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Behavioral/Cognitive

Deep Brain Stimulation of Different Pedunculopontine Targets in a Novel Rodent Model of Parkinsonism

Nadine K. Gut1,2 and Philip Winn1

1Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G4 0RE, Scotland, United Kingdom, and 2School of Psychology and Neuroscience, University of St. Andrews, St. Andrews KY16 9JP, Scotland, United Kingdom

The pedunculopontine tegmental nucleus (PPTg) has been proposed as a target for deep brain stimulation (DBS) in parkinsonian patients, particularly for symptoms such as gait and postural difficulties refractory to dopaminergic treatments. Several patients have had electrodes implanted aimed at the PPTg, but outcomes have been disappointing, with little evidence that gait and posture are improved. The PPTg is a heterogeneous structure. Consequently, exact target sites in PPTg, possible DBS mechanisms, and potential benefits still need systematic investigation in good animal models. We have investigated the role of PPTg in gait, developed a refined model of parkinsonism including partial loss of the PPTg with bilateral destruction of nigrostriatal dopamine neurons that mimics human pathophysiology, and investigated the effect of DBS at different PPTg locations on gait and posture using a wireless device that lets rats move freely while receiving stimulation. Neither partial nor complete lesions of PPTg caused gait deficits, underlining questions raised previously about the status of PPTg as a motor control structure. The effect of DBS in the refined and standard model of parkinsonism were very different despite minimal behavioral differences in nonstimulation control conditions. Anterior PPTg DBS caused severe episodes of freezing and worsened gait, whereas specific gait parameters were mildly improved by stimulation of posterior PPTg. These results emphasize the critical importance of intra-PPTg DBS location and highlight the need to take PPTg degeneration into consideration when modeling parkinsonian symptoms. They also further implicate a role for PPTg in the pathophysiology of parkinsonism.

Key words: basal ganglia; cognition; deep brain stimulation; dopamine; freezing of gait; posture

Introduction

The pedunculopontine tegmental nucleus (PPTg) has a role in parkinsonism: there is cholinergic and noncholinergic cell loss (Hirsch et al., 1987; Rinne et al., 2008; Pienaar et al., 2013) and altered activity in remaining neurons (Mitchell et al., 1989; Orive et al., 2000; Breit et al., 2001; Aravamuthan et al., 2008). Since 2005, PPTg deep brain stimulation (DBS) has been used to address symptoms such as gait, postural disturbances, and freezing of gait (FOG). Despite initial positive reports (Mazzone et al., 2005; Plaha and Gill, 2005; Stefani et al., 2007), subsequent studies were disappointing (Ferraye et al., 2010). The hypothesis that PPTg DBS could correct axial symptoms came from two assumptions: (1) dysfunction of basal ganglia (BG) output nuclei has a pathological effect on PPTg that might be corrected by local low-frequency stimulation; and (2) PPTg is a locomotor control structure (Pahapill and Lozano, 2000; Nandi et al., 2002). This second assumption is based on primate studies that report frank motor effects of lesions centered on (but including more than) the PPTg (Kojima et al., 1997; Munro-Davies et al., 1999). However, other primate studies show that firing PPTg neuron firing relates more to cognitive functions, not movement (Kobayashi and Okada, 2007). Studies in cats, rats, and mice are not consistent with PPTg being a motor control center (Inglis et al., 1994a,b; Alderson et al., 2003; Homs-Ormo et al., 2003; Steiniger and Kretschmer, 2004; Winn, 2006). Rather, it has a role in learning and decision making, with subtle deficits in motor control emerging only as task difficulty increases (Olmstead et al., 1998; Corrigall et al., 2001; Samson and Chappell, 2001; Alderson et al., 2004, 2006; Diederich and Koch, 2005; Kobayashi and Okada, 2007; Wilson et al., 2009; Thompson and Felsen, 2013; MacLaren et al., 2014). Differences in anatomical organization are unlikely to account for different experimental outcomes: PPTg structure and connectivity is highly conserved from teleost fish through to humans (Rye et al., 1987; Brantley and Bass, 1988; Mesulam et al., 1989; Medina and Reiner, 1994; Marin et al., 1997; Stephenson-Jones et al., 2011).

The PPTg contains populations of cholinergic, GABAergic, and glutamatergic neurons, with different distributions and patterns of connections (Ros et al., 2010; Martinez-Gonzalez et al., 2011); for example, BG output is directed to anterior and sensory input to posterior parts (Winn, 2008), creating functional differ-
ences across the PPTg (Alderson et al., 2008). This raises questions about whether the consequences of PPTg DBS depend on location and what kinds of effects to expect. Given the variable nature of PPTg DBS in parkinsonian patients and uncertainty about functional differentiation within the PPTg, we undertook experiments in rodents models to define the therapeutic potential for PPTg DBS. We did this by doing the following: (1) assessing the effect of both full and partial PPTg lesions on gait and posture, (2) combining a partial PPTg lesion with bilateral destruction of substantia nigra pars compacta (SNC) dopamine (DA) neurons to model better the pathology of parkinsonism; and (3) examining the effects on gait of DBS in different PPTg locations.

Materials and Methods

Subjects
One hundred eight adult male Lister Hooded rats (Harlan Olac) weighing 317–396 g were housed singly under a 12 h light/dark cycle and with food control. Body weight was monitored daily and food adjusted (never less than 15 g/d per rat) to maintain stable weight; water was available ad libitum. Experiments were performed in compliance with the United Kingdom Animals (Scientific Procedures) Act 1986 and European Communities Council Directive of 24/11/86 (86/609/EEC).

