SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
BIOMEDICAL ENGINEERING
PARKINSON'S DISEASE

Received
11 June 2013

Accepted
6 January 2014

Published
23 January 2014

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# Chronic Spinal Cord Electrical Stimulation Protects Against 6-hydroxydopamine Lesions

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**Although L-dopa continues to be the gold standard for treating motor symptoms of Parkinson's disease (PD), it presents long-term complications. Deep brain stimulation is effective, but only a small percentage of idiopathic PD patients are eligible. Based on results in animal models and a handful of patients, dorsal column stimulation (DCS) has been proposed as a potential therapy for PD. To date, the long-term effects of DCS in animal models have not been quantified. Here, we report that DCS applied twice a week in rats treated with bilateral 6-OHDA striatal infusions led to a significant improvement in symptoms. DCS-treated rats exhibited a higher density of dopaminergic innervation in the striatum and higher neuronal cell count in the substantia nigra pars compacta compared to a control group. These results suggest that DCS has a chronic therapeutical and neuroprotective effect, increasing its potential as a new clinical option for treating PD patients.**

Parkinson's disease (PD) is a debilitating neurodegenerative disorder caused by progressive loss of the dopaminergic neurons of the nigrostriatal pathway[1]. A variety of pharmacological approaches, of which L-dopa administration is the most effective, have been used to alleviate PD motor symptoms by supplementing the dopamine (DA) deficiency observed in the striatum[2]. Despite its initial efficacy, long-term administration of L-dopa results in fluctuations in the clinical response and in L-dopa-induced dyskinesia (LID), a condition which is difficult to treat[2].

The therapeutic approach involving electrical stimulation of subcortical nuclei, known as deep brain stimulation (DBS), is also very effective in alleviating the motor symptoms of PD[3]. The best candidates for this treatment are idiopathic PD patients with motor fluctuations and L-dopa-induced dyskinesia[3]. Depending on the selection criteria, 1.6 to 4.5% of PD patients are eligible for subthalamic nucleus DBS[4], making DBS available only to a small percentage of the overall population of PD patients.

Based on previous results obtained in three different rodent models of PD[5], we have proposed that a new neuromodulation procedure that does not invade the brain tissue, known as dorsal column stimulation (DCS), has the potential to emerge as an additional therapeutic option for PD patients. In our hands, the most effective DCS effects in rodent PD models were obtained when continuous high frequency electrical stimulation was delivered to the large superficial fibers, running through the dorsal columns of the spinal cord, by a transversally oriented stimulating electrode, implanted at the high thoracic segments of the cord.

Following the publication of our animal study, a hasty testing of DCS in two patients with advanced PD was carried out. This study did not produce any improvement in motor function[6]. However, these initial negative results were easily explained by the significant methodological differences between the stimulation protocols employed in the animal and clinical studies. These included: the localization of the electrodes (high cervical in the human study vs. high thoracic in the rodent study), and the orientation of the electrode poles relative to the spinal cord (longitudinal in the clinical study vs. transversal in the animal experiments). These differences caused the effective surface of contact between the electrodes and the spinal cord to be around seven times larger in the rodent protocol when compared to the human study. Such a marked difference could account for the activation of substantially more ascending dorsal column fibers in the rodents, explaining why such a potent therapeutic effect was observed in the animal study and absent in the clinical one[7].

Soon after, however, support for our hypothesis that DCS can be effective as a PD therapy was subsequently obtained with the publication of four other clinical reports. These studies clearly indicated that DCS, originally intended for treating chronic pain, led to significant alleviation of motor symptoms in a total of 18 PD patients. For example, a PD patient, who had been previously implanted with a quadripolar spinal cord stimulator at low thoracic level to treat low back pain, experienced significant improvement of his motor symptoms during high frequency (130 Hz) DCS[8]. Furthermore, DCS also produced significant improvement of gait, posture, stability, and bradykinesia in 15 PD patients who received a spinal cord implant designed to treat low back and leg pain[9]. Next, a patient with advanced PD and other sensory symptoms also experienced improvement in gait and posture with quadripolar DCS applied at thoracic level[10]. Lastly, a female patient with PD and chronic neuropathic pain, experienced increasing improvement of motor PD symptoms over a two year period after initiating the DCS treatment. This improvement included alleviation of tremor and rigidity, and an improvement in gait and posture[11].

In our view, the pro-kinetic effect of DCS described in animal models and in several PD patients could be explained by the activation of somatosensory fibers, running through the dorsal column of the spinal cord, which led subsequently to the modulation of the ongoing activity of somatosensory and motor supraspinal structures[5,12,13]. Accordingly, in our original study, we observed that the motor effect of DCS is almost instantaneous and lasts as long as the electrical stimulation is maintained (i.e., see the supplementary video from previous studies[5,8]).

Up to now, however, the results reported with spinal cord stimulation in animal models of PD have been limited to its acute effect. Nonetheless, DCS is also known to cause changes in gene expression at supraspinal structures, which in turn may lead to long-term sustained effects[14,15]. In the present study, therefore, we explored, for the first time, the potential effects of chronic delivery of DCS in rats with bilateral intrastriatal 6-hydroxydopamine (6-OHDA) lesions. While the lesioned rats exhibited sustained weight loss, postural and gait abnormalities, paralleled by destruction of dopaminergic striatal projections, a group of DCS-treated animals showed a dramatic and consistent reversal of these signs, including significant gain of body weight gain, marked improvement in motor functions, and less dopaminergic neuronal loss throughout the nigrostriatal system. These results are compatible with a neuroprotective effect of DCS against chemically induced dopaminergic lesions, and suggest that DCS could have long-term benefits as a potential new therapy for PD.

## Results

**Chronic DCS prevents severe body weight loss in 6-OHDA lesioned rats.** Rats received a bilateral 6-OHDA striatal or a sham lesion, and their weight and motor symptoms were evaluated for a period of 6 weeks (See Fig. 1a for time course of the experiment). While the sham lesion surgery, conducted on a group of control rats, caused minor weight loss that was recovered almost immediately, bilateral intrastriatal 6-OHDA lesions resulted in sustained weight loss (Fig. 1b). Twice a week DCS treatment (30 minutes per session) caused a dramatic recovery of body weight in 6-OHDA lesioned rats. Indeed, DCS treated animals not only recovered significantly faster than non-treated 6-OHDA rats; their weights were significantly higher from the 11th day post lesion to the end of the experiment (Day 11, $p < 0.05$; days 12–14, $p < 0.01$; days 15–42, $p < 0.001$, Bonferroni multiple comparisons, DCS treated compared to non-treated Fig. 1b).

