-E**) Group averaged instantaneous velocity graphs showing no increase in a sham eYFP (**B**) or 6-OHDA eYFP mouse (**C**), with increases in velocity during stimulation in a sham ChR2 (**D**) and 6-OHDA ChR2 (**E**) mouse. (**F-I**) Effects of photoactivation on open field metrics for sham eYFP (n = 5), sham ChR2 (n = 6), 6-OHDA eYFP (n = 5), and 6-OHDA ChR2 (n = 5) groups (three-way MM ANOVAs, *post hoc* Bonferroni pairwise). *Photoactivation increased in both sham and 6-OHDA ChR2 groups:* (**F**) distance travelled (ChR2 vs. eYFP: $p < 0.01$), (**G**) locomotor bouts (ChR2 vs. eYFP: $p < 0.01$), (**H**) animal movement speed (ChR2 vs. eYFP: $p < 0.001$) and (**I**) duration of locomotion in the open field (ChR2 vs. eYFP: $p < 0.001$). (**J**) The graph presents animal rotational bias using the turn angle sum. There was a significant increase in 6-OHDA ChR2 rotational bias during A13 region photoactivation (6-OHDA ChR2 vs. 6-OHDA eYFP: $p < 0.001$). (**K**) Diagram depicting the pole test. A mouse is placed on a vertical pole facing upwards time for release is derived from time the experimenter removes their hand from the animal's tail to when it touches the ground. (**L, M**) Photoactivation of the A13 region led to shorter descent times in 6-OHDA ChR2 mice compared to 6-OHDA eYFP mice ($p < 0.01$). A pre-op baseline was performed, followed by post-op three weeks later. On experiment day, performance with no stim (Exp - NS) was compared with photostimulation (Exp - Stim). (**M**) 6-OHDA ChR2 mice showed a greater reduction in descent time compared to 6-OHDA eYFP mice (6-OHDA ChR2 vs. 6-OHDA eYFP: $p < 0.001$). ***$p < 0.001$, **$p < 0.01$. Error bars indicate SEMs.

Next, we examined the reliability of photoactivation to initiate locomotion. The percentage of trials with at least one bout of locomotion was compared for the pre- and stim time points. 6-OHDA ChR2 animals showed a reliable pro-locomotion phenotype with A13 region photoactivation (Figure S3A, $p < 0.05$). As was expected in the control 6-OHDA eYFP group, there was no effect of photoactivation on the probability of engaging in locomotion (Figure S3A, $p = 0.71$).

Animal movement speed also factors into the total distance travelled measure and can be discussed in regard to a bradykinetic phenotype in 6-OHDA lesioned mice (Magno et al., 2019; Sanders and Jaeger, 2016). The 6-OHDA and sham ChR2 groups displayed increases in average speed in comparison to the 6-OHDA and sham eYFP groups during photostimulation (Figure 3H, $p < 0.001$). When we examined the time taken to initiate locomotion, there was no significant difference between sham or 6-OHDA ChR2 groups (Figure S3B, $p = 0.95$).

Photoactivation of the A13 region increases ipsilesional turning in the open field test

Unilateral 6-OHDA lesions drive asymmetric rotational bias (Boix et al., 2015; L.-X. Li et al., 2021; Magno et al., 2019; Thiele et al., 2012). We were interested in whether this persisted with stimulation and noted upon observation of photoactivation that animals appeared to have increased ipsilesional rotation. As observed, 6-OHDA ChR2 animals had an increase in turn angle sum (TAS), indicating an increase in their rotational bias with photoactivation in the ipsilesional direction (Figure 3J, $p < 0.001$). As expected, 6-OHDA eYFP animals showed consistent rotational bias throughout time. The rotational bias of sham ChR2 was also compared to determine whether the increased rotational bias was due to photoactivation or the interaction of photoactivation and the lesion. The sham ChR2 group showed no significant change in TAS with photoactivation (Figure 3J, $p = 0.06$). Next, we examined whether the increased turning angle sum in the 6-OHDA ChR2 group was observed during periods of locomotion. When the TAS was calculated only during periods of locomotion, the rotational bias in the animal orientation in the 6-OHDA ChR2 animals was not observed. The rotational bias was observed between bouts of locomotor activity.

Skilled vertical locomotion is improved in the pole test with photoactivation of the A13 region.

The pole test is a classic 6-OHDA behavioral paradigm (Figure 3K) that involves skilled locomotor abilities for an animal to turn and descend a vertical pole (Matsuura et al., 1997; Ogawa et al., 1985). Improvements in function can be inferred if the time taken to complete the test decreases (Matsuura et al., 1997; Ogawa et al., 1985). 6-OHDA mice demonstrated significantly greater descent times than sham mice (Figure 3L, $p < 0.01$). Photoactivation of the A13 region reduced descent times for both 6-OHDA and sham groups on the pole test (Figure 3L, $p < 0.01$, Movie S3). Neither of the eYFP groups showed any changes in the time to complete the pole test.

To further understand the effects of photoactivation on the ability of mice to descend the pole, the time taken for mice to descend after turning was analyzed to remove any influence of animals spending time investigating their environment on the top of the pole. While all groups showed reduced total pole test descent time with photoactivation, considering just the time to descend from turn alone, there was a larger improvement with A13 region photoactivation in the 6-OHDA ChR2 mice compared to 6-OHDA eYFP mice (Figure 3M, $p < 0.001$). These results indicate that photoactivation has the effect of reducing bradykinesia by improving the ability of mice to descend the pole during the PT.

Dopaminergic Cells in the A13 region are preserved in the unilateral 6-OHDA mouse model

While photoactivation of the A13 region increased locomotor activity in both sham and 6-OHDA lesioned mice, we observed differences in speed and directional bias between the two groups. We hypothesized that these differences might be due to changes in the A13 region's connectome as previous research has shown that 6-OHDA lesions can lead to increases in firing frequency and metabolic activity in this brain region, over a period of one month (Périer et al., 2000). To investigate this possibility, we used whole brain imaging approaches (Hansen et al., 2020; Zhan et al., 2021) to examine changes in the connectome following 6-OHDA lesions of the nigrostriatal pathway.

As expected (Iancu et al., 2005), our whole brain imaging results showed that TH^+ cells in SNc (Figure S4A-C) were more vulnerable to the 6-OHDA neurotoxin than those in the ventral tegmental area (VTA) and A13 (Figure S4D-F). Specifically, we found that 6-OHDA-treated mice showed a significantly greater percentage of TH^+ cell loss in SNc compared to the VTA and A13 (VTA vs. SNc: $p < 0.01$; A13 vs. SNc: $p < 0.01$). In contrast, sham animals showed no significant difference in TH^+ cell loss across SNc, VTA and A13 (Figure S4G, $p > 0.05$). These findings are consistent with observations in the human brain, where the A13 region is preserved even in the presence of extensive nigrostriatal degeneration (Matzuk and Saper, 1985). Our results confirm that the 6-OHDA mouse model effectively replicates this aspect of Parkinson's disease pathology.

Extensive Remodeling of the A13 Region Connectome Following Unilateral Nigrostriatal Degeneration

Although photoactivation of the A13 region was effective in restoring speed in 6-OHDA lesioned mice (Figure 3), we observed an increase in circling behavior. This suggested that additional changes possibly reflecting alterations in the A13 connectome may be occuring. To investigate these potential changes without the potential confounds of an implanted optrode over the area, we conducted separate experiments to examine the changes in the input and output patterns of the A13 region. We did this by co-injecting anterograde (AAV8-CamKII-mCherry) and retrograde AAV (AAVrg-CAG-GFP) tracers into the A13 nucleus (Paxinos and Franklin, 2008) (Figure S4H). The injection core and spread were determined in the rostrocaudal direction from the injection site (Figure S5). The viral spread was centered around the zona incerta containing the A13, with minor spread to adjacent areas in some cases (Figure S5. To assess whether unilateral nigrostriatal degeneration led to changes in the organization of motor-related inputs and outputs from the A13, we first visualized interregional correlations of afferent and efferent proportions for each condition using correlation matrices (Figure 4A and B; 18 regions in a pairwise manner). Correlation matrices were organized using the hierarchical anatomical groups from the Allen Brain Atlas (Figure 4C-F). To control for experimental variations in the total labeling of neurons and fibers, we calculated the proportion of total inputs and outputs by dividing the afferent cell counts or efferent fiber areas in each brain region by the total number found in the brain. The data were then normalized to a $\log_{10}$ value to reduce variability and bring brain regions with high and low proportions of cells and fibers to a similar scale (Kimbrough et al., 2020). Comparing the afferent and efferent proportions pairwise between mice showed good consistency with an average correlation of 0.91 ± 0.02 (Spearman's correlation, Figure S6).