Experimental design
At the start of each experiment, rats were trained for 10–12 d on the Noldus CatWalk to measure gait and posture. Rats were then tested for 3–4 d to obtain a presurgical baseline. Experiment 1 examined the role of the PPTg in the symptomatology of parkinsonism. Experiment 2 tested a novel model of parkinsonism, and DBS of the anterior PPTg (aPPTg; Experiment 3) and posterior PPTg (pPPTg; Experiment 4) was examined to determine whether there were beneficial effects on gait and posture. Experimental protocols are shown in Figure 1.

Surgical procedures
All surgical procedures were performed with rats secured in a stereotaxic frame (David Kopf Instruments) under isoflurane anesthesia (2–3% isoflurane and 1.4 L/min O2) and pretreated with an analgesic (Rimadyl); 0.05 ml/rat carprofen, 5% w/v, i.p.; Pfizer). After surgery, animals received intraperitoneal injections of Hartmann’s solution (~1 ml/h surgery; Baxter Healthcare).

PPTg lesions. Excitotoxic lesions of the PPTg were made following an established protocol (Wilson et al., 2009). In brief, in two surgical procedures separated by 1 week, the PPTg was lesioned bilaterally by pressure injection of 180 nl of 0.09–0.12 M ibotenic acid for full lesions and 200 nl of 0.06 M ibotenic acid for partial lesions into the aPPTg or pPPTg at these coordinates [incisor bar elevated to create a horizontal angle of 8°29’ between it and the interaural line (IAL) (Whishaw et al., 1977)]; in Experiments 1 and 2: aPPTg, +0.6 mm IAL, ±2.0 mm midline, and −7.0 mm from dura; pPPTg, −0.8 mm IAL, ±1.9 mm midline, and −6.5 mm from dura; in Experiments 3 and 4: aPPTg, +0.5 mm IAL, ±2.0 mm midline, and −7.0 mm from dura; pPPTg, −0.7 mm IAL, ±1.9 mm midline, and −6.7 mm from dura. Sham-operated controls underwent identical procedures but received only vehicle (sterile phosphate buffer).

DA depletion using 6-OHDA. Seven micrograms (free base weight) of 6-OHDA-hydrobromide dissolved in 2 μl of sterile 0.1% l-ascorbic acid/0.9% saline shortly before infusion were delivered bilaterally at four sites in the striata at the following coordinates (incisor bar at 0 mm): (1) +1.3 mm anteroposterior (AP; from bregma), ±2.6 mm mediolateral (ML; from the midline at the skull surface), −5.0 mm dorsodental (DV; from dura); (2) +0.4 mm AP, ±3.0 mm ML, −5.0 mm DV; (3) −0.4 mm AP, ±4.2 mm ML, −5.0 mm DV; and (4) −1.3 mm AP, ±4.5 mm ML, −5.0 mm DV. Infusions were made (1 μl/min) via 30 gauge stainless steel cannula connected via polyethylene tubing to 10 μl syringes mounted in a Harvard microdrive pump. Cannulae were left in situ for 2 min after each infusion before retracting them. Control rats received infusions only of vehicle.

Combined 6-OHDA and PPTg lesions. Rats receiving combination DA depletion and partial bilateral PPTg lesions underwent three separate surgeries. During the first two, bilateral partial PPTg lesions were created; after an additional 7 d recovery, the DA-depleting procedure was performed.

DBS electrode implantation. For DBS experiments, rats received bilateral electrode implants into either the aPPTg or pPPTg. These procedures were conducted during the same surgery as infusion of 6-OHDA. After the last infusion of 6-OHDA, the incisor bar was elevated to create a horizontal angle of 8°29’ between it and the IAL. Concentric bipolar electrodes were lowered into place at the same coordinates used for toxin infusion. Electrodes were made from Teflon-coated platinum/iridium (90:10) wire (50 μm uncoated diameter, 114 μm coated diameter; Science Products) threaded through 30 gauge stainless steel cannula (Plastics One) insulated with polyolefin 2:1 heat shrink (total diameter, 300 μm + 50 μm; wire protruding, 500 μm; length of bare tip of cannula and wire, 200 μm). Anode and cathode of the electrodes were then connected to a connector head stage that was then secured to the skull with dental cement (methyl methacrylate-based dental acrylic; Simplex Rapid) anchored by stainless steel screws (0–80 × ½ and 0–80 × ½; Plastics One).

Intracranial stimulation
Chronic stimulation was performed by attaching and securing a wireless DBS device to the head stage. The home cages were modified to protect animals from damaging the device and head cap. DBS devices were programmed to deliver biphasic constant-current square-wave pulses at a frequency of 25 Hz and pulse width of either 160 μs (DBS1 settings) or 500 μs (DBS2 settings). First, the threshold for stimulation-induced behavioral side-effects, such as slowing or an arrest of exploratory behavior up to “freezing” and “staring” or erected whiskers and/or ears, was determined for each rat individually in a stepwise procedure (starting at ~30 μA, increases of 20 μA). Each stimulation epoch was no longer than ~20 s with at least 1 min between epochs. The intensity for chronic stimulation was set at 20% of the side-effect-inducing intensity and retested and further adjusted if animals still appeared disturbed by stimulation. Final average stimulation intensities were as follows: in Experiment 3: DBS1, 78 μA (range, 56–120 μA); DBS2, 61 μA (range, 4072 μA); in Experiment 4: DBS1, 107 μA (range, 72–136 μA); DBS2, 105 μA (range, 72–152 μA; here, for both settings, the intensity was lower for the 6-OHDA group than for the other groups).