Even though the body weight of both 6-OHDA and 6-OHDA + DCS rats was significantly lower than that of the sham control animals throughout the experiment (ANOVA, two factor experiment with repeated measures, groups × days interaction: $p < 0.0001$), by the 6th week, the weight of DCS treated rats approached that of the control rats much more than non-treated animals.

In addition to faster weight recovery, DCS also prevented severe weight loss in lesioned rats during the early period after lesioning. 6-OHDA + DCS treated rats had a maximum weight loss ([30.94 ± 1.96]% ~9 days post lesion) that was significantly lower than non-treated 6-OHDA rats ([40.35 ± 1.68]% ~15 days post lesion, $p < 0.05$, Mann-Whitney test, Fig. 1c). Weight recovery of 6-OHDA + DCS rats began in week 2 and continued to improve until week 6, while non-treated 6-OHDA rats started weight recovery only in the 6th week, implying that chronic DCS almost immediately reversed the trend of weight loss. Fig. 1d shows that after initiation of DCS treatment, weight change (normalized to week 1) was significantly higher at every time point for treated rats as compared to non-treated subjects: week 2 ($0.68 \pm 3.37$, $-10.38 \pm 2.08$, $p < 0.05$), week 3 ($7.92 \pm 3.95$, $-8.87 \pm 3.26$, $p < 0.05$), week 4 ($12.26 \pm 4.52$, $-8.46 \pm 2.57$, $p < 0.01$), week 5 ($21.19 \pm 3.52$, $-3.42 \pm 2.3$, $p < 0.001$) and week 6 ($28.45 \pm 3.23$, $6.32 \pm 1.66$, $p < 0.001$; all measurements obtained with Mann-Whitney test).

The slope of body weight recovery, calculated from a line connecting the minimum weight value with the final day value, was also higher for DCS treated rats than non-treated rats, ($3.15 \pm 0.21$ vs. $2.2 \pm 0.33$, $p < 0.05$, Mann-Whitney test, Fig. 1e) confirming that chronic DCS not only prevented sustained weight loss by initiating weight recovery earlier, but it also accelerated the process of weight recovery in the 6-OHDA lesioned rats.

**Long term DCS restores motor function in PD rats.** Bilateral intrastriatal lesioning with 6-OHDA resulted in a significant loss of motor function. Lesioned rats developed a characteristic crouched posture, which we quantified from video images by measuring the length of the major axis of an ellipse fitting the animal's body. The crouched posture of the 6-OHDA rats resulted in an axis length shorter than in control rats. Quantitative analysis revealed that there was a significant interaction between groups and weeks (ANOVA, two factor experiment with repeated measures, groups × weeks: $p < 0.05$). Thus, starting at week 1.5, a gradual reversal of posture abnormalities was observed in the chronic DCS treated rats. Fig. 2a shows that axis length was significantly higher for 6-OHDA + DCS rats ($n = 6$) for week 3.5 ($63.59 \pm 0.74$ versus $56.54 \pm 2.10$, $p < 0.05$) and 4.5 ($64 \pm 1.79$ versus $56.37 \pm 1.79$, $p < 0.05$) as compared to non-treated rats, $n = 8$ (Bonferroni multiple comparisons). Overall, 30 min continuous DCS twice a week was sufficient to restore normal posture in 6-OHDA lesioned rats.

Lesioned rats in this study showed no significant differences in the distance traveled during a 30 min open field session between the control and the DCS treated animals (Fig. 2b). Nonetheless, control rats showed a progressive decrease in traveled distance over the weeks, while 6-OHDA rats, both treated and non-treated, showed an irregular pattern (ANOVA, two factor experiment with repeated measures, groups × weeks interaction: $p < 0.0001$, but no differences were seen on post-hoc analysis). In the same way, the average speed displayed in the open field did not show significant differences between the groups (Fig. 2c, ANOVA, two factor experiment with repeated measures, groups × week interaction: $p < 0.01$, no differences on post-hoc analysis). Yet, the untreated lesioned rats displayed a striking symptom related to locomotion: a rigid gait resulting in a non-smooth locomotion (see Supplementary Video). This symptom could be quantified by calculating the spectral power at the frequency range of 0.5–4.75 Hz of the instant acceleration vector of the rat displacement: high power values represent a jerky, non-smooth gait (see Supplementary Figure 1 for an example), compatible with rigidity and crouched posture. DCS treated rats, on the other hand, showed a much lower spectral power in the same frequency (0.5–4.75 Hz) of instant acceleration vector. This allowed DCS-treated rats to exhibit a smoother gait, suggesting that DCS