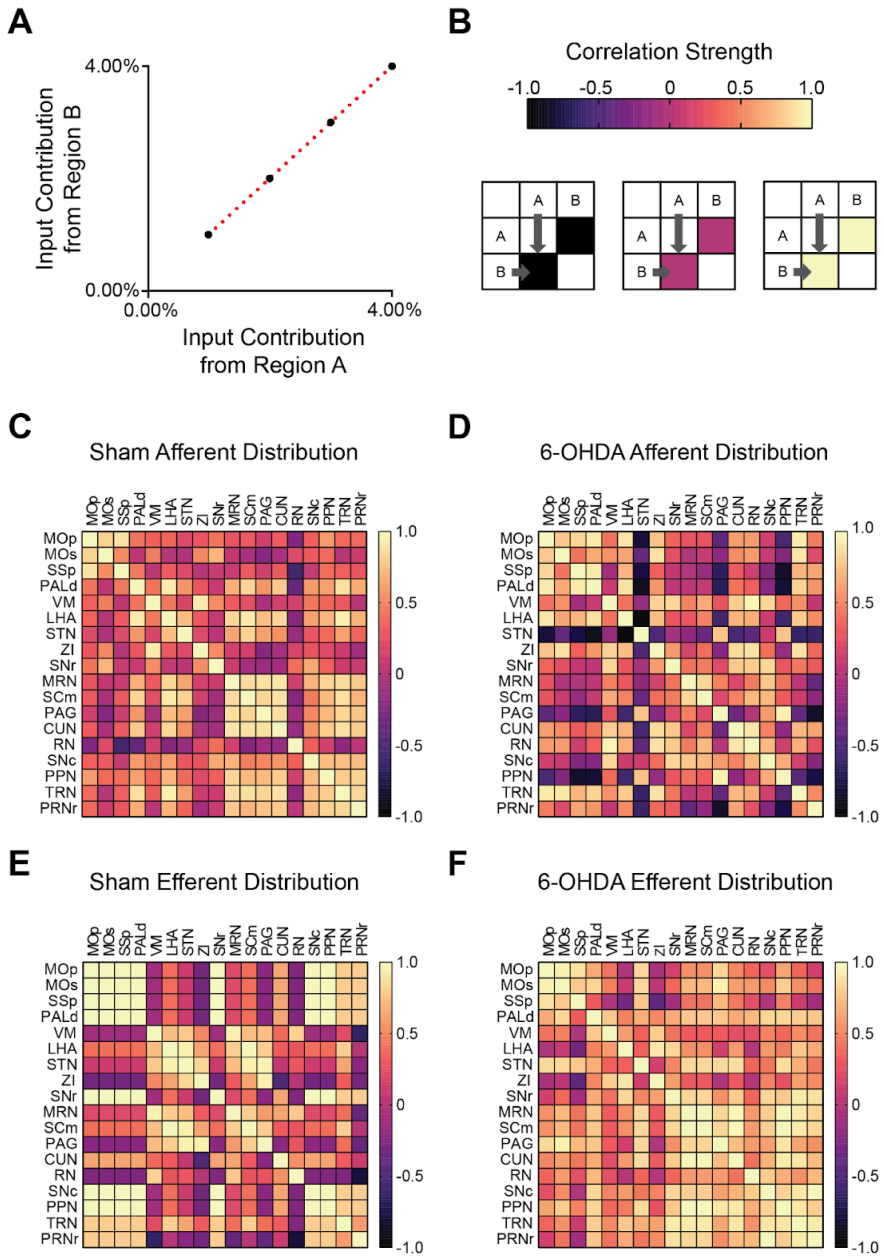

Figure 4. Nigrostriatal degeneration causes widespread changes in A13 region input and output connections. Correlation matrices were used to visualize input and output patterns of the A13 region. Here, we focus on motor-related pathways. **(A)** Brain regions with similar input patterns show high correlation. **(B)** Correlation strength is shown by cell color in the matrix: yellow represents strong positive correlations, magenta indicates no correlation, and black represents strong negative correlations. (C, D) Sham animals displayed stronger interregional correlations of inputs from motor-related regions across the neuroaxis to the A13 region compared to 6-OHDA-lesioned mice. This suggests a broader distribution of inputs among motor related cortical, subcortical, and brainstem regions in sham condition. (D) In 6-OHDA-lesioned mice, inputs to the A13 region from the STN, PAG, and PPN became negatively correlated, unlike inputs from other motor-related regions. In contrast, inputs from motor-related pallidal and incertohypothalamic areas showed stronger positive correlations with cortical inputs, suggesting these regions had a relatively greater influence on A13 activity. (E, F) In contrast, output patterns from the A13 region showed strong interregional correlation among cortical and brainstem motor related regions in 6-OHDA-lesioned mice compared to sham animals. (E) In sham animals, A13 outputs to cortical regions were negatively correlated with outputs to thalamic, hypothalamic, and midbrain regions. This pattern disappeared after nigrostriatal degeneration, suggesting a more widespread distribution of A13 outputs. MOp (primary motor cortex), MOs (secondary motor cortex), SSp (primary somatosensory area), PALd (pallidum, dorsal), VM (ventral medial thalamic nucleus), LHA (lateral hypothalamus), STN (subthalamic nucleus), ZI (zona incerta), SNr (substantia nigra pars reticular), MRN (midbrain reticular nucleus), SCm (superior colliculus, motor), PAG (periaqueductal gray), CUN (cuneiform), RN (red nucleus), SNc (substantia nigra pars compacta), PPN (pedunculopontine nucleus), TRN (tegmental reticular nucleus), and PRNr (pontine reticular nucleus).

We observed changes in afferent input patterns to the A13 region between the sham and the 6-OHDA groups. Using a correlation matrix, we found that inputs from motor related brain regions to the A13 region in sham animals showed overall higher positive correlations across cortical, subcortical and brainstem areas than in 6-OHDA mice (Figure 4C). In 6-OHDA-lesioned mice, input distribution from dorsal pallidum, lateral hypothalamus, zona incerta, and tegmental reticular nucleus became positvely correlated to somatomotor (MOp, MOs) and somatosensory (SSp) cortical areas (Figure 4D). These regions were notably different (anti-correlated) compared to other brain regions. These data suggest a shift in the A13 inputs after 6-OHDA lesions, with a relative increase in cortical, pallidal, and hypothalamic influence compared to from other motor related brain regions.

In contrast, output patterns from the A13 region showed a higher overall correlation between brain regions in 6-OHDA-lesioned mice. In sham animals, A13 outputs to cortical regions were negatively correlated with outputs to thalamic, hypothalamic, and midbrain regions (Figure 4E). This pattern disappeared (Figure 4F) after nigrostriatal degeneration, suggesting a broader, less biased distribution of A13 outputs.

Differential remodeling of A13 region connectome ipsi- and contra-lesion following 6-OHDA-mediated nigrostriatal degeneration

We used whole-brain imaging to further investigate how the A13 connectome is affected by a unilateral 6-OHDA lesion. Our analysis revealed distinct patterns of remodelling on the lesioned (ipsilesional) and intact (contralesional) sides of the brain (Figure 5B-E). On the ipsilesional side, we observed a decrease in A13 afferent density in 6-OHDA mice compared to sham animals from several key regions (Figure 5A), including the primary motor cortex (MOp), primary somatosensory area (SSp), and secondary motor cortex (MOs). In contrast, ipsilesional compensatory increases in A13 afferents were observed, for example, in lateral hypothalamic area (LHA), Substantia Nigra pars reticulata (SNr), Superior colliclus (SCm), and PAG.

Interestingly, the contralesional side showed a more conservative pattern of afferent remodeling, with a modest decrease in A13 afferent density in primary motor cortex (MOp) and primary somatosensory cortex (SSp) of 6-OHDA mice, with a slight increase in the secondary motor cortex (MOs). For hypothalamic and midbrain structures only LHA and PAG showed evidence for an increase. Several regions, including the hypothalamus, midbrain, and pons showed bilateral upregulation of A13 afferents suggesting a more global compensatory response to the unilateral lesion.

Ipsilesional A13 efferents were decreased in 6-OHDA mice (Figure 5A) mainly in the somatosensory cortex (SSp), with some modest increases in midbrain structures, whereas, contralesional efferent projection patterns showed increases in efferent density 6-OHDA mice compared to sham in cortical structures (MOp, MOs, SSp). This was accompanied by modest decreases in efferent density in 6-OHDA contralesional midbrain structures compared to sham.

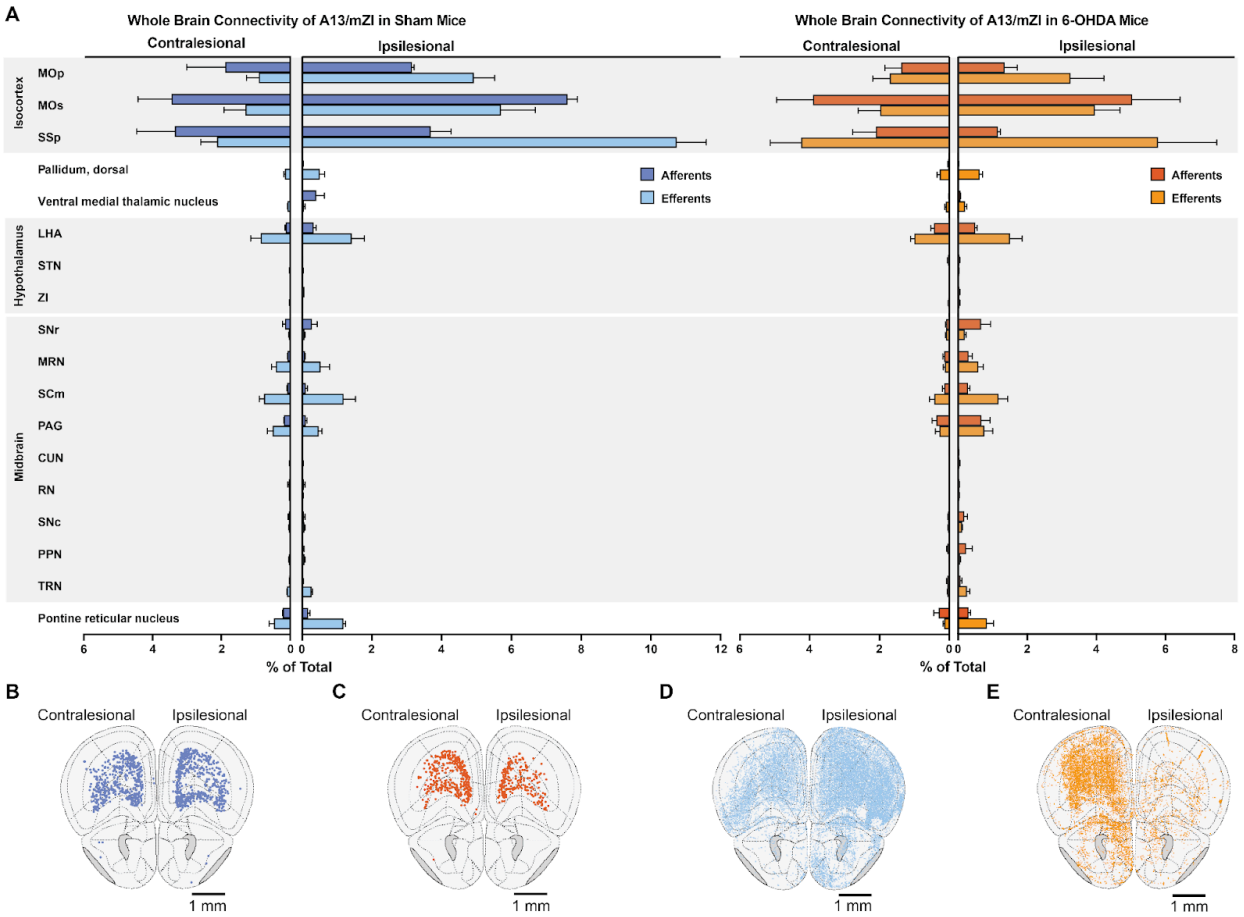

Figure 5. Unilateral nigrostriatal degeneration causes distinct changes in A13 connectivity.
(A) A13 input and output connections to motor-related brain areas. Anterograde and retrograde viruses were injected into the A13 region on the lesioned side (see methods). The graph shows the relative change in input (afferents) and output (efferents) connections in 6-OHDA-lesioned mice compared to sham controls. **(B, C)** Brain regions showing changes in A13 input connections in sham **(B)** and 6-OHDA-lesioned **(C)** mice**. (D, E)** Brain regions showing changes in A13 output connections in sham **(D)** and 6-OHDA-lesioned **(E)** mice. Error bars represent SEMs. 6-OHDA: n = 3; sham: n = 2. Brain region abbreviations are from the Allen Brain Atlas. MOp (primary motor cortex), MOs (secondary motor cortex), SSp (primary somatosensory area), LHA (lateral hypothalamus), STN (subthalamic nucleus), ZI (zona incerta), SNr (substantia nigra pars reticulata), MRN (midbrain reticular nucleus), SCm (superior colliculus, motor), PAG (periaqueductal gray), CUN (cuneiform), RN (red nucleus), SNc (substantia nigra pars compacta), PPN (pedunculopontine nucleus), and TRN (tegmental reticular nucleus).