Behavioral testing
CatWalk. Gait testing was conducted on the CatWalk 7.1 (Noldus). The principal working mechanisms of the CatWalk are explained in the study by Hamers et al. (2006). In brief, rats traverse a 100 × 12 × 0.6 cm glass walkway, motivated by their home cage being present at the far end for them to return to after testing. A camera mounted below the runway captures footfall: the spacing and size of each print is assessed to derive measures of gait. Before presurgery testing, rats were trained for 10–12 d on the CatWalk to ensure an acceptable performance of three consecutive non-interrupted runs. The following parameters were analyzed: (1) for gait stability: base of support (BOS; distance between the two forepaws and two hindpaws), duty cycle (stance duration expressed as a percentage of the duration of the step cycle), support (percentage of contact of two diagonal paws (diagonal) or three paws (three) with the ground of all combinations of numbers of paws); (2) for velocity: swing speed; (3) for stride: stride length; and (4) for coordination: print position (distance between the position of the hindpaw and the position of the previously placed forepaw on the ipsilateral side within the same step cycle).

FOG. During Experiments 3 and 4, rats showed moderate to severe freezing during testing. Unlike gait hesitation in the middle of a run (slowing down and coming to a stop) reflecting destination hesitation according to Fahn’s classification of Fahn (1995) (see Schaufelma et al., 2003), which had been observed previously, in the DBS studies, some rats suffered from severe start hesitation. If no run at all (or not the required three runs per testing session) could be recorded, the performance was...
recorded as such (freezing leading to less than three runs and freezing leading to complete lack of data for the session).

L-DOPA administration. As part of the DBS experiments, rats received injections of L-DOPA [12 mg · kg⁻¹ · ml⁻¹ 1-3,4-dihydroxyphenylalanine methylester (free base weight) plus 15 mg · kg⁻¹ · ml⁻¹ benserazide hydrochloride (free base weight) in sterile 0.9% saline, i.p.; Fig. 1C]. Immediately after injection, rats were returned to their home cage and kept under close observation to classify and quantify changes in behavior. The severity of L-DOPA-induced dyskinesias was assessed by determining the time for which rats displayed abnormal involuntary movement (AIM) using a six-point scale (Creese and Iversen, 1973) and presented in minutes. Behavior was rated as follows: 1, normal; 2, active with bursts of sniffing and rearing; 3, continuous sniffing and rearing over the entire area of the cage; 4, continuous abnormal behavior in one place (e.g., up and down head movements, crossing and lifting of the forelegs); 5, additional bursts of licking or gnawing of paws, legs, or sides (including self-mutilation, which was always intercepted by experimenters providing alternative substrates for gnawing) and/or gnawing of the cage; and 6, continuous licking and/or gnawing.

Histological analysis
All rats were given a lethal intraperitoneal injection of 200 mg/ml sodium pentobarbitone (0.6–0.8 ml/rat Euthatal; Merial Animal Health) and transcardially perfused with 0.1% PBS, followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer; ~350 ml). Brains were stored in 20% sucrose solution in 0.1 M PB at 4°C before coronal 30 μm sections were cut on a freezing microtome. Parallel sections (1:6) were immunohistochemically processed (free-floating) for the following: (1) neuron-specific nuclear protein (NeuN) in the upper brainstem using a mouse-derived anti-NeuN monoclonal antibody (Merck Millipore); (2) ChAT of the upper brainstem using goat anti-ChAT polyclonal antibody (Merck Millipore); and (3) tyrosine hydroxylase (TH) and calbindin-D₂₈k (CB; double stain) of the midbrain using mouse-derived monoclonal anti-CB (Sigma-Aldrich) and mouse-derived monoclonal anti-TH. Vector Laboratories Elite ABC kits providing biotinylated secondary antibody, avidin, and biotinylated horseradish peroxidase reagent were used, as well as 3,3-diaminobenzidine tetrahydrochloride (Sigma Fast DAB; Sigma-Aldrich) for NeuN and CB and slate gray peroxidase substrate (ImmPACT SG; Vector Laboratories) for visualizing TH-containing neurons.
cells. Sections were mounted onto slides, and sections were stained for NeuN counterstained with cresyl violet.