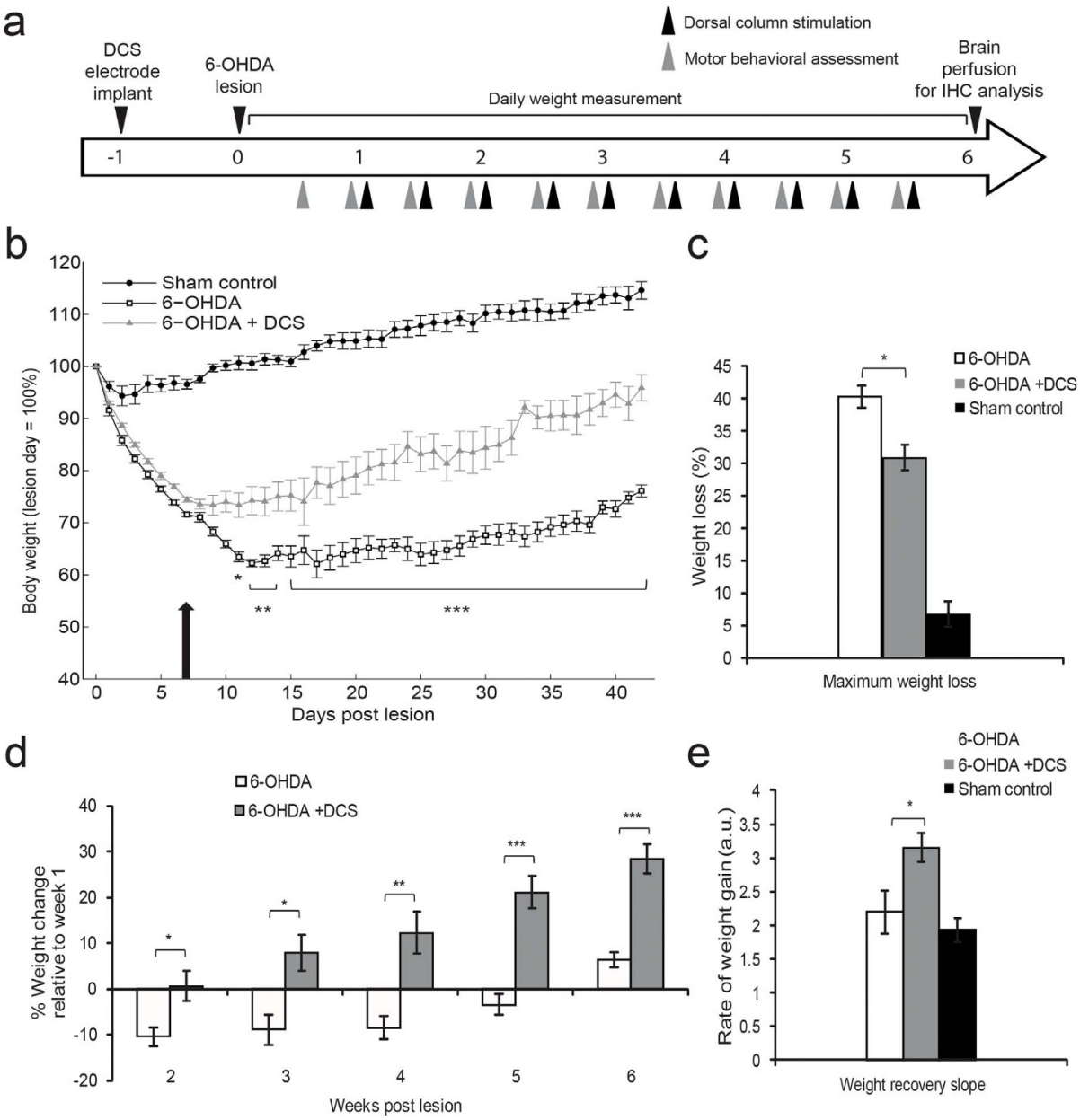


**Figure 1 | DCS improves weight recovery of 6-OHDA lesioned rats.** (a) Time course of the experiment. Numbers indicate experimental weeks from onset at time 0 (6-OHDA lesion). Rats in 6-OHDA + DCS group sustained epidural DCS electrode implantation (week −1) 1 week before the 6-OHDA lesion (week 0). Rats in 6-OHDA and sham control groups underwent only bilateral lesion procedure; 6-OHDA group (total 52.5 μg 6-OHDA), sham control group (only vehicle solution). Timing of motor assessment and 30 minute DCS sessions is illustrated by grey and black upward arrows respectively. Six weeks post lesion (week 6), brains were collected and processed for immunohistochemistry (IHC). (b) Changes in body weight after bilateral intrastriatal 6-OHDA lesion with or without DCS treatment. Lesioned, non-treated rats (n = 8) suffered sustained weight loss with little-to-none recovery. Lesioned rats with DCS treatment (n = 7, 30 min, 333 Hz continuous DCS during 30 min twice a week, starting 7th day, black arrow) recovered body weight significantly faster than non-treated rats ($p < 0.0001$, two-way repeated measure ANOVA). *:$p < 0.05$(day 11), **:$p < 0.01$(days 12–14), ***:$p < 0.001$(days 15–42), Bonferroni multiple comparisons. c) Maximum weight loss is significantly larger for non-treated rats compared to DCS treated rats. *:$p < 0.05$, Mann-Whitney test. d) DCS treatment reverses the trend of weight loss almost immediately, while non-treated rats continue to lose weight till week 5 as shown by weight change relative to week 1, which is significantly higher for treated rats as compared to non-treated. *:$p < 0.05$, **:$p < 0.01$, ***:$p < 0.001$, Mann-Whitney test. (e) DCS treatment results in accelerated weight recovery. Rate of weight gain is significantly higher in treated rats compared to non-treated. *:$p < 0.05$, Mann-Whitney test, a.u. (arbitrary unit). All error bars are s.e.m.

had an effect in improving animal locomotion. Overall, there was a significant interaction between groups and weeks in the spectral power of the acceleration vector (Fig. 2d, ANOVA, two factor experiment with repeated measures, groups × weeks: $p < 0.05$). At week 5, lesioned rats (2.46 ± 0.66) had higher spectral power than both DCS treated (0.91 ± 0.17) and control animals (0.96 ± 0.13), $p < 0.05$, Bonferroni multiple comparisons. Again, this finding suggests that the DCS treatment had a significant effect in improving motor behavior in lesioned animals.

Further, the length of the stride was measured at week 4 for animals in the three groups. Untreated lesioned rats (58.74 ± 2.59) had shorter strides as compared to both DCS treated (69.12 ± 2.77) and