DISCUSSION

Photoactivating the A13 region enhances locomotion in lesioned and sham mice, with increased distance travelled, locomotion duration, and speed. Particularly in 6-OHDA mice, it rescued the number of locomotor bouts and significantly improved bradykinesia. Additionally, we observed remodeling of the A13 connectivity post-nigrostriatal lesions. The zona incerta is a thin structure that extends from the rostral thalamus to the rostral portion of the red nucleus in rodents, and zona incerta regions around the subthalamic nucleus, situated substantially caudally to the A13, have shown clinical significance (Ossowska, 2019).

The role of the A13 region in locomotion in sham mice

We provide the first direct evidence that photoactivation of the A13 region can drive locomotion, suggesting the pro-locomotor functions of the zona incerta extend further rostrally in the mouse (Mitrofanis, 2005): photoactivation of cZI neurons increased animal movement speed in prey capture (Zhao et al., 2019) and active avoidance (Hormigo et al., 2020). Interestingly, early research on the cat suggested a role for the zona incerta in locomotion, particularly in the region adjacent to the subthalamic nucleus, but the effectiveness of rostral zona incerta regions containing the A13 was not reported (Grossman, 1958). Recent work shows that the A13 is involved in forelimb grasping (Garau et al., 2023), suggesting other motor control functions, while a positive valence associated with motivated food seeking behavior has also been reported (Ye et al., 2023). Previous work targeting the mZI region, including somatostatin (SOM^+), calretinin (CR^+), and $vGlut2^+$ neurons, did not change locomotor distance travelled in the OFT (Z. Li et al., 2021). However, multiple populations being photostimulated or targeting more medial populations in the ZI may contribute to the differences. Our findings align with mini-endoscope recordings from $CaMKII\alpha^+$ rostral ZI cells, which overlap the A13 showing subpopulations whose activity correlates with either movement speed or anxiety-related locations (Z. Li et al., 2021). Other work has found that photoactivation of GABAergic mZI pathways which project to the cuneiform promote exploratory activity by inhibiting cuneiform vGlut2 neurons (Sharma et al., 2024). The prolocomotory patterns following activation of mZI GABA populations and the more general activation of the region found in this work differ. The activity patterns with CamII kinase promotor transfection of the region produced a marked effect on locomotor speed, accompanied by thigmotactic behaviors not observed when mZI GABAergic populations are activated. Prolocomotory observed from the mZI region by us and others differ from the effects seen when lateral GABAergic ZI populations (dorsal and ventral ZI) are stimulated. Microinjection of $GABA_A$ receptor agonists, into the ZI results in a significant reduction in locomotor distance and velocity or cataplexy (Chen et al., 2023; Wardas et al., 1988). Alternatively, suppression of GABAergic ZI activity can increase locomotion following microinjection of $GABA_A$ receptor antagonists (Périer et al., 2002) or induce bradykinesia and akinesia by chemogenetic or optogenetic inhibition in healthy naive mice (Chen et al., 2023). The differing results likely are due to the projection patterns of GABAergic populations.

Because the A13 region photoactivation produces increased locomotor speed and improved descent times on the pole test, it highlights its role in movement control. Since A13 stimulation did not alter coordination during the task, it suggests a complex behavioral role consistent with its upstream location from the brainstem and its extensive connectome. A13 photoactivation increases animal speed, duration, and distance travelled. Taken together, these findings represent a rescue of function in the 6-OHDA model. Interestingly, in 6-OHDA and sham mice there was a latency of between 10 and 15 s on average following photostimulation before locomotion was initiated. Such delays are typical of stimulating sites upstream of the cuneiform, such as the dlPAG, which has a delay of several seconds (Tsang et al., 2021).

Photoactivation of the A13 reduces bradykinesia and akinesia in mouse PD models

While much work has targeted basal ganglia structures to address PD symptoms (DeLong and Wichmann, 2015), our research demonstrates that photoactivation of the A13 region can alleviate both bradykinesia and akinesia in 6-OHDA mice. Our work shows that A13 projections are affected at cortical and striatal levels following 6-OHDA, consistent with our observed changes in locomotor function. Over 28 days, there was a remarkable change in the afferent and efferent A13 regional connectome, despite the preservation of TH^+ ZI cells. This is consistent with previous reports of widespread connectivity of the ZI

(Mitrofanis, 2005). The preservation of A13 is expected since A13 lacks DAT expression (Bolton et al., 2015; Negishi et al., 2020; Sharma et al., 2018) and is spared from DAT-mediated toxicity of 6-OHDA (Dauer and Przedborski, 2003; Konnova et al., 2018; Simola et al., 2007). While A13 cells were spared following nigrostriatal degeneration, our work demonstrates its connectome was rewired. The ipsilateral afferent projections were markedly downregulated, while contralesional projecting afferents showed upregulation. In contrast, efferent projections showed less downregulation in the cortical subplate regions and bilateral upregulation of thalamic and hypothalamic efferents. Similar timeframes for anatomical and functional plasticity affecting neurons and astrocytes following an SNc or MFB 6-OHDA have been previously reported (Bosson et al., 2015; Perović et al., 2005; Requejo et al., 2020). Human PD brains that show degeneration of the SNc have a preserved A13 region, suggesting that our model, from this perspective, is externally valid (Matzuk and Saper, 1985).

Combined with photoactivation of the A13 region, we provide evidence for plasticity following damage to SNc. A previous brain-wide quantification of TH levels in the MPTP mouse model identified additional complexity in regulating central TH expression compared to conventional histological studies (Roostalu et al., 2019). These authors reported decreased SNc TH^+ cell numbers without a significant change in TH^+ intensity in SNc and increased TH^+ intensity in limbic regions such as the amygdala and hypothalamus (Roostalu et al., 2019). Still, there was a downstream shift in the distribution pattern of A13 efferents following nigrostriatal degeneration with a reduction in outputs to cortical and striatal subregions. This suggests A13 efferents are more distributed across the neuraxis than in sham mice. One hypothesis arising from our work is that the preserved A13 efferents could provide compensatory innervation with collateralization mediated contralesionally and, in some subregions, ipsilesionally to increase the availability of extracellular dopamine.

Several A13 efferent targets could be responsible for rotational asymmetry. In a unilateral 6-OHDA model, ipsiversive circling behaviour is indicative of intact striatal function on the contralesional side (Carey, 1991; Schwarting et al., 1991; Ungerstedt, 1971; Zetterström et al., 1986). Instead, the predictive value of a treatment is determined by contraversive circling mediated by increased dopamine receptor sensitivity on the ipsilesional striatal terminals (Costall et al., 1976; Lane et al., 2006). Our findings show that A13 stimulation enhances ipsiversive circling and may represent a gain of function on the intact side, but this may be simply due to 6-OHDA mice having reduced locomotion overall. Given the preservation of A13 cells in PD, bilateral stimulation of A13 could potentially reduce motor asymmetry and alleviate bradykinesia and akinesia.

With the induction of a 6-OHDA lesion, there is a change in the A13 connectome, characterized by a reduction in bidirectional connectivity with ipsilesional cortical regions. In rodent models, the motor cortices, including the M1 and M2 regions, can shape rotational asymmetry (Gradinaru et al., 2009; Magno et al., 2019; Sanders and Jaeger, 2016; Valverde et al., 2020). Activation of M1 glutamatergic neurons increases the rotational bias (Valverde et al., 2020), while M2 neuronal stimulation promotes contraversive rotations (Magno et al., 2019). Our data suggest that A13 photoactivation may have resulted in the inhibition of glutamatergic neurons in the contralesional M1. An alternative possibility is the activation of the contralesional M2 glutamatergic neurons, which would be expected to induce increased ipsilesional rotations (Magno et al., 2019). The ZI could generate rotational bias by A13 modulation of cZI glutamatergic neurons via incerto-incertal fibers (Ossowska, 2019; Power and Mitrofanis, 1999),

which promotes asymmetries by activating the SNr (L.-X. Li et al., 2021). The incerto-incertal interconnectivity has not been well studied, but the ZI has a large degree of interconnectivity (Sharma et al., 2018; Tsang et al., 2021) along all axes and between hemispheres (Power and Mitrofanis, 1999). However, this may only contribute minimally given that unilateral photoactivation of the A13 cells in sham mice failed to produce ipsiversive turning behavior while unilateral photoactivation of cZI glutamatergic neurons in sham animals was sufficient in generating ipsiversive turning behavior (L.-X. Li et al., 2021). Another possibility involves the A13 region projections to the MLR. With the unknown downstream effects of A13 photoactivation, there may be modulation of the PPN neurons responsible for this turning behavior (Masini and Kiehn, 2022). The thigmotactic behaviors suggest some effects may be mediated through dlPAG and CUN (Tsang et al., 2021), and recent work suggests the CUN as a possible therapeutic target (Fougère et al., 2021; Noga and Whelan, 2022). Since PD is a heterogeneous disease, our data provide another therapeutic target providing context-dependent relief from symptoms. This is important since PD severity, symptoms, and progression are patient-specific.

Towards a preclinical model

To facilitate future translational work applying DBS to this region, we targeted the A13 region using AAV8-CamKII-mCherry viruses. The CaMKIIα promoter virus is beneficial because it is biased towards excitatory cells (Haery et al., 2019), narrowing the diversity of the transfected A13 region and when combined with traditional therapies, such as L-DOPA, it could be a translatable strategy (Watakabe et al., 2015; Watanabe et al., 2020). Optogenetic strategies have been used to activate retinal cells in humans, partially restoring visual function and providing optimism that AAV-based viral strategies can be adapted in other human brain regions (Sahel et al., 2021). A more likely possibility for stimulation of deep nuclei is that DREADD technology could be adapted, which would not require any implants, however, this remains speculative. Near-term, our work suggests the A13 is a possible target for DBS. Gait dysfunction in PD is particularly difficult to treat, and indeed when DBS of subthalamic nucleus is deployed, a mixture of unilateral and bilateral approaches have been used (Lizarraga et al., 2016), along with stimulation of multiple targets (Stefani et al., 2007). This represents the heterogeneity of PD and underlines the need for considering multiple targets. DBS does not always have the same outcomes as optogenetic stimulation (Neumann et al., 2023). Alternative stimulation strategies such as cyclic burst DBS (Fischer et al., 2020) may provide ways to augment or circumvent the need for optogenetic approaches to be deployed. Future work may want to consider a multipronged strategy to hone burst stimulation parameters with identification of cell populations to deploy DBS in a more targeted manner (Spix et al., 2021).