For lesion assessment, all sections were assessed using a light microscope (Leica DM LB2) at 1.6× magnification. The extent of nonselective damage caused by ibotenic acid in and around the PPTg was assessed on the NeuN-stained sections, judged by lack of cell bodies. Lesion outlines were drawn on rat brain atlas schematics (Paxinos and Watson, 2005). Lesions were assessed visually and rated semiquantitatively from 0 to 4 (0, no PPTg lesion; 1, small/partial PPTg lesion (>50% of NeuN-stained neurons remaining); 2, partial to full PPTg lesion (<50% of NeuN-positive (NeuN⁺) neurons remaining); 3, fully lesioned PPTg (no neurons stained for NeuN in the PPTg area); and 4, extensive lesion covering PPTg and surrounding tissue). Animals with either a lack of PPTg lesion on at least one side (0; PPTg not hit) or lesions that were so extensive that they damaged the SN (4 on both sides) were excluded. For full lesions, histology had to show at least lesion in one hemisphere scoring 4 and the other side scoring 2. For partial lesions (including combined lesion groups), a score of 1 was required as the minimum and no more than a score of 3 on either side. To judge the degree of damage to cholinergic cells, surviving ChAT⁺ neurons within the entire PPTg were counted and expressed as a percentage of the average total cell count taken from sham-lesioned animals; in the case of Experiments 3 and 4 (with all rats bearing electrodes, regardless of lesion condition) brains with track lesions from the implanted electrodes, neurons of the nontargeted part of the PPTg (anterior or posterior, respectively) were counted; the percentage reflects half of what was counted in controls. To determine the extent of DA neuron loss, all TH⁺ neurons within the dorsal and ventral tier of the SNc, SN pars medialis, and SN pars lateralis were counted manually. The additional CB stain was performed to distinguish DA-containing neurons of the SN from the VTA (including the parabrachial pigmented area) neurons. All animals of the 6-OHDA groups of Experiments 2–4 were included in this analysis (16 with 6-OHDA only; 15 with 6-OHDA and PPTg lesion). These data also formed the basis for correlating the relationship between DA neuron and PPTg loss.

Statistical analysis
All statistical analyses were performed using SPSS version 21 (IBM). Mixed ANOVAs were performed to compare within either postsurgery testing days or testing conditions and between groups. Significant interactions were explored with univariate ANOVAs and repeated-measures ANOVAs. Greenhouse–Geisser corrections were used when indicated. Post hoc analyses were performed with Tukey’s HSD tests. Frequency counts of FOG were analyzed by χ² tests for association. Statistically significant effects were accepted with a p value ≤0.05. Correlations between the amount of cholinergic neuron loss from the PPTg and motor parameters on the CatWalk were run using Pearson’s correlation.

Results
Histological analysis
In all experiments, only rats with PPTg lesions as described above (Fig. 2), a loss of TH immunoreactivity of at least 90%, and correct electrode placements without excessive damage (Fig. 3) were included in behavioral analyses. Thirteen rats were excluded because they did not meet criteria, 25 rats died after ibotenic acid infusions (there are respiratory difficulties after infusion of the excitotoxin into the PPTg; Wilson et al., 2009), and three rats did not meet performance criteria in CatWalk training. The final group sizes were as follows: in Experiment 1: full PPTg lesion, n = 6; sham lesion, n = 4; in Experiment 2: partial PPTg lesion, n = 7; combined lesion, n = 7; 6-OHDA lesion, n = 7; sham lesion, n = 8; in Experiment 3: combined lesion, n = 4; 6-OHDA lesion, n = 5; sham lesion, n = 5; in Experiment 4: combined lesion, n = 4; 6-OHDA, n = 4; sham lesion, n = 4.

Cell loss after ibotenic acid infusions
PPTg lesion locations and sizes from all experiments are shown in Figure 2, with representative sections stained to show NeuN and ChAT immunohistochemistry. Figure 4 shows counts of cholinergic cells in PPTg. Rats in the full PPTg lesion group showed a mean loss of 68.80% of ChAT⁺ neurons (range of 49.92–93.10%); the partial PPTg lesion group of Experiment 2 lost 56.08% (range of 33.32–72.65%). Cholinergic cell loss in the combined lesion group of Experiment 2 reached 73.27% (range of 56.45–95.65%), in Experiment 3 (PPTg cell count) reached 55.82% (range of 37.46–80.15%), and in Experiment 4 (aPPTg cell count) reached 50.59% (range of 34.69–67.35%).

Effect of DA depletion on cholinergic cell count in the PPTg
DA loss from the SNc was assessed with TH and CB immunohistochemistry; representative lesions are shown in Figure 2G. The traditional parkinsonian model (6-OHDA group) in all experiments was assessed for possible PPTg cholinergic cell loss caused by a chronically DA-depleted SNc. Did a lack of functioning target neurons and altered afferent input from the BG contribute to PPTg damage? In Experiments 2–4, cholinergic cell survival was 97.26% (range of 83.83–108.05%), 93.38% (range of 77.76–106.22%), and 99.69% (range of 73.90–122.45%), respectively, and did not differ from sham controls (p = 0.979, p = 0.758, and p = 1.000, respectively). Loss of SNc DA neurons did not affect PPTg cholinergic neuron number.

Effect of PPTg lesions on DA cell survival in the SNc (Experiment 2)
To test the hypothesis that lack of afferent stimulation of nigral DA neurons after PPTg degeneration might contribute to DA cell loss, DA cell counts were examined in rats bearing partial PPTg lesions. TH⁺ cell count was, on average, 89.88% of sham controls (range of 68.86–112.33%). Statistically, there was no difference between the PPTg lesion group and sham controls (p = 0.230).

Behavior
Effect of PPTg lesions on gait: Experiment 1
Full PPTg lesions did not impair rats’ gait in any parameters (Table 1, Experiment 1). PPTg-lesioned rats did not differ from sham-operated animals in gait stability, speed, stride, or coordination (BOS forepaw, F(1,8) = 2.224, p = 0.174; BOS hindpaw, F(1,8) = 0.144, p = 0.714; duty cycle forepaw, F(1,8) = 4.782, p = 0.060; duty cycle hindpaw, F(1,8) = 0.223, p = 0.650; support diagonal paw, F(1,8) = 0.003, p = 0.955; support three paws, F(1,8) = 1.870, p = 0.209; swing speed forepaw, F(1,8) = 2.938, p = 0.125; swing speed hindpaw, F(1,8) = 2.745, p = 0.136; stride length forepaw, F(1,8) = 3.606, p = 0.094; stride length hindpaw, F(1,8) = 2.233, p = 0.173; print position right, F(1,8) = 0.226, p = 0.648; print position left, F(1,8) = 4.619, p = 0.064). Likewise, rats bearing partial PPTg lesions (Experiment 2) showed no differences from controls.