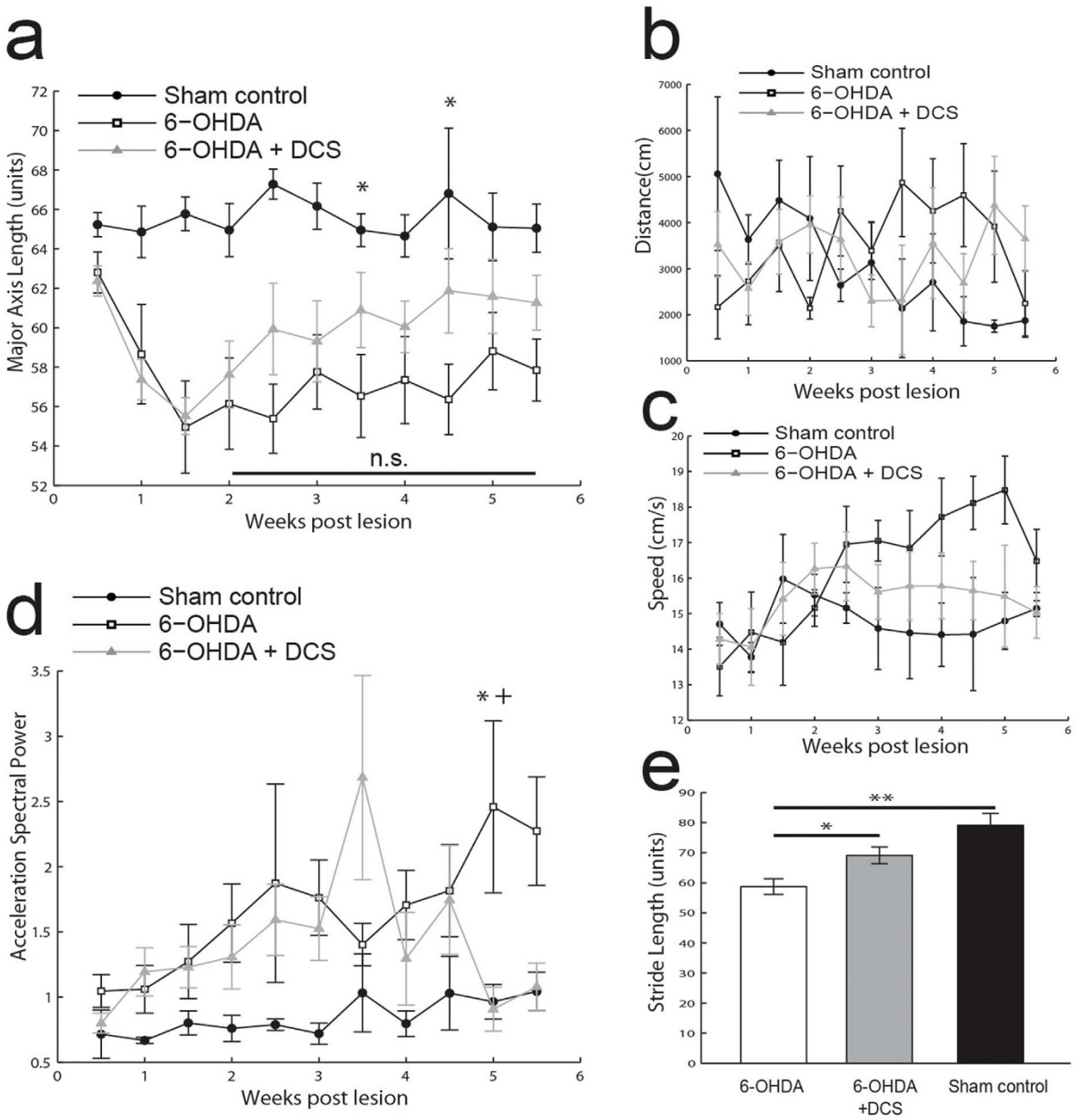


**Figure 2 | DCS restores motor functions in PD rats.** (a) Changes in rat posture (measured as major axis length, greater length implying better posture) with or without DCS treatment. Lesioned rats develop crouched posture resulting in shorter major axis length. DCS treatment restores posture significantly faster than non-treated rats [groups × weeks interaction: $p < 0.05$, two-way repeated measure ANOVA, *:$p < 0.05$ at week 3.5 and 4.5 between DCS treated ($n = 6$) and non-treated rats ($n = 8$), n.s.: DCS treated rats were not significantly different from controls ($n = 4$) from week 2, Bonferroni multiple comparisons]. (b) Distance travelled and (c) average speed during a 30 min open field session was not significantly different between the groups (groups × weeks interaction: $p < 0.0001$ for distance, groups × weeks interaction: $p < 0.01$ for speed, but no differences on post-hoc analysis for both). (d) Spectral power of the acceleration vector in frequency range 0.5–4.75 Hz (indicating jerky non-smooth locomotion) was significantly higher in lesioned rats than DCS treated and controls towards the end [$p < 0.05$, two-way repeated measure ANOVA, *:$p < 0.05$ (6-OHDA + DCS compared to 6-OHDA), +:$p < 0.05$ (6-OHDA compared to controls), Bonferroni multiple comparisons]. (e) Stride length measured at week 4 was significantly higher for DCS treated rats as compared to non-treated ($p < 0.01$, one-way ANOVA, *:$p < 0.05$, **:$p < 0.01$-Tukey's multiple comparison test). All error bars are s.e.m.

control animals (79.05 ± 4.02), however the stride length of treated rats was not significantly different from that of controls (One-way ANOVA, $p < 0.01$, Tukey's Multiple Comparison test, Fig. 2e).

**Long-term DCS protects nigrostriatal dopaminergic system.** 6-OHDA lesions resulted in severe damage to nigrostriatal dopaminergic striatal projections as compared to sham controls, as evidenced by the loss of TH immunoreactivity in these areas (Fig. 3a, bottom and middle panels). Quantification of the striatal TH immunoreactivity by a contrast index (CI) showed significant differences when the three groups were compared (one-way ANOVA, $p < 0.05$, Fig. 3c, top panel). 6-OHDA lesioning caused a 67% decrease of striatal TH levels when compared to the sham control group (6-OHDA CI 0.114 ± 0.022, sham lesion 0.34 ± 0.024; Bonferroni's Multiple Comparison, $p < 0.05$). However, DCS treatment of the 6-OHDA lesioned rats resulted in a 35% decrease of striatal TH levels. The difference