Limitations

Currently, few PD animal models are available that adequately model the progression and the extent of SNc cellular degeneration while meeting the face validity of motor deficits (Dauer and Przedborski, 2003; Konnova et al., 2018). While the 6-OHDA models fail to capture the age-dependent chronic degeneration observed in PD, they lead to robust motor deficits with acute degeneration and allow for compensatory changes in connectivity to be examined. Moreover, the 6-OHDA lesions resemble the unilateral onset (Hughes et al., 1992) and persistent asymmetry (Lee et al., 1995) of motor dysfunction in PD. Another option could be the MPTP mouse model, which offers the ease of systemic administration and translational value to primate models; however, the motor deficits are variable and lack the asymmetry observed in human patients (Hughes et al., 1992; Jagmag et al., 2015; Lee et al., 1995; Meredith and

Rademacher, 2011). Despite these limitations, the neurotoxin-based mouse models, such as MPTP and 6-OHDA, offer greater SNc cell loss than genetic-based models; in the case of the 6-OHDA model, it captures many aspects of motor dysfunctions in PD (Dauer and Przedborski, 2003; Jagmag et al., 2015; Konnova et al., 2018; Simola et al., 2007). As in human PD, we found no significant change in A13 TH^+ cell counts (Matzuk and Saper, 1985). Our work suggests a possibility that glutamatergic neurons may be involved, however future work will be required to tease apart the relative contributions of neuronal populations within the A13 region contributing to the effects shown here. A more comprehensive approach with opto-tagging specific cell classes and specific labeling of glutamatergic versus dopaminergic classes could be an option, especially considering work showing the presence of multiple classes of dopamine cells in the hypothalamus (Romanov et al., 2017) . Another limitation is that since A13 neurons remained intact following a lesion, it is possible that changes in connectome reflected secondary effects from other regions impacted by the 6-OHDA lesion. However, the fact that there was a significant change in the connectome post-6-OHDA injection and striatonigral degeneration is in and of itself important to document.

Conclusions

Our research underscores the role of the A13 region beyond the classic nigrostriatal axis in Parkinson's disease, driving locomotor activity and mitigating bradykinetic and akinetic deficits linked to impaired DAergic transmission. This observation indicates a rescue of locomotion loss in 6-OHDA-lesioned mice, as well as bradykinesia. Additionally, it produced possible gain-of-function effects, such as circling behavior, which may be attributed to plasticity changes induced by the 6-OHDA lesions. Widespread remodelling of the A13 region connectome is critical to our understanding of the effects of dopamine loss in PD models. In summary, our findings support an exciting role for the A13 region in locomotion with demonstrated benefits in a mouse PD model and contribute to our understanding of heterogeneity in PD.

MATERIALS AND METHODS

Animals

All care and experimental procedures were approved by the University of Calgary Health Sciences Animal Care Committee (Protocol #AC19-0035). C57BL/6 male mice 49 - 56 days old (weight: M = 31.7 g, SEM = 2.0 g) were group-housed (< five per cage) on a 12-h light/dark cycle (07:00 lights on - 19:00 lights off) with *ad libitum* access to food and water, as well as cat's milk (Whiskas, Mars Canada Inc., Bolton, ON, Canada). Mice were randomly assigned to the groups described.

Surgical Procedures

We established a well-validated unilateral 6-OHDA mediated Parkinsonian mouse model (Thiele et al., 2012) (Figure 1, Movie S4). 30 minutes before stereotaxic microinjections, mice were intraperitoneally injected with desipramine hydrochloride (2.5 mg/ml, Sigma-Aldrich) and pargyline hydrochloride (0.5 mg/ml, Sigma-Aldrich) at 10 ml/kg (0.9% sterile saline, pH 7.4) to enhance selectivity and efficacy of 6-OHDA induced lesions (Thiele et al., 2012). All surgical procedures were performed using aseptic techniques, and mice were anesthetized using isoflurane (1 - 2%) delivered by 0.4 L/min of medical-grade oxygen (Vitalair 1072, 100% oxygen).

Mice were stabilized on a stereotaxic apparatus. Small craniotomies were made above the medial forebrain bundle (MFB) and the A13 nucleus within one randomly assigned hemisphere. Stereotaxic

microinjections were performed using a glass capillary (Drummond Scientific, PA, USA; Puller Narishige, diameter 15 – 20 mm) and a Nanoject II apparatus (Drummond Scientific, PA, USA). 240 nL of 6-OHDA (3.6 µg, 15.0 mg/mL; Tocris, USA) was microinjected into the MFB (AP -1.2 mm from bregma; ML ±1.1 mm; DV -5.0 mm from the dura). Sham mice received a vehicle solution (240 nL of 0.2% ascorbic acid in 0.9% saline; Tocris, USA).

Whole Brain Experiments

For tracing purposes, a 50:50 mix of AAV8-CamKII-mCherry (Neurophotonics, Laval University, Quebec City, Canada, Lot #820, titre 2×10^{13} GC/ml) and AAVrg-CAG-GFP (Addgene, Watertown, MA, Catalogue #37825, Lot #V9234, titre $\geq 7\times10^{12}$ vg/mL) was injected ipsilateral to 6-OHDA injections at the A13 nucleus in all mice (AP -1.22 mm from bregma; ML ±0.4 mm; DV -4.5 mm from the dura, the total volume of 110 nL at a rate of 23 nL/s). Post-surgery care was the same for both sham and 6-OHDA injected mice. The animals were sacrificed 29 days after surgery.

Photoactivation Experiments

36.8 nL of AAVDJ-CaMKIIα-hChR2(H134R)-eYFP (UNC Stanford Viral Gene Core; Stanford, CA, US, Catalogue #AAV36; Lots #3081 and #6878, titres 1.9×10^{13} and 1.7×10^{13} GC/mL, respectively) or eYFP control virus (AAVDJ-CaMKIIα-eYFP; Lots #2958 and #5510, titres 7.64×10^{13} and 2.88×10^{13} GC/mL, respectively) were injected into the A13 (AP: -1.22 mm; ML -0.5 mm from the Bregma; DV -4.5 mm from the dura). A mono-fiber cannula (Doric Lenses, Quebec, Canada, Catalogue #B280-2401-5, MFC_200/230-0.48_5mm_MF2.5_FLT) was implanted slowly 300 µm above the viral injection site. Metabond® Quick Adhesive Cement System (C&B, Parkell, Brentwood, NY, US) and Dentsply Repair Material (Dentsply International Inc., York, PA, USA) were used to fix the optical fiber in place. Animals recovered from the viral surgery for 19 days before follow-up behavioral testing. Figure 1 shows a timeline of behavioral tests.

ChR2 photoactivation

A Laserglow Technologies 473 nm laser and driver (LRS-0473-GFM-00100-05, North York, ON, Canada) were used to generate the photoactivation for experiments. The laser was triggered by TTL pulses from an A.M.P.I. Master-8 stimulator (Jerusalem, Israel) or an Open Ephys PulsePal (Sanworks, Rochester, NY, US) set to 20 Hz, 10-ms pulse width and 5 mW power. All fiber optic implants were tested for laser power before implantation (Thorlabs, Saint-Laurent, QC, Canada; optical power sensor (S130C) and meter (PM100D)). The Stanford Optogenetics irradiance calculator was used to estimate the laser power for stimulation ("Stanford Optogenetics Resource Center," n.d.). A 1×2 fiber-optic rotary joint (Doric Lenses, Quebec, Canada; FRJ_1x2i_FC-2FC_0.22) was used. The animals' behaviors were recorded with an overhead camera (SuperCircuits, Austin, TX, US; FRJ_1x2i_FC-2FC_0.22; 720 x 480 resolution; 30 fps). The video was processed online (Cleversys, Reston, VA; TopScan V3.0) with a TTL signal output from a National Instruments 24-line digital I/O box (NI, Austin, TX, US; USB-6501) to the Master-8 stimulator.

Behavioral Testing

Open Field Test

Each mouse was placed in a square arena measuring 70 (W) x 70 (L) x 50 (H) cm with opaque walls and recorded for 30 minutes using a vertically mounted video camera (Model PC165DNR, Supercircuits,

Austin, TX, USA; 30 fps). 19 days following surgery, mice were habituated to the open field test (OFT) arena with a patch cable attached for three days in 30 minute sessions to bring animals to a common baseline of activity. On experimental days, after animals were placed in the OFT, a one-minute-on-three-minutes-off paradigm was repeated five times following an initial ten minutes baseline activity. Locomotion was registered when mice traveled a minimum distance of 10 cm at 6 cm/s for 20 frames over a 30-frame segment. When the mouse velocity dropped below 6 cm/s for 20 frames, locomotion was recorded as ending. Bouts of locomotion relate to the number of episodes where the animal met these criteria. Velocity data were obtained from the frame-by-frame results and further processed in a custom Python script to detect instantaneous speeds greater than 2 cm/s (Masini and Kiehn, 2022). All animals that had validated targeting of the A13 region were included in the OFT data presented in the results section, except for one sham ChR2 animal, which showed grooming rather than the typical locomotor phenotypes.

Pole Test

Mice were placed on a vertical wooden pole (50 cm tall and 1 cm diameter) facing upwards and then allowed to descend the pole into their home cage (Glajch et al., 2012). Animals were trained for three days and tested 2-5 days pre-surgery. Animals were acclimatized 21-22 days post-surgery under two conditions: without a patch cable and with the patch cable attached without photoactivation. On days 24-27, experimental trials were recorded with photoactivation. Video data were recorded for a minimum of three trials (Canon, Brampton, ON, Canada; Vixia HF R52; 1920 x 1080 resolution; 60 fps). A blinded scorer recorded the times for the following events: the hand release of the animal's tail, the animal fully turning to descend the pole, and the animal reaching the base of the apparatus. Additionally, partial falls, where the animal slipped down the pole but did not reach the base, and full falls, where the animal fell to the base, were recorded separately. All validated animals were included in the quantified data, including the sham ChR2 animal that began grooming in the OFT upon photoactivation. This animal displayed proficiency in performing the PT during photoactivation. It started grooming upon completion of the task when photoactivation was on. One sham ChR2 animal was photostimulated at 1 mW since it would jump off the apparatus at higher stimulation intensities.

Immunohistochemistry

A13 and SNc region

Post hoc analysis of the tissue was performed to confirm the 6-OHDA lesion and validate the targeting of the A13 region. Following behavioral testing, animals underwent a photoactivation protocol to activate neurons below the fiber optic tip (Koblinger et al., 2018). Animals were placed in an OFT for ten minutes before receiving three minutes of photoactivation. Ten minutes later, the animals were returned to their home cage. 90 minutes post photoactivation, animals were deeply anesthetized with isoflurane and then transcardially perfused with room temperature PBS followed by cold 4% paraformaldehyde (PFA) (Sigma-Aldrich, Catalogue #441244-1KG). The animals were decapitated, and the whole heads were incubated overnight in 4% PFA at 4°C before the fiber optic was removed and the brain removed from the skull. The brain tissue was post-fixed for another 6 - 12 hours in 4% PFA at 4°C then transferred to 30% sucrose solution for 48 - 72 hours. The tissue was embedded in VWR® Clear Frozen Section Compound (VWR International LLC, Radnor, PA, US) and sectioned coronally at 40 or 50 µm using a Leica cryostat set to -21°C (CM 1850 UV, Concord, ON, Canada). Sections from the A13 region (-0.2 to -2.0 mm past bregma) and the SNc (-2.2 to -4.0 mm past bregma) were collected and stored in PBS containing 0.02%

(w/v) sodium azide (EM Science, Catalogue #SX0299-1, Cherry Hill, NJ, US) (Paxinos and Franklin, 2008).