Gait and posture in a combined DA-depletion and PPTg lesion: Experiments 2–4
The goal of Experiment 2 was to establish a model in rodents to reflect better the pathophysiology of parkinsonism: did the addition of partial PPTg lesions change gait and postural impairments seen after bilateral DA depletion? Data are presented in Table 1 (Experiment 2).

Cholinergic neuron loss and gait patterning
There was a range in the extent of cholinergic neuron survival after full PPTg lesions. However, there was no consistent correlation between cholinergic survival and activity on the CatWalk. The deficit in BOS for the forelimbs only (not the hindlimbs) correlated with cholinergic survival (−0.85, p = 0.03), but no other measure of speed or stride showed significant correlation (with either two-tailed or one-tailed tests).
Figure 2. A, Representation of lesion extension along the rostrocaudal axis in rats of each experiment after PPTg ibotenic acid infusion. The position of the selected schematics (adapted from Paxinos and Watson, 2005) on the rostrocaudal axis is represented as the distance from the IAL, in millimeters). Sections IAL +2.88 mm through to IAL +1.68 mm were identified as sections containing aPPTg neurons (identified by PPTg cholinergic neurons in shams and partially lesioned rats), and those from IAL +1.08 mm to IAL +0.36 mm represent sections containing pPPTg. Lesion damage is represented in colored shading: blue for lesions in rats of PPTg lesion groups and red for lesions in rats of combined lesion groups. B–G, Representative histological photomicrographs of lesions in the aPPTg and pPPTg of rats bearing full lesions in Experiment 1 (B, NeuN stained; C, ChAT stained) and of rats bearing partial lesions in Experiments 2–4 (D, NeuN stained; E, ChAT stained; F, ChAT stained sections of sham-lesioned rat). G shows midbrain sections at the level of the SN in 6-OHDA-lesioned rats and shams; sections are double stained for anti-CB and anti-TH. Scale bars: B, D, 1 mm; C, E, F, 250 μm; G, short, 1 mm and long, 500 μm.
Gait stability. Changes in gait parameters indicate increased instability in DA-depleted rats: BOS of forelimbs and hindlimbs was significantly increased in both parkinsonian groups compared with controls (forelimbs, $F_{(3,25)} = 27.154, p < 0.001$; hindlimbs, $F_{(3,25)} = 20.219, p < 0.001$; Tukey’s HSD: forelimbs, combined lesion group, $p < 0.001$; 6-OHDA group, $p < 0.001$; hindlimbs, combined lesion group, $p < 0.001$; 6-OHDA group, $p < 0.001$); there was no significant difference between Parkinson’s disease models ($p = 0.683$). The use of diagonal two-legged support during the gait cycle decreased in parkinsonian rats, compensated for by an increase of three-legged support (diagonal, $F_{(3,25)} = 7.991, p = 0.001$; three, $F_{(3,25)} = 5.536, p = 0.005$).

Speed. Swing speed of forelimbs and hindlimbs of both 6-OHDA and refined models of parkinsonism decreased significantly compared with shams (forelimbs, $F_{(3,25)} = 26.540, p < 0.001$; hindlimbs, $F_{(3,25)} = 66.856, p < 0.001$; Tukey’s HSD tests, all $p$ values < 0.001). The two models did not differ from each other (forelimbs, $p = 0.492$; hindlimbs, $p = 0.719$).

Stride. Parkinsonian rats took shorter steps than shams (forelimbs, $F_{(3,25)} = 13.7795, p < 0.001$; hindlimbs, $F_{(3,25)} = 11.156, p < 0.001$; Tukey’s HSD: forelimbs, combined lesion group, $p = 0.021$; 6-OHDA group, $p < 0.001$; hindlimbs, combined lesion group, $p = 0.068$; 6-OHDA group, $p < 0.001$). There was no significant difference between the lesion groups (forelimbs, $p = 0.095$; hindlimbs, $p = 0.134$).

FOG: Experiments 3 and 4
In Experiments 3 and 4, it was often difficult for rats to complete non-interrupted runs or to complete all three required runs because of severe FOG (starting hesitation). Instances of FOG were reported as a percentage of all available testing occasions of each condition (Fig. 5) and analyzed as frequency counts. In Experiment 3, the combined lesion group was more likely to freeze in all four conditions (postsurgery baseline, $\chi^2_{(2)} = 13.680, p = 0.001$; DBS1, $\chi^2_{(2)} = 27.756, p < 0.001$; DBS2, $\chi^2_{(2)} = 24.333, p < 0.001$; DBS-OFF, $\chi^2_{(2)} = 7.200, p = 0.027$). In Experiment 4, no FOG occurred during DBS-OFF, but, in DBS1, the combined lesion group was more likely to freeze than the 6-OHDA group ($\chi^2_{(2)} = 8.649, p = 0.013$).