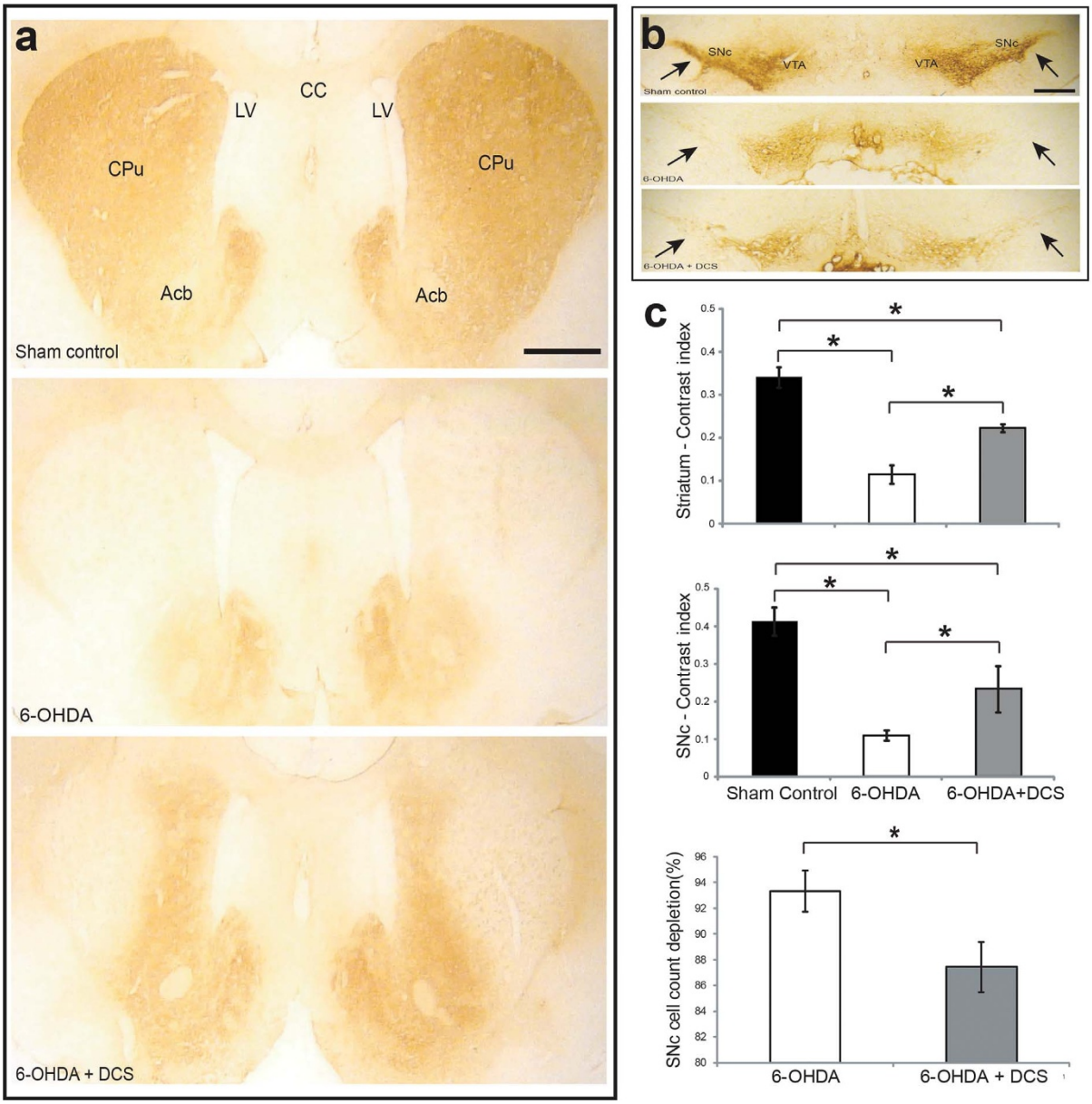


**Figure 3 | DCS protects nigrostriatal dopaminergic system.** (a) Representative immunostaining for tyrosine hydroxylase (TH) in striatum (DAB stain). Note the higher dopaminergic innervation in the striatum (CPu, Acb) of DCS treated rat as compared to non-treated, scale bar = 1 mm, CC = Corpus Callosum, LV = Lateral Ventricle, CPu = Caudate Putamen, Acb = Nucleus Accumbens. 6-OHDA lesion caused a 67% decrease of striatal TH levels (measured by contrast index), with respect to the sham control group, while treatment of the 6-OHDA lesioned rats with DCS resulted only in a 35% decrease, (top panel, 3c). The difference between the TH levels of 6-OHDA (n = 6) and 6-OHDA + DCS (n = 6) groups was significant (*:$p < 0.05$, Bonferroni's Multiple Comparison, (b) Representative immunostaining for tyrosine hydroxylase in substantia nigra pars compacta (SNc), scale bar = 500 um, SNc = substantia nigra pars compacta, VTA = ventral tegmental area. 6-OHDA lesion resulted in a severe loss of TH immunoreactivity (measured by contrast index) in the SNc. 6-OHDA rats showed 74% loss in TH CI as compared to sham controls while DCS treated rats showed only 44%. There was significant difference between the TH levels of 6-OHDA (n = 6) and 6-OHDA + DCS (n = 6) groups in the SNc (*:$p < 0.05$, Bonferroni's Multiple Comparison, middle panel, 3c. Dopaminergic neuronal cell loss in SNc (expressed as % of neuronal count in sham controls) was significantly higher in 6-OHDA (93.32 ± 1.62, n = 8) rats as compared to 6-OHDA + DCS (87.43 ± 1.95, n = 6) rats (*:$p < 0.05$, t-test, 2 tailed). All error bars are s.e.m.

between the TH levels of the 6-OHDA and 6-OHDA + DCS groups (around 32% of the TH control levels) was significant (6-OHDA + DCS CI 0.222 ± 0.018, 6-OHDA CI 0.114 ± 0.022, Bonferroni's Multiple Comparison, $p < 0.05$).

Similarly, 6-OHDA lesioning resulted in a severe loss of TH immunoreactivity, measured by the contrast index in the subtantia nigra pars compacta (SNc), as shown in the example in Fig. 3b. Six weeks after lesioning, 6-OHDA rats showed a significant decrease in TH CI (0.106 ± 0.014) as compared to sham lesion levels (0.410 ± 0.038; one-way ANOVA followed by Bonferroni's Multiple Comparison, $p < 0.05$), which represents a 74% loss. Meanwhile, 6-OHDA rats treated with DCS exhibited only a 44% loss, which was significantly different from non-treated rats (6-OHDA + DCS CI 0.230 ± 0.061, 6-OHDA CI 0.106 ± 0.014, $p < 0.05$, Bonferroni's Multiple Comparison, Fig. 3c, middle panel). 6-OHDA lesioning also resulted in severe loss of dopaminergic neurons from the SNc. Neuronal cell count depletion (expressed as % of sham control cell count) was significantly higher in non-treated rats [(93.32 ± 1.62)%, n = 8] as