Immunohistochemistry staining was done on free-floating sections. The A13 sections were labelled for c-Fos, TH, and GFP (to enhance eYFP viral signal), and received a DAPI stain to identify nuclei. The SNc sections were stained with TH and DAPI (Table 1). Sections were washed in PBS (3 x 10 mins) then incubated in a blocking solution comprised of PBS containing 0.5% Triton X-100 (Sigma-Aldrich, Catalogue #X100-500ML, St. Louis, MO, US) and 5% donkey serum (EMD Millipore, Catalogue #S30-100ML, Billerica, MA, USA) for 1 hour. This was followed by overnight (for SNc sections) or 24-hour (for A13 sections) incubation in a 5% donkey serum PBS primary solution at room temperature. On day 2, the tissue was washed in PBS (3 x 10 mins) before being incubated in a PBS secondary solution containing 5% donkey serum for 2 hours (for SNc tissue) or 4 hours (for A13 tissue). The secondary was washed with a PBS solution containing 1:1000 DAPI for 10 mins, followed by a final set of PBS washes (3 x 10 mins). Tissue was mounted on Superfrost® micro slides (VWR, slides, Radnor, PA, US) with mounting media (Vectashield®, Vector Laboratories Inc., Burlingame, CA, US), covered with #1 coverslips (VWR, Radnor, PA, US) then sealed.

Whole Brain

Mice were deeply anesthetized with isoflurane and transcardially perfused with PBS, followed by 4% PFA. To prepare for whole brain imaging, brains were first extracted and postfixed overnight in 4% PFA at 4°C. The next day, a modified iDISCO method (Renier et al., 2014) was used to clear the samples and perform quadruple immunohistochemistry in whole brains. The modifications include prolonged incubation and the addition of SDS for optimal labelling. Antibodies used are listed in Table 1 and the protocol is provided in Table 2.

Image Acquisition and Analysis

Photoactivation Experiments

All tissue was initially scanned with an Olympus VS120-L100 Virtual Slide Microscope (UPlanSApo, 10x and 20x, NA = 0.4 and 0.75). Standard excitation and emission filter cube sets were used (DAPI, FITC, TRITC, Cy5), and images were acquired using an Orca Flash 4.0 sCMOS monochrome camera (Hamamatsu, Bridgewater Township, NJ, US). For c-Fos immunofluorescence, A13 sections of the tissue were imaged with a Leica SP8 FALCON (FAst Lifetime CONtrast) scanning confocal microscope equipped with a tunable laser and using a 63x objective (HC PlanApo, NA = 1.40).

SNc images were imported into Adobe Illustrator, where the SNc (Fougère et al., 2021), including the pars lateralis (SNl), was delineated using the TH immunostaining together with the medial lemniscus and cerebral peduncle as landmarks (bregma -3.09 and -3.68 mm) (Iancu et al., 2005; Paxinos and Franklin, 2008; Stott and Barker, 2014). Cell counts were obtained using a semi-automated approach using an Ilastik (v1.4.0b15) (Berg et al., 2019) trained model followed by corrections by a blinded counter (Fougère et al., 2021; Iancu et al., 2005). Targeting was confirmed on the 10x overview scans of the A13 region tissue by the presence of eYFP localized in the mZI around the A13 TH^+ nucleus, the fiber optic tip being visible near the mZI and A13 nucleus, and the presence of c-Fos positive cells in $ChR2^+$ tissue. C-Fos expression colocalization within the A13 region was performed using confocal images. The mZI &

A13 region was identified with the 3rd ventricle and TH expression as markers (Paxinos and Franklin, 2008).

Whole Brain Experiments

Cleared whole brain samples were imaged using a light-sheet microscope (LaVision Biotech UltraMicroscope, LaVision, Bielefeld, Germany) with an Olympus MVPLAPO 2x objective with 4x optical zoom (NA = 0.475) and a 5.7 mm dipping cap that is adjusted for the high refractive index of 1.56. The brain samples were imaged in an ethyl cinnamate medium to match the refractive indices and illuminated by three sheets of light bilaterally. Each light sheet was 5 µm thick, and the width was set at 30% to ensure sufficient illumination at the centroid of the sample. Laser power intensities and chromatic aberration corrections used for each laser were as follows: 10% power for 488 nm laser, 5% power for 561 nm laser with 780 nm correction, 40% power for 640 nm laser with 960 nm correction, and 100% power for 785 nm laser with 1,620 nm correction. Each sample was imaged coronally in 8 by 6 squares with 20% overlap (10,202 µm by 5,492 µm in total) and a z-step size of 15 µm (xyz resolution = 0.813 µm x 0.813 µm x 15 µm). While an excellent choice for our work, confocal microscopy offers better resolution at the expense of time. To gain a better resolution using a light-sheet microscope in select regions (eg. SNc and A13 cells), we increased the optical zoom to 6.3x.

A13 Connectome Analysis

Images were processed using ImageJ software (Schneider et al., 2012). Raw images were stitched, and a z-encoded maximum intensity projection across a 90 µm thick optical section was obtained across each brain. 90 µm sections were chosen because the 2008 Allen reference atlas images are spaced out at around 100 µm. Brains with insufficient quality in labeling were excluded from analysis (n = 1 of three sham and n = 3 of six 6-OHDA mice). Instructions for identifying YFP^+ or TH^+ cells to annotate were provided to the manual counters. YFP^+ and TH^+ cells were manually counted using the Cell Counter Plug-In (ImageJ). $mCherry^+$ fibers were segmented semi-automatically using Ilastik software (Berg et al., 2019) and quantified using particle analysis in ImageJ. Images and segmentations were imported into WholeBrain software to be registered with the 2008 Allen reference atlas (Fürth et al., 2018). The TO-PRO™-3 and TH channels were used as reference channels to register each section to a corresponding atlas image. ImageJ quantifications of cell and fiber segmentations were exported in XML formats and registered using WholeBrain software. To minimize the influence of experimental variation on the total labeling of neurons and fibers, the afferent cell counts or efferent fiber areas in each brain region were column divided by the total number found in a brain to obtain the proportion of total inputs and outputs. Connectome analyses were performed using custom R scripts (L. H. Kim et al., 2021). For interregional correlation analyses, the data were normalized to a $\log_{10}$ value to reduce variability and bring brain regions with high and low proportions of cells and fibers to a similar scale. The consistency of afferent and efferent proportions between mice was compared in a pairwise manner using Spearman's correlation (Figure S6).

Quantification of 6-OHDA mediated TH^+ cell loss.

The percentage of TH^+ cell loss was quantified to confirm 6-OHDA mediated SNc lesions. TH^+ cells within ZI, VTA and SNc areas from 90 µm thick optical brain slice images (AP: -0.655 to -3.88 mm from bregma) were manually counted by two blinded counters (n = 3 sham and n = 6 6-OHDA mice; ZI region in 2 of 6 6-OHDA mice were excluded due to presence of abnormal scarring/healing at the injection site

of viruses). Subsequently, WholeBrain software (Fürth et al., 2018) was used to register and tabulate TH^+ cells in the contralesional and ipsilesional brain regions of interest. Counts obtained from the two counters were averaged per region. The percentage of TH^+ cell loss was calculated by dividing the difference in counts between contralesional and ipsilesional sides by the contralesional side count and multiplying by 100%.

Statistical analyses

All data were tested for normality using a Shapiro-Wilk test to determine the most appropriate statistical tests. The percent ipsilesional TH^+ neuron loss within the SNc as defined above using a Pearson correlation (Fougère et al., 2021) was used to ascertain the effect of the 6-OHDA lesion on behavior. A Wilcoxon rank-sum test was performed for comparisons within subjects at two timepoints where normality failed, and the central limit theorem could not be applied. The two groups were compared using an unpaired t-test with Welch's correction. A mixed model ANOVA (MM ANOVA) was used to compare the effects of group type, injection type and time. Additionally, Mauchly's test of sphericity was performed to account for differences in variability within the repeated measures design. A Greenhouse Geisser correction was applied to all ANOVAs where Mauchly's test was significant for RM and MM ANOVAs. The *post hoc* multiple comparisons were run when the respective ANOVAs reached significance using Dunnett's or Dunn's tests for repeated measures of parametric and non-parametric tests, respectively. The pre-stimulation timepoints were used as the control time point to determine if stimulation altered behavior. A Bonferroni correction was added for *post hoc* comparisons following a MM ANOVA between groups at given time points to control for alpha value inflation. All correlations, t-tests, and ANOVAs were performed, and graphs were created using Prism version 9.3.1 (Graphpad) or SPSS (IBM, 28.0.1.0). Full statistical reporting is in Supplemental Statistics.xls.

Figures

Figures were constructed using Adobe Photoshop, Illustrator, and Biorender.

Primary Antibody	Species	Type	Supplier	Catalogue #	Dilution	Tissue Region
c-Fos	Rabbit	Polyclonal	Synaptic Systems (Göttingen, Germany)	226 003	1:1000	A13
GFP	Chicken	Polyclonal	Aves Lab (Tigard, OR)	GFP-1010	1:1000 1:5000	Whole brain A13
mCherry	Rat	Monoclonal	Invitrogen, Thermo Fisher Scientific (Waltham, MA)	M11217	1:500	Whole brain
TH	Rabbit	Polyclonal	Abcam (Cambridge, UK)	AB112	1:500 1:1000	Whole brain SNc
TH	Sheep	Polyclonal	Millipore Sigma (Burlington, MA)	AB1542	1:500	A13
Secondary Antibody			Supplier	Catalogue #	Dilution	Tissue Region
Alexa Fluor® 488 Donkey Anti-Chicken			Jackson ImmunoResearch Inc. (Westgrove, PA)	703-545-155	1:200 1:1000	Whole brain A13
Alexa Fluor® 594 Donkey Anti-Rabbit			Invitrogen, Thermo Fisher Scientific	A-21207	1:500	A13
Alexa Fluor® 647 Donkey Anti-Rabbit			Invitrogen, Thermo Fisher Scientific	A-31573	1:1000	SNc
Alexa Fluor® 647 Donkey Anti-Sheep			Invitrogen, Thermo Fisher Scientific	A-21448	1:1000	A13
Alexa Fluor® 790 Donkey Anti-Rabbit			Invitrogen, Thermo Fisher Scientific	A-11374	1:200	Whole brain
Cy™3 AffiniPure Donkey Anti-Rat			Jackson ImmunoResearch Inc.	712-165-153	1:200	Whole brain
Nuclear Stain			Supplier	Catalogue #	Dilution	Tissue Region
DAPI			Invitrogen, Thermo Fisher Scientific	D1306	1:1000	A13, SNc
TO-PRO™-3 Iodide (642/661)			Invitrogen, Thermo Fisher Scientific	T3605	1:5000	Whole brain

Table 1. List of antibodies used for immunohistochemical staining of the A13 and SNc regions, as well as the whole brain.