The effect of PPTg DBS on gait: Experiments 3 and 4
aPPTg DBS. Gait data collection was impaired in this experiment because of a high incidence of FOG. Figure 5 shows FOG in this experiment and the subsequent one involving DBS in the pPPTg. Analysis of gait measures in the aPPTg DBS rats was made using...
the available data. Figure 6 presents the principal measures. Repeated-measures ANOVAs across testing conditions showed that, in the combined lesion group, there was no difference between conditions in any parameter; DBS in the aPPTg had no effect on these rats’ gait (BOS: forelimbs, \( F_{(3,3)} = 3.711, p = 0.155 \); hindlimbs, \( F_{(3,3)} = 3.337, p = 0.174 \); duty cycle: forelimbs, \( F_{(3,3)} = 0.184, p = 0.901 \); hindlimbs, \( F_{(3,3)} = 2.166, p = 0.271 \); support: diagonal, \( F_{(3,3)} = 0.498, p = 0.709 \); three, \( F_{(3,3)} = 0.294, p = 0.829 \); swing speed: forelimbs, \( F_{(3,3)} = 0.682, p = 0.682 \); hindlimbs, \( F_{(3,3)} = 1.183, p = 0.447 \); stride length: forelimbs, \( F_{(3,3)} = 0.377, p = 0.778 \); hindlimbs, \( F_{(3,3)} = 1.174, p = 0.449 \); print position right: \( F_{(3,3)} = 6.667, p = 0.077 \); print position left: \( F_{(3,3)} = 1.376, p = 0.400 \).

In the 6-OHDA group, aPPTg DBS had a significant detrimental effect on a number of gait parameters. In many cases, these only came about in DBS2. BOS of the forelimbs increased compared with the postsurgical baseline deficit with DBS1 and DBS2. The stance duration of the forelimbs also increased: the stance duration of the forelimbs increased compared with the postsurgical baseline deficit with DBS1 and DBS2. The stance duration of the forelimbs also increased: the stance duration of the forelimbs increased compared with the postsurgical baseline deficit with DBS1 and DBS2. The stance duration of the forelimbs also increased: the stance duration of the forelimbs increased compared with the postsurgical baseline deficit with DBS1 and DBS2. The stance duration of the forelimbs also increased: the stance duration of the forelimbs increased compared with the postsurgical baseline deficit with DBS1 and DBS2. The stance duration of the forelimbs also increased: the stance duration of the forelimbs increased compared with the postsurgical baseline deficit with DBS1 and DBS2. The stance duration of the forelimbs also increased: the stance duration of the forelimbs increased compared with the postsurgical baseline deficit with DBS1 and DBS2. The stance duration of the forelimbs also increased: the stance duration of the forelimbs increased compared with the postsurgical baseline deficit with DBS1 and DBS2. The stance duration of the forelimbs also increased: the stance duration of the forelimbs increased compared with the postsurgical baseline deficit with DBS1 and DBS2.
The effect of l-DOPA administration. The effects of l-DOPA in Experiments 3 and 4 are shown in Figure 7. The effect of l-DOPA on gait could not be assessed because the drug at the dose administered induced dyskinesia-like AIMs, which made testing on the CatWalk impossible. At a mean time of 11.8 min (range of 7.0–20.8 min) after l-DOPA administration, the parkinsonian rats showed the first AIM, which then became more severe. Sham-lesioned controls did not show any signs of these. The rate of progression differed between groups and testing condition. The total amount of time the lesioned animals showed AIMs did not differ between groups (anterior DBS-OFF, $F_{(1,7)} = 3.506, p = 0.103$; DBS-ON, $F_{(1,5)} = 3.241, p = 0.122$; posterior DBS-OFF, $F_{(1,5)} = 4.057, p = 0.091$; DBS-ON, $F_{(1,5)} = 0.086, p = 0.781$); it was the quality of AIMs that changed rather than the duration (Fig. 7).

Figure 7 shows the time for which rats in the different groups and conditions were dyskinetic. Both parkinsonian groups showed AIMs after intraperitoneal l-DOPA injections. Irrespective of the position of the stimulation electrode, the group with combined DA depletion and PPTg lesions showed more severe AIMs than the 6-OHDA group and mostly of a different quality (Fig. 7A, B; licking and biting rather than stereotyped head and body movements; for details, see the figure legend).

Focusing on these differences (AIMs scores 4 and 6), the analyses showed an amelioration of the severe AIMs in the combined lesion group with posterior DBS (Fig. 7D) but not with anterior DBS (Fig. 7C): pPPTg DBS (ON) caused a shift of the l-DOPA-induced AIMs. The appearance of continuous licking and biting was reduced, with AIMs instead taking the form of stereotyped up and down head movements and crossing and lifting of the forelegs [aPPTg DBS experiment, no interaction of group $\times$ DBS condition (ON/OFF): score 4, $F_{(1,6)} = 4.591, p = 0.076$; score 6, $F_{(1,6)} = 0.005, p = 0.944$; no effect of condition: score 4, $F_{(1,6)} = 0.000, p = 1.000$; score 6, $F_{(1,6)} = 1.212, p = 0.313$; pPPTg DBS experiment: significant effect of condition: score 4, $F_{(1,5)} = 9.791, p = 0.026$; score 6, significant interaction of group $\times$ DBS condition, $F_{(1,5)} = 8.504, p = 0.033$].