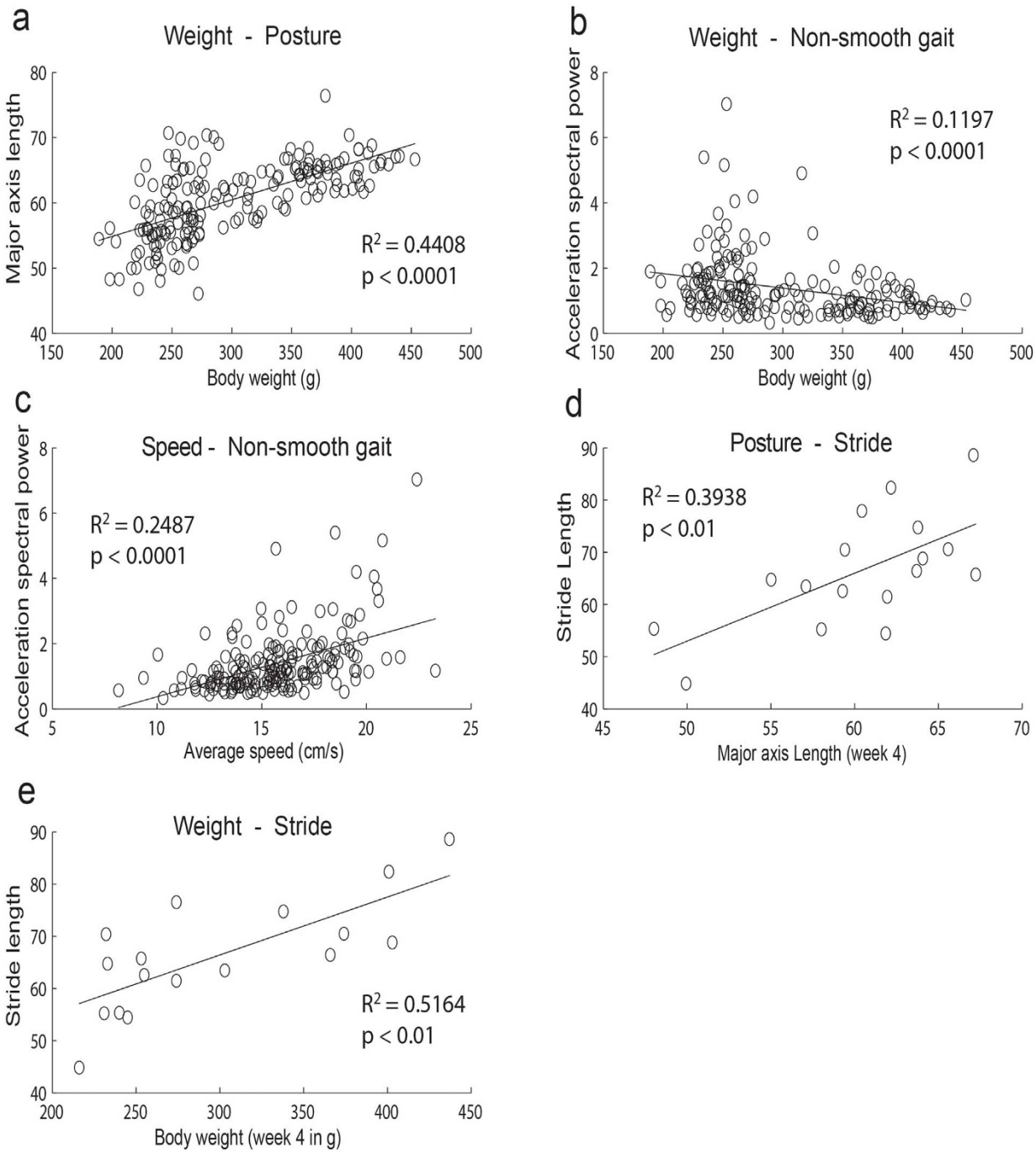


**Figure 4 | Neuroprotective effect of DCS on weight and PD symptoms.** (a) There was significant correlation between weight and major axis length (measure of posture) throughout the experiment (Spearman test, $p < 0.0001$). (b) Body weight had a significant negative correlation with spectral power of acceleration vector (indicating jerky non-smooth locomotion) – Spearman test, $p < 0.0001$. (c) Spectral power of acceleration vector and speed were significantly correlated throughout the experimental period (Spearman test, $p < 0.0001$). Stride length measured at week 4 had significant correlation with major axis length ((d), Spearman test, $p < 0.01$) and body weight ((e), Spearman test, $p < 0.01$) indicating that recovery of body weight was related to an overall improvement in motor symptoms.

compared to DCS treated [(87.43 ± 1.95)%, n = 6] animals ($p < 0.05$, t-test, Fig. 3c, bottom panel).

**Global effects of neuroprotection.** We also investigated the potential relation between body weight and multiple motor symptoms by calculating the linear correlations between them. This analysis revealed a positive correlation between weight and major body axis length (Spearman test: $r = 0.6639$, $p < 0.0001$, Fig. 4a). Likewise, a significant negative correlation was found between weight and non-smooth gait (Spearman test: $r = 0.346$, $p < 0.0001$, Fig. 4b), indicating that rats with greater weight loss had severe motor symptoms. Body weight also exhibited a significant correlation with stride length measured at week 4 (Spearman test: $r = 0.7186$, $p < 0.01$, Fig. 4e), indicating that an improvement in body weight was correlated with an improvement in posture and gait. There was a strong correlation between non-smooth gait and speed (Spearman test: $r = 0.4987$, $p < 0.0001$, Fig. 4c), confirming our claim that the non-smooth gait was predominantly observed during high speed