Day #	Instructions
1	Washed 2 x 30 minutes in PBS on a shaker at room temperature.
2	Dehydrated tissue in methanol/H_2O series of 20%, 40%, 60%, 80%, 100%, 100% (1 hour each at room temperature) then left overnight in a 66% dichloromethane/33% methanol solution.
3	Washed twice in 100% methanol at room temperature and then chilled the samples at 4°C. Bleached the samples in chilled fresh 5% H_2O_2 in methanol (1 volume 30% H_2O_2 to 5 volumes methanol), overnight at 4°C.
4	Rehydrated in methanol/water series of 80%, 60%, 40%, 20%, PBS (1 hour each at room temperature). Washed twice in PTx.2 (0.2% Triton™ X-100 in PBS). Incubated the samples in Permeabilization Solution at 37°C for 2 days.
6	Washed 3 x 1-2 hours in 0.5mM SDS/PBS at 37°C then incubated for 3 days.
9	Incubated with the primary antibody in 0.5mM SDS/PBS at 37°C for 3 days.
12	Refreshed with the primary antibody in PTx.2 at 37°C then incubated for 4 days.
17	Washed 5 x 2 hours in PTwH (0.2% Tween® 20 and 10 mg/mL Heparin stock solution in PBS) and incubated at 37°C overnight.
18	Incubated with secondary antibody in PTwH/3% Donkey Serum at 37°C for 3 days.
21	Refreshed with secondary antibody in PTwH/3% Donkey Serum at 37°C for 4 days.
25	Washed 5 x 2 hours in PTwH then incubated at 37°C overnight.
26	Dehydrated in methanol/water series of 20%, 40%, 60%, 80%, 100% (1 hour each at room temperature). Refreshed the samples with 100 % methanol and left overnight at room temperature.
27	Incubated in 66% DCM/33% methanol for 3 hours on a shaker at room temperature. Then incubated in 100% DCM (Sigma 270997-12X100ML) for 2 x 15 minutes (with shaking) to wash away methanol. Incubated samples with ethyl cinnamate for 3 hours at room temperature with shaking. Refreshed ethyl cinnamate then left at room temperature for imaging.

Table 2. Protocol for Whole Brain Clearing.

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DATA AVAILABILITY
All datasets and code will be available in a public repository.

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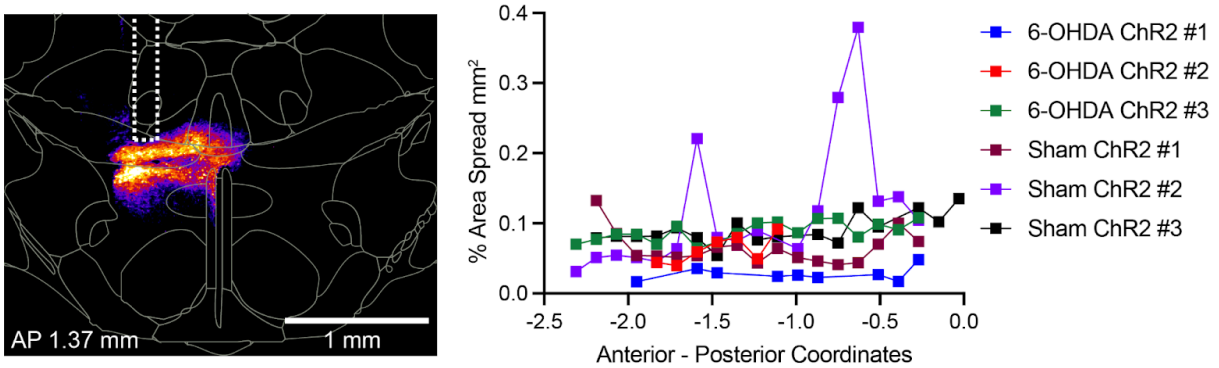

Figure S1. Quantification of channelrhodopsin viral spread in the rostral-caudal direction from the injection site in 6-OHDA-treated and sham animals. The left panel shows a representative image of viral spread, including the optic fibre track, visualized using the "fire" lookup table in FIJI/ImageJ software, with targeting precision confirmed by coronal overlays from the Mouse Allen Brain Atlas to the A13/Mzi region. The right panel displays a graph illustrating the percentage of viral spread across anterior-posterior coordinates for all ChR2-infected mice, calculated as the ratio of viral expression area to total tissue section area. Data were obtained from mouse brain sections injected with AAVDJ-CaMKIIα-ChR2 into the A13/Mzi region, sectioned at 50 µm, and analyzed using the VS120 Virtual Slide Scanner. Quantification was performed by blinded analysts using FIJI/ImageJ, with defined regions of interest (ROIs).

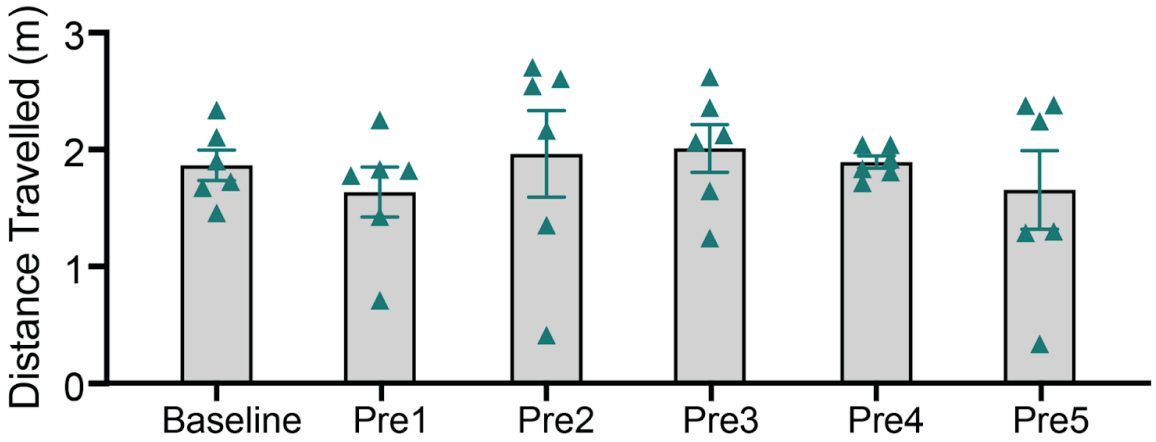

Figure S2. Time course of open field locomotion distance travelled over 30 minutes. Locomotion distance travelled for the six sham ChR2 animals at baseline and at the five pre-stimulation timepoints compared using a 1-way RM ANOVA ($F_{5,25} = 0.49$, $p = 0.78$). Data indicate mean ± SEM bars.

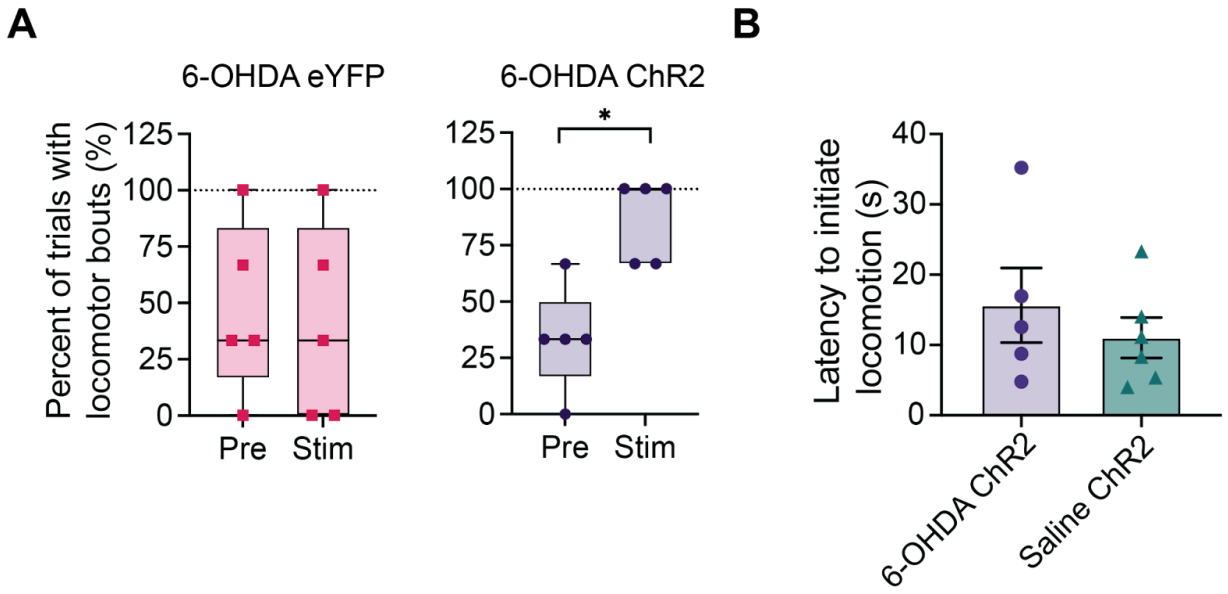

Figure S3. Characterization of A13 region photoactivation temporal dynamics on locomotion initiation. (A) Percent of trials where there was at least one bout of locomotion. Data are plotted as box and whiskers with the horizontal line through the box indicating the group median, interquartile range indicated by the limits of the box, and group minimum and maximum indicated by the whiskers. Asterisks indicate significant comparisons using the Wilcoxon signed-rank test: * $p < 0.05$. **(B)** The average time for the ChR2 group animals to begin locomotion after the onset of photoactivation was not different from sham mice ($p = 0.953$). Means plotted with error bars indicating ± SEM.