Discussion

Key findings

First, the PPTg is not critical for normal gait: neither partial nor full PPTg lesions affected stability, speed, stride, or coordination. Addition of a partial PPTg lesion to a DA-depleting lesion did not affect gait differently from DA depletion alone. However, compared with controls, rats with additional PPTg lesions had no deficits in hindlimb stride length or the distribution of support, unlike those with only DA-depleting lesions. Second, loss of PPTg neurons did not affect SNc DA neuron number or vice versa. In parkinsonism, in which mechanisms of toxicity and progression differ from animal models, different events might occur, but there is no a priori reason to believe that PPTg loss damages SNc DA neurons. Third, FOG was not seen in rats with only 6-OHDA lesions but was marked in rats with a 6-OHDA lesion plus a partial PPTg lesion, as well as an electrode implanted in the aPPTg but not the pPPTg. Activation of aPPTg electrodes exaggerated FOG; neither PPTg electrodes nor DBS had the same effect. Fourth, effects on gait depended on the parkinsonian model and target site. In DA-depleted rats with an intact PPTg, pPPTg DBS ameliorated gait deficits, but aPPTg DBS made them worse. However, with partial PPTg damage (as in parkinsonism), DBS was ineffective. It was not the case that partial PPTg lesion made DBS altogether ineffective: rats with partial PPTg and DA-depleting lesions developed stronger l-DOPA-induced AIMs than DA-depleted rats with an intact PPTg. pPPTg DBS (but not aPPTg DBS) attenuated these; it was possible to activate surviving neurons for therapeutic benefit.
Clinical position

We used stimulation frequencies relevant to clinical work in humans (and monkeys). Clinically, lower frequencies are consistently most beneficial: higher frequencies cause impairment (Nandi et al., 2002; Plaha and Gill, 2005). Our pilot experiments using 130 Hz produced akinesia or freezing, regardless of how low the intensity. Initial encouraging clinical results after PPTg DBS (Mazzone et al., 2005; Plaha and Gill, 2005; Stefani et al., 2007) were not confirmed (Stefani et al., 2013). Follow-up studies reported a decline of the transient gait amelioration, and double-blind trials showed no motor improvements with PPTg DBS measured by Unified Parkinson’s Disease Rating Scale (UPDRS) or objective measures of freezing (Ferraye et al., 2010). The initial focus on gait and posture diverted to cognitive deficits: (1) start-react and reaction time in attentional tasks (Thevathasan et al., 2010); (2) working memory, verbal fluency, executive functioning, and delayed recall (Alessandro et al., 2010; Ceravolo et al., 2011); and (3) rapid eye movement sleep (Lim et al., 2009). Were initial clinical expectations based on a flawed hypothesis? The majority of PPTg DBS publications refer to the PPTg as the major component of the MLR, a notion that derives from stimulation studies of rostral brainstem in decerebrate cats and rats (García-Rill et al., 1987; Skinner et al., 1990) and lesion studies in monkeys (Kojima et al., 1997; Aziz et al., 1998; Munro-Davies et al., 1999). This is not entirely supported now (García-Rill et al., 2011): the lesion studies are problematic to interpret (Winn, 2006), and recent primate electrophysiological data emphasize nonmotor PPTg functions (Okada and Kobayashi, 2013). The literature in mice, rats, and cats is consistent: loss or inactivation of PPTg effects tasks involving learning and decision making but not movement (Swerdlow and Koob, 1987; Dllu et al., 1991; Inglis et al., 1994a,b; Olmstead and Franklin, 1994; Keating and Winn, 2002; Alderson et al., 2003; Homs-Ormo et al., 2003; Steiniger and Kreitschmer, 2004; Taylor et al., 2004; Wilson et al., 2009). It has been argued that PPTg organization and projections differ in primates but, although there are differences in the strength of particular PPTg connections (Alam et al., 2011), the pattern of connectivity is highly conserved across species (Winn, 2008; Martinez-Gonzalez et al., 2011). What is interesting is the increasing clinical focus on cognitive deficits; this aligns with experimental studies, reinforcing the belief that PPTg function is not simple motor control and validating the utility of rat models for exploring PPTg function.

Interpretation and conclusion

The animal literature shows that the PPTg has a role in cognitive functions and sleep (but is not a master switch for sleep–wake transitions; Saper et al., 2010; Petrovic et al., 2013). What does this mean for its contribution to movement disorders? Cholinergic, GABAergic, and glutamatergic neurons in the PPTg are distributed along different rostrocaudal gradients (Mesulam et al., 1983; Wang and Morales, 2009). Its anatomical, neurochemical, and electrophysiological heterogeneity explains the functional diversity of the structure, with lesions in different parts having different effects (Alderson et al., 2008; Wilson et al., 2009; Ros et al., 2010; Martinez-Gonzalez et al., 2012). Consequently, the effects of PPTg DBS will depend on the exact location: of the few cases reporting benefits from PPTg DBS, the best effects were achieved with posterior stimulation (Ferraye et al., 2010) or outside the PPTg (Zrinzo et al., 2007). The pPPTg receives fast sensory input, responding to its meaning and value. In contrast, the aPPTg is the principal site of BG input, which brakes PPTg by GABA-mediated inhibition. Both parts project to thalamocortical and corticostratial systems via the thalamus and midbrain DA neurons, as well as connecting with structures below, such as the pontine reticular formation and spinal cord (Pan and Hyland, 2005; Kobayashi et al., 2007; Maskos, 2008; Mena-Segovia et al., 2008a; Okada et al., 2009; Thompson and Felsen, 2013).