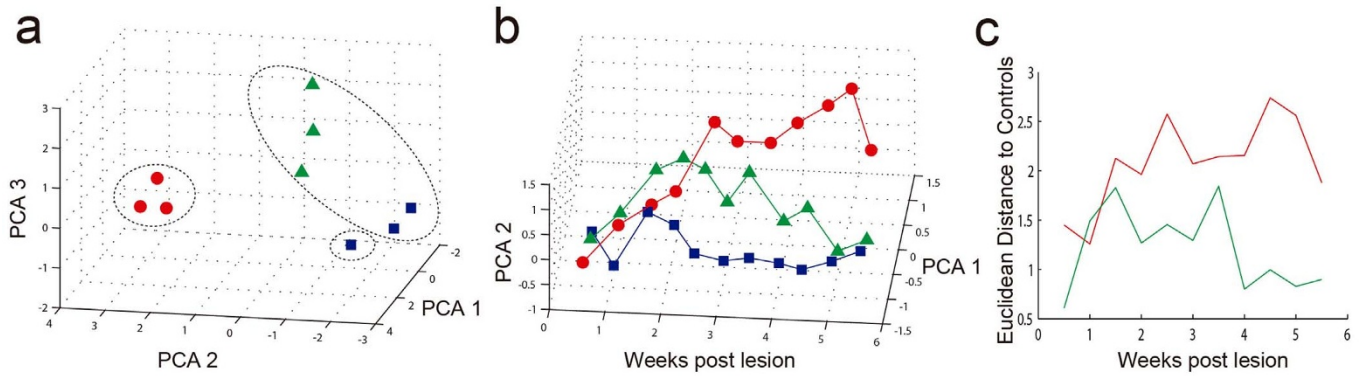


**Figure 5 | DCS reverts weight, behavioral, and cellular parameters back to normality.** (a) Principal component analysis (PCA) was performed using 9 rats having all the data variables at a single time point at the end of the experiment [weight (day 42); axis length, acceleration power, distance and speed at week 5.5; stride (week 4); TH immunoreactivity of striatum and SNc and SNc cell count). Representation of the individual rat data points, using the first three PCs on a 3D space, shows that the rats tend to cluster according to their experimental group. Agglomerative cluster analysis of the data (dotted ellipsoids) correctly identified the 6-OHDA rats (red dots), but failed to separate the control (blue squares) and the 6-OHDA + DCS (green triangles) rats. (b) PCA was performed with four motor parameters [speed, distance, non-smooth gait (acceleration power), and posture (axis length)] for all time points and the first two PC scores of all groups were averaged for each time point and plotted against time post lesion. The resulting graph shows the progression of the groups throughout the 6 weeks of the experiment. While the three experimental groups start close to each other (week 0.5–1.5), the 6-OHDA and 6-OHDA + DCS groups split from control after week 2. By the end of the experimental time, the 6-OHDA + DCS group joins the control group (c) Euclidean distances of the average PCs of each group shows that the motor parameters of the 6-OHDA + DCS group started to drift from the untreated 6-OHDA group after week 1.5 and became close to control parameters after week 4.

displacement. A significant correlation between major body axis length and stride length (Spearman test: $r = 0.6275$, $p < 0.01$ Fig. 4d) was also observed, indicating that rats with better posture had improved gait at the end of the experimental period. Overall, all these linear correlations supported our contention that chronic DCS treatment improved the clinical effects of 6-OHDA lesions in rats.

Finally, we performed a cluster analysis using the principal component scores derived from a set of motor variables (posture, non-smooth gait, distance and speed at week 5.5, stride at week 4), histological variables (TH immunoreactivity of striatum and SNc, and SNc cells/slide) and weight at day 42. The cluster analysis correctly grouped and isolated the data points from the 6-OHDA rats but failed to separate the control and the 6-OHDA + DCS treated rats (Fig. 5a). Next, using only the motor variables (speed, distance, posture, non-smooth gait), we analyzed how the three groups of animals behaved over time, by plotting the progression of the first two principal components which account for 79% of the total variance. This analysis revealed that, immediately after the lesion, the DCS treated group initially clustered together with the 6-OHDA lesioned group. Yet, 1.5 weeks afterwards, the DCS treated group started to separate from the lesioned group and move towards the control group. By the end of the experimental period (week 6), the 6-OHDA + DCS group joined the control group's space and became indistinguishable from it (Fig. 5b). This effect can be clearly documented by plotting the Euclidean distances of 6-OHDA and 6-OHDA + DCS groups from the control group against time (Fig. 5.c). At week 1.5 the 6-OHDA + DCS group separates from 6-OHDA and starts approaching the control's trajectory, reaching it by week 4 (Fig. 5c).

## Discussion

In this study we quantified for the first time the long-term effects of electrical stimulation of the dorsal column of the spinal cord (DCS) on body weight, motor symptoms and survival of nigrostriatal dopaminergic neurons in a chronic rat model of Parkinson's disease. We found that chronic DCS applied on a regular basis was associated with progressive improvement in characteristic PD motor symptoms and accelerated recovery of lost weight. This improvement in clinical signs was paralleled with the maintenance of a higher density of dopaminergic innervation in the striatum and neuronal cell count in the SNc of DCS-treated rats when compared to a group of untreated 6-OHDA lesioned animals. These results show that long-term DCS is associated with functional and structural recovery in a classic animal model of PD, suggesting that this method may be considered in the future as a potential therapy for PD patients.

Comparison between the three groups of animals utilized in this study - sham control, 6-OHDA, and 6-OHDA + DCS - revealed a progressive weight increase in the control and the chronic DCS groups after the initial procedures (sham lesion and 6-OHDA lesion respectively). The control group reached its initial weight within 2 weeks post-surgery, as expected[16–18]. Afterwards, this group presented a normal weight increase of approximately 3% per week. Animals treated with chronic DCS showed a weight increase of 6% per week, following the 6-OHDA lesion, which was more prominent after 2 weeks of treatment. It is not clear if the increase was only an effect of improved motor function (i.e. restoring the ability of the animal to feed itself) or increased appetite (due to overall effects of treatment) or both. For example, dysphagia caused by oropharyngeal dysfunction and hyposmia, which could be responsible for weight loss, are common findings in advanced PD patients[19,20]. It is also suggested that neuroendocrinological dysregulation or lower concentrations of orexins could play an important role in the feeding behavior of PD patients[21]. Consistent with our present findings, data from advanced PD patients subjected to subthalamic (STN) DBS shows increased appetite and an average weight gain of 13% within ~16 months of treatment[22], weight gain of $9.7 \pm 7$ kg within $12.7 \pm 7.8$ months with 60% improvement in UPDRS-III motor scores[23] and a positive correlation between motor symptom improvement and weight gain[24]. Our findings indicate that this latter correlation was also obtained when DCS was applied to our PD animals. Future studies in our laboratory will address how the activity of neural ensembles controlling motor and feeding functions is affected in Parkinsonian states.

The 6-OHDA lesion did not cause quantitative decrease in the animal's average speed and traveled distance. This could be explained by the phenomenon known as starvation-induced hyperactivity[25], which may have masked or compensated for the expected hypokinetic symptoms. Yet the lesioned animals exhibited clear motor symptoms, such as crouched posture, short strides, and non-smooth displacement across the open field, all of which were significantly

reduced by the DCS treatment delivered only twice a week. Using the current protocol for deep brain stimulation (DBS) as a benchmark, one can postulate that a more frequent treatment or even continuous DCS could very likely lead to even larger motor effects.