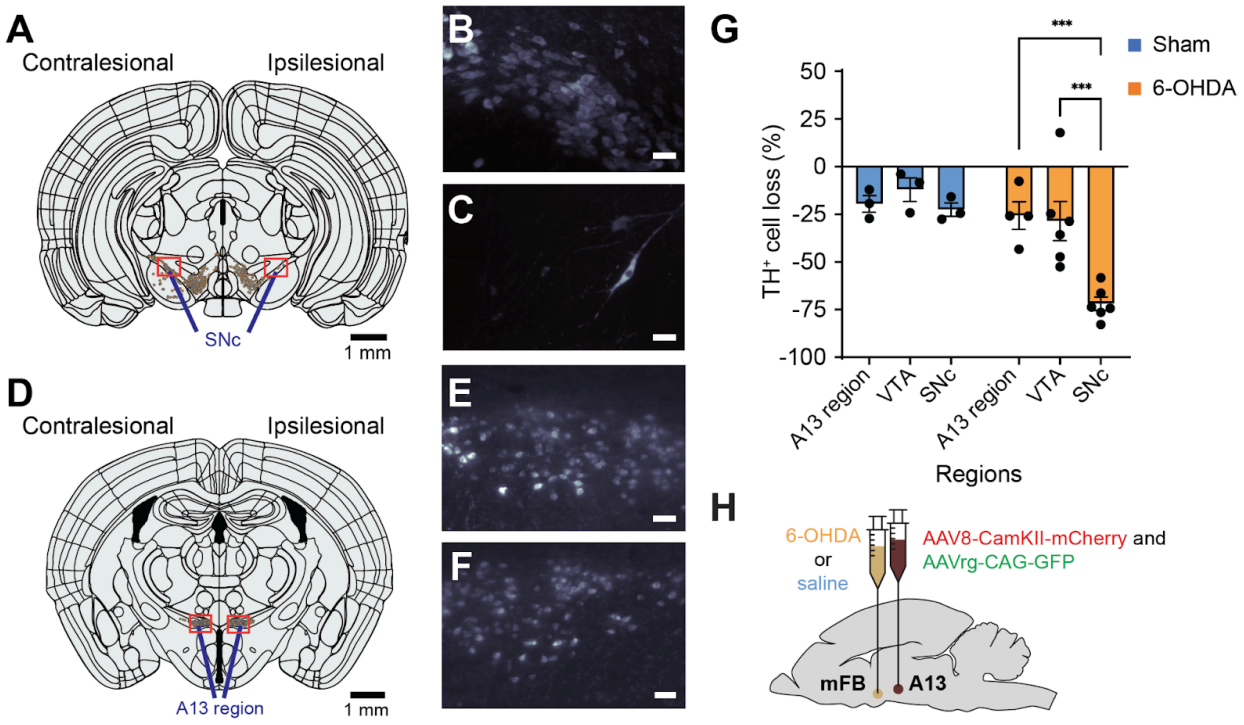

Figure S4. **Preservation of TH$^+$ A13 cells in Parkinsonian mouse models.** Representative slices of SNc (AP: -3.08 mm, **A**) and A13 region (AP: -1.355 mm, **D**) following registration with WholeBrain software. Full 3D brain is available (see Movie S4). There was a lack of TH$^+$ SNc cells following 6-OHDA injections at the MFB **(A)**. **(B, C)** Zoomed sections (90 µm thickness) of red boxes in panel A in left to right order. Meanwhile, TH$^+$ VTA cells were preserved bilaterally. In addition, TH$^+$ A13 cells were present ipsilesional to 6-OHDA injections **(D)**. **(E, F)** Zoomed sections (90 µm thickness) of red boxes in panel D in left to right order. When calculating the percentage of TH$^+$ cell loss normalized to the intact side, there was a significant interaction between the condition group and brain regions (repeated measures two-way ANOVA with *post hoc* Bonferroni pairwise, sham: $n = 3$, 6-OHDA: $n = 6$) **(G)** 6-OHDA-treated mice showed a significantly greater percentage of TH$^+$ cell loss in SNc compared to VTA and A13 region (VTA vs. SNc: $p = 0.005$; A13 region vs. SNc: $p < 0.05$). In contrast, sham showed no significant difference in TH$^+$ cell loss across SNc, VTA and A13 region regions ($p > 0.05$). *$p < 0.05$, and **$p < 0.01$. (**H**) Dual ipsilateral stereotaxic injection into the MFB and A13 region. Scale bars are 50 µm unless otherwise indicated.

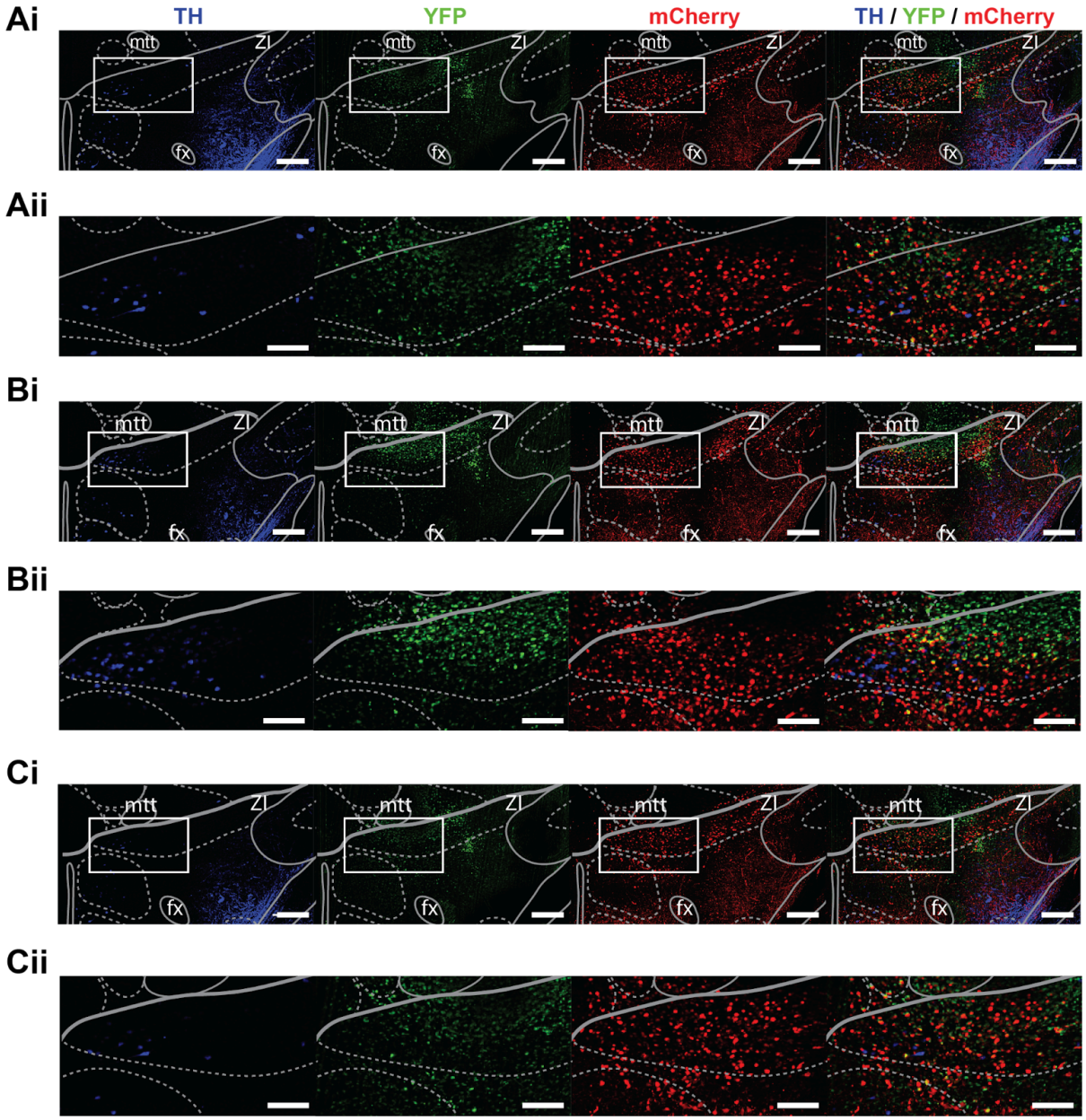

Figure S5. **Example of the injection core in a sham brain for viral tracers and the rostral and caudal spread to the injection site (A13).** Viral tracers (AAV8-CamKII-mCherry and AAVrg-CAG-GFP) were mixed 50:50. Light-sheet images around the injection site were obtained with 2x objective, 6.3x optical zoom, and a z-step size of 2 µm (xyz resolution = 0.477 µm x 0.477 µm x 2 µm). Background filtering (median value of 20 pixels and Gaussian smoothing with a sigma value of 10) was performed in ImageJ software 1 and visualized in IMARIS 9.8 (Belfast, United Kingdom). 2008 Allen reference atlas images were overlaid on top of 90 µm maximum intensity projections taken from IMARIS 9.8 (Belfast, United Kingdom): -1.26 mm **(A)**, -1.36 mm **(B)**, and -1.46 mm **(C)**. Zoomed in sections of each white rectangular area at each coordinate (rows '**i**') are displayed below for each fluorophore (rows '**ii**'). Scale bars for rows 'i' are 200 µm and for rows 'ii are 100 µm.