aPPTg DBS in this study worsened gait in the 6-OHDA model and caused marked FOG in the combined lesion group. FOG as a gait disturbance is not a pure motor problem, and, as in patients, FOG here was contextual: it did not appear in the home cage, but, within the confined space of the CatWalk, animals showed pronounced start hesitation. Two possible explanations for aPPTg DBS-
induced FOG are as follows. (1) It could activate local BG-projecting GABA neurons (Mena-Segovia et al., 2008a). This is plausible but does not sit easily with the fact that aPPTg damage itself promoted FOG. (2) Alternatively, FOG might be a consequence of aPPTg DBS, exacerbating a problem with automatic or habitual control of behavior, a BG function disrupted in parkinsonism. Replacing the automatic control of action effected by the BG (dorsolateral striatum/posterior lateral putamen) that is disturbed in parkinsonism with ventral striatal goal-directed processes would account for slower execution of movements and susceptibility to interference from other goal-directed tasks (Redgrave et al., 2010). Redgrave and colleagues hypothesize that output from ventral striatal goal-directed systems has to overcome “noisy” output from stimulus–response habitual control circuits at points at which the two systems converge, such as the aPPTg. Therefore, loss of functionality here is disabling in situations with increased need for goal-directed processes to override automatic and habitual ones. MacLaren et al. (2014) suggest that motor impairment in complex motor tasks after PPTg lesions might be attributable to a lack of integration of sensory information—including nonphysical attributes such as salience and value—supporting the hypothesis of integration failure at the level of the PPTg in more complex motor behavior.

Posterior stimulation improved gait parameters in the 6-OHDA lesion group. Through thalamic connections, the PPTg influences cortical activity. Mena-Segovia and colleagues (Mena-Segovia et al., 2008b; Mena-Segovia and Bolam, 2011) have shown that neurochemically identified PPTg neurons differentially effect cortical oscillatory activity. Increased activity is seen in several cortical areas after PPTg stimulation, including the medial sensorimotor area (Ballanger, 2009). Underactivity here is important in parkinsonian motor symptoms (Berardelli et al., 2001), and increased activation is associated with motor improvement (Brooks and Samuel, 2000). Furthermore, in the parkinsonian rodent model, the relation between cortical and PPTg activity was shown to be altered (Aravamuthan et al., 2008). It is likely that benefits seen here after pPPTg DBS can be attributed to an influence on cortical structures, which will help ameliorate deficiencies in corticostratial activity produced by DA depletion. However, for clinical application, it needs to be highlighted that this effect was absent in the model with PPTg degeneration. This suggests that, for patients with advanced parkinsonism, PPTg DBS is not a promising option. Most studies of PPTg DBS (including the early ones reporting most improvement) describe severe gait deficits and high UPDRS scores. The effect of symptom severity (and likely linked degree of degeneration) on PPTg DBS outcome is difficult to compare across studies, because factors, such as electrode placement, number of electrodes, stimulation parameters, and diagnosis, also contribute. PPTg DBS is only discussed for patients with pronounced gait impairment, a condition developed in more advanced stages when DBS in the PPTg, according the results reported

Figure 7. l-DOPA-induced stereotypies. A, Experiment 3. During DBS-OFF, the combined lesion group displayed very severe AIMs (score 6) for longer than those of other severity levels (effect of score [F(5,15) = 15.890, p < 0.001]), Tukey’s corrected pairwise comparisons (score 1, q = 8.089, p < 0.05; score 3, q = 8.632, p < 0.05). Univariate ANOVAs comparing both groups directly revealed that the combined lesion group showed AIMs of the most severe stage (score 6) significantly longer that the 6-OHDA group (F(1,7) = 7.151, p = 0.032), although this relation was inverse regarding dyskinesia score 4 (F(1,7) = 15.890, p = 0.005). B, Experiment 4. The 6-OHDA group displayed less severe stages of dyskinesia for longer than the combined lesion group (score 2, F(1,6) = 7.377, p = 0.035; score 3, F(1,6) = 8.828, p = 0.025; score 5, F(1,6) = 7.573, p = 0.033), whereas the combined lesion group showed significantly more continuous licking and biting (F(1,6) = 9.220, p = 0.023). C, D, DBS-ON. The effect of l-DOPA did not change in Experiment 3. There was no interaction of group × condition (ON vs OFF), regarding neither score 4 (F(1,6) = 4.591, p = 0.076) nor score 6 (F(1,6) = 0.005, p = 0.944). However, pPPTg DBS (Experiment 4) caused a shift of the l-DOPA-induced AIMs in the combined lesion group from continuous licking and biting to dyskinetic behavior in the form of up/down head movements and crossing/lifting of the forelegs (score 4) (significant interaction of group × condition (ON vs OFF; F(1,6) = 8.904, p = 0.033) on score 6).
here, is not beneficial. However, because the pPPTg can affect corticostriatal activity, it might be an appropriate target to address cognitive domains, as the clinical literature suggests (Potter-Nerger and Volkman, 2013). Future studies will have to address the role of the aPPTg in FOG (could this be ameliorated by targeted deactivation of GABA input?) and the precise effects of pPPTg DBS on corticostriatal function. Using model systems like this systematically might yet reveal the PPTg to be a valuable therapeutic target in Parkinsonism.

References


Maskos U (2008) The cholinergic mesopontine tegmental nucleus is a relatively novel rodent model of PPTg DBS.