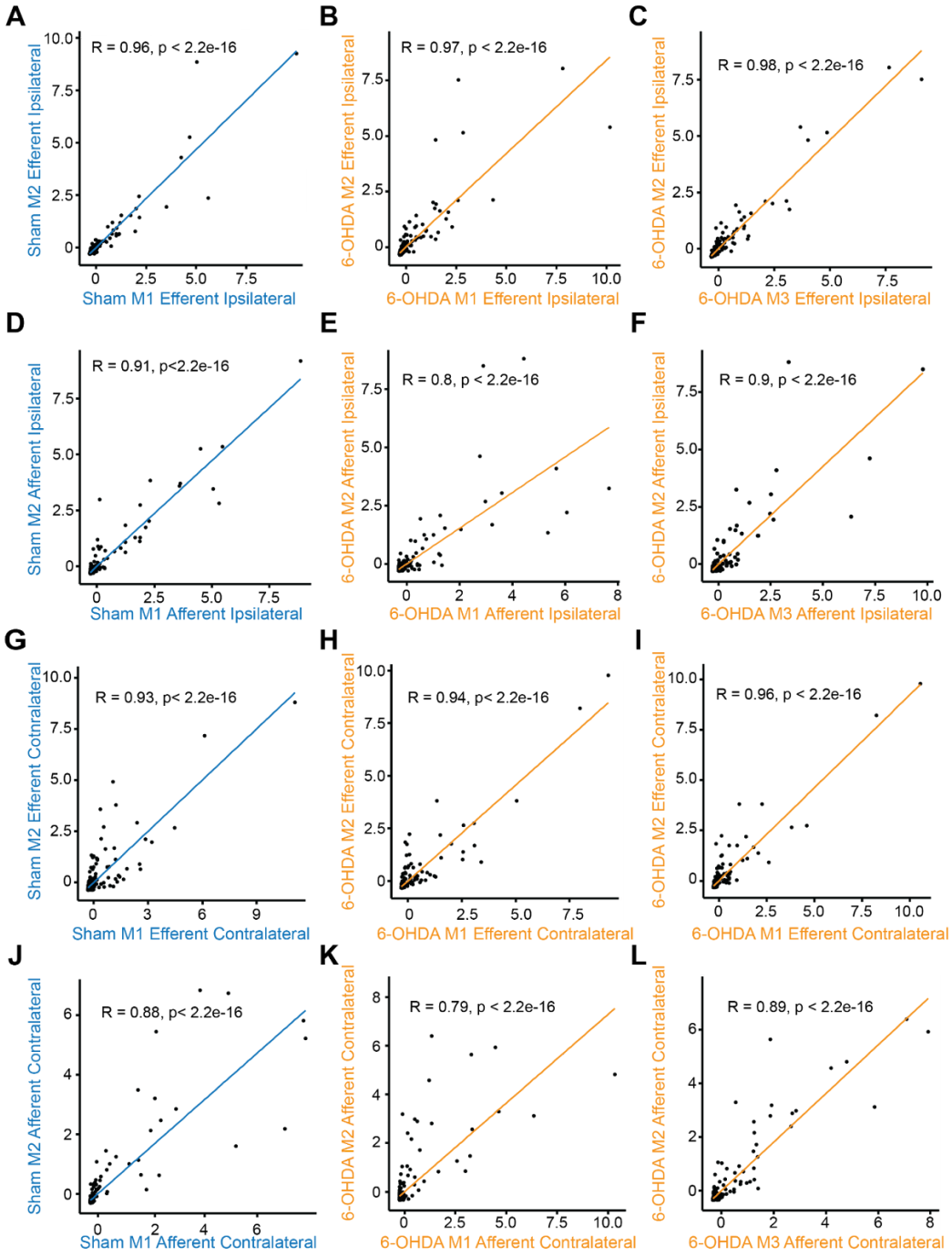

Figure S6. The ipsilateral (A-F) and contralateral (G-L) afferent and efferent proportions across Sham (blue) and 6-OHDA (orange) mice were consistent when compared in a pairwise manner. An experimental variation on the total labeling of neurons and fibers was minimized by dividing the afferent cell counts or efferent fiber areas in each brain region by the total number found in a brain to obtain the proportion of total inputs and outputs. Using Spearman's correlation analysis, we found afferent and efferent proportions across animals to be consistent among each other with an average correlation of 0.91 (SEM = 0.02). M1 = mouse #1, M2 = mouse #2, M3 = mouse #3.

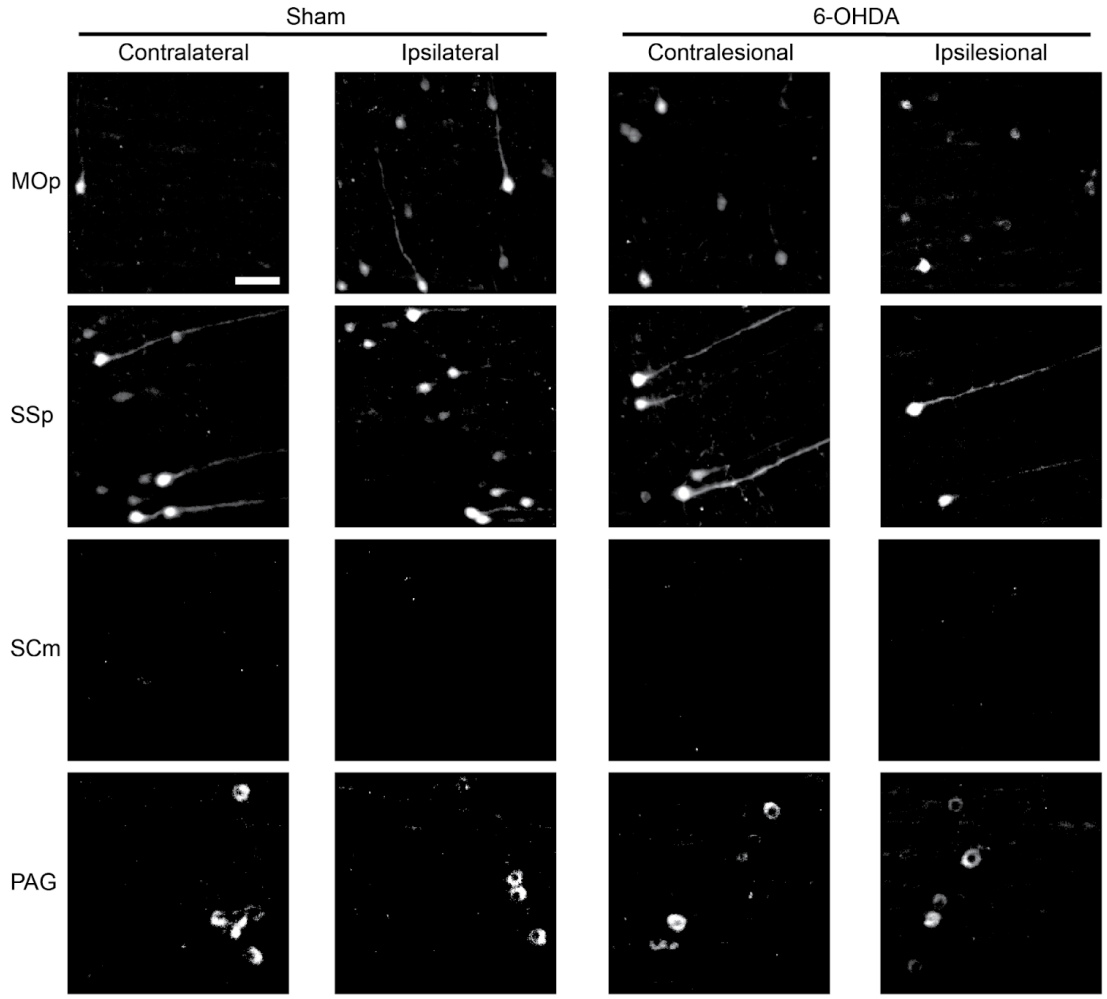

Figure S7. Examples of retrogradely labelled GFP positive fibers and cells from selected regions illustrating cell bodies from whole brain imaging that project to the A13 region. Expressions of GFP detected using light-sheet microscopy with 2x objective, 6.3x optical zoom, and a z-step size of 2 µm (xyz resolution = 0.477 µm x 0.477 µm x 2 µm). Brain regions were delimited by registration with Allen Brain Atlas (see Methods) and cropped out of selected 90 µm image stack in ImageJ software 1. A background filter (Gaussian smoothing with a rolling ball radius of 20 pixels) and a minimum filter with radius of 1 pixel were performed in ImageJ software 1. Scale bar = 50 µm.

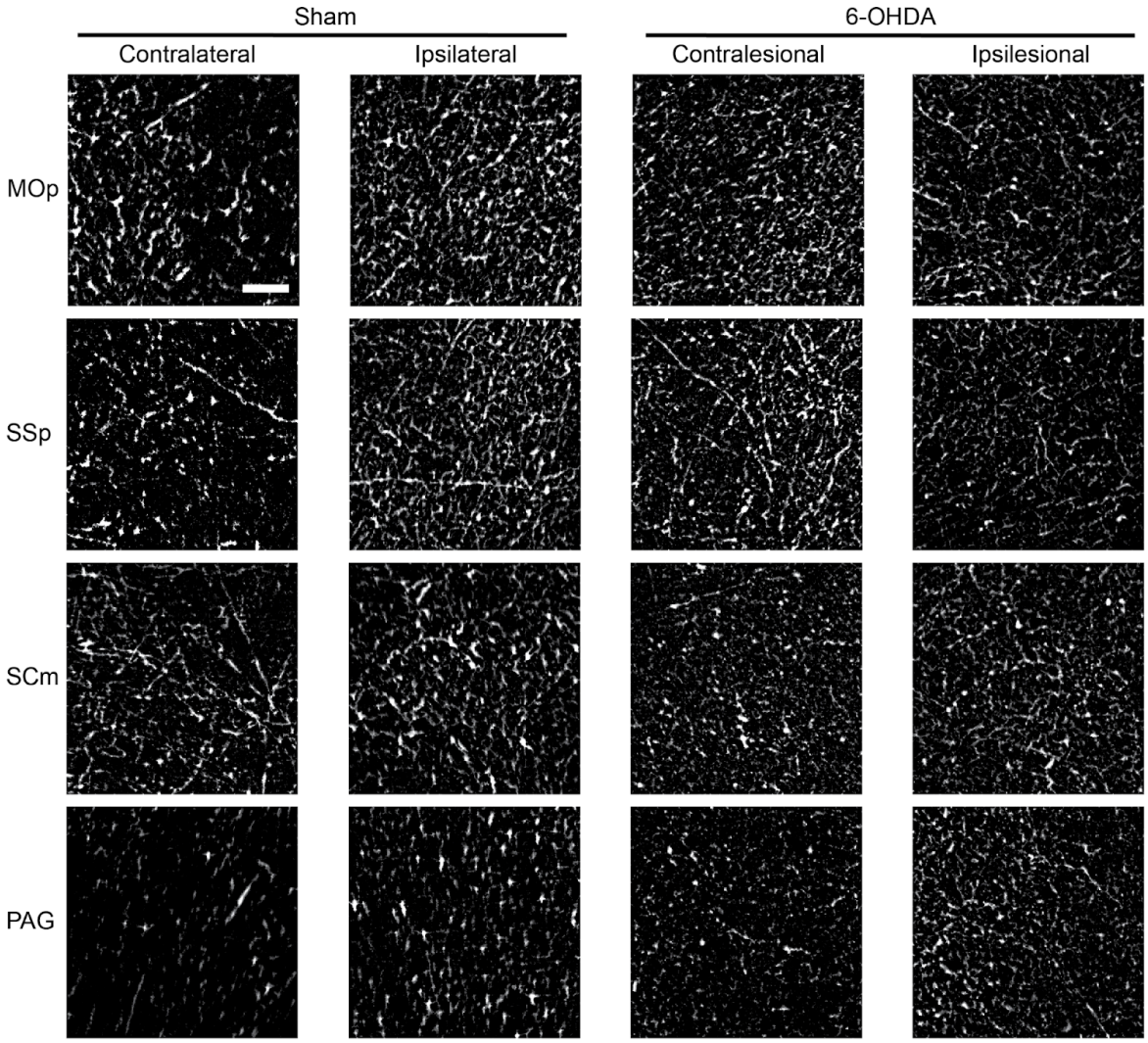

Figure S8. Examples of anterogradely labelled mCherry positive fibers from selected regions illustrating cell bodies from whole brain imaging that project to the A13 region. Expressions of mCherry detected using light-sheet microscopy with 2x objective, 6.3x optical zoom, and a z-step size of 2 µm (xyz resolution = 0.477 µm x 0.477 µm x 2 µm). Brain regions were delimited by registration with Allen Brain Atlas (see Methods) and cropped out of selected 90 µm image stack in ImageJ software 1. A background filter (Gaussian smoothing with a rolling ball radius of 5 pixels) and a minimum filter with a radius of 1 pixel were applied in ImageJ software 1. Scale bar = 50 µm.

Movie S1. Photoactivation of the A13 region in a 6-OHDA model mouse producing increased locomotion in the OFT (2x speed).

Movie S2. Photoactivation of the A13 region in a sham mouse producing increased locomotion in the OFT (2x speed).

Movie S3. Photoactivation of the A13 region during the pole test in a 6-OHDA model mouse decreases pole descent time (0.5x speed).

Movie S4. Video showing TH staining following whole brain imaging and staining in a 6-OHDA model mouse brain. Focus on TH expression in the A13 and SNc regions.