At this point it is important to mention what mechanisms could account for the motor effects of DCS in PD. Both experimental evidence and modeling, obtained to explain the mechanism of DCS to treat chronic pain conditions, indicate that electrical stimulation delivered in the dorsal epidural space, as we did in the current work, activates mainly the superficial fibers of the dorsal columns and the dorsal roots of the corresponding spinal segment[26]. Thus, the consensus reached by this literature proposes that the mechanisms underlying the DCS effects reported here should emerge from the exclusive activation of ascending somatosensory fibers running through the dorsal portion of the spinal cord. This activation could, in turn, modulate the activity of multiple supraspinal structures, including thalamic, striatal and cortical areas[5,12]. We should, therefore, emphasize that even though higher DCS intensities can additionally recruit deeper structures of the dorsal columns, such as the corticospinal tract (CST) (which in rodents is located in the ventral part of the dorsal columns) we never observed any sign, such as muscle twitching of proximal or distal myotomes, that could support the thesis that the CST was recruited by our experimental protocol to stimulate the spinal cord. Even in humans, where CST are located more laterally, DCS at intensities above therapeutic levels can recruit fibers of the CST or local spinal motorneurons or interneurons[27,28]. However, since our experiments were conducted at a stimulation intensity that did not cause any muscle twitching, it is highly unlikely that activation of CST has contributed in any way to the effects described hereby. Yet, given the anatomical differences between rodent and primate nervous system (i.e. position of the CST within the spinal cord[29]) it is crucial to confirm these results in non-human primate models. To address that very issue, we have recently concluded a series of studies showing that DCS produces the same beneficial effects, in terms of improvement of motor symptoms, in a primate model of PD. Such results are currently being prepared for publication (personal observation).

Degeneration of the nigrostriatal dopaminergic system is a common finding in postmortem studies of PD patients, and also a good indicator of the stage of the disease[30]. After long term treatment with DCS twice a week, we found that 6-OHDA rats exhibited a moderate yet significant reduction in the depletion of striatal TH-staining and TH-IR neuronal cells in SNc, as compared to non-treated lesioned animals. Although the mechanisms underlying such a reduction in dopaminergic degeneration have not been determined, we think they might be mediated by increased production or delivery of neurotrophic factors. Previous studies have shown that intrastriatal injections of brain derived neurotrophic factor (BDNF) can attenuate the effect of 6-OHDA lesions[31,32]. A recent study involving STN-DBS has shown proof of a neuroprotective effect on the SNc neurons in a rodent 6-OHDA model[33], while subsequent experiments from the same group showed an increase in levels of nigrostriatal BDNF following STN DBS[34]. This could be the case with our DCS treated animals. Although clinical studies conducted with advanced PD patients to measure disease progression using 18F-fluorodopa PET failed to confirm a neuroprotective effect of clinically effective STN-DBS[35], it would be interesting to investigate in the future whether neuroprotective effects of chronic DCS can be observed in a clinical population.

Considering the increased longevity of the population worldwide, the introduction of novel treatments that address both the symptoms and progressive nature of PD constitute a major priority for the management of Parkinsonian patients. Based on recently described preliminary evidence showing efficacy of DCS in a series of PD patients worldwide[8–11] and our own results in both acute and chronic PD animal models, we propose that chronic epidural DCS, a procedure that does not invade the brain, does not have serious side effects and can be carried out at much lower costs and risks for patients, could be employed at the early clinical stages of PD to manage some of its cardinal motor symptoms. This conclusion is further supported by our preliminary data showing that DCS can also alleviate motor symptoms in a genetic model of PD in rats and in a primate model of PD (R.F. and M.A.L.N. personal observations).

At this point, there are very few reports of DCS in PD patients. Thus, it is difficult to predict if the eligibility of DCS will be substantially better than for DBS. However, the lack of major side effects, the relative ease with which the surgical procedure can be performed, and the fact that there is no need to penetrate into brain tissue suggest that DCS could become an early stage therapy in the future management of PD patients.

## Methods

**Animals.** A total of 41 male Long-Evans rats (body weight ranging from 310 to 450 g) were housed individually with ad-libitum food and water in a temperature controlled room on a 12 h light/12 h dark cycle. Animal procedures were performed according to prior approved protocols by Duke University Institutional Animal Care and Use Committee and in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23). Rats were divided into 3 groups (sham control, 6-OHDA lesion and 6-OHDA lesion + DCS).

**6-OHDA lesion and stimulation electrode implant procedures.** Rats in the 6-OHDA + DCS group underwent two separate surgical procedures. First, they were implanted with spinal stimulation electrodes under anesthesia induced with 5% halothane, ketamine (100 mg/kg), xylazine (10 mg/kg) and atropine (0.05 ml). Postoperative weight was monitored daily. The implantation procedure was adapted from previous studies[5,36]. The electrodes were inserted in the epidural space under T2 (thoracic vertebra) and tied to it with surgical suture. This prevented electrode migration and facilitated stimulation over a long period. One week later, after recovery of initial weight, rats were anesthetized for a second surgery, with 5% halothane, followed by intramuscular injections of ketamine (100 mg/kg), xylazine(10 mg/kg) and atropine (0.05 ml). A total of 52.5 ug 6-OHDA hydrobromide (Sigma Company, USA - 3.5 mg/ml in 0.05% ascorbate saline) was injected bilaterally into the striatum, at 3 locations on each side, using a needle, driven by a syringe pump (Sage, Model 361, Firstenberg Machinery Co Inc., USA) via 10 uL Hamilton syringe, at 1 uL/min. The needle was left in situ for 5 minutes and withdrawn slowly, to prevent backtracking of the drug. Anteroposterior, mediolateral and dorsoventral coordinates for the injections were: +1.0, +/−3.0, −5.0; −0.1, +/−3.7, −5.0 and −1.2, +/−4.5, −5.0[37] from bregma. Destruction of noradrenergic fibers and terminals was prevented by 1,3-Dimethyl-2-imidazolidinone (DMI, Sigma Company, 25 mg/kg), administered IP, 30 minutes prior to 6-OHDA treatment[38].

Rats belonging to the 6-OHDA lesion and sham control groups underwent only the surgical procedure required to perform the lesion; animals in the 6-OHDA lesion group received bilateral injections of 6-OHDA, while rats in the sham control group received only vehicle solution (0.05% ascorbate saline). Time course of the entire experiment is shown in Fig. 1a. The lesion procedures were performed by the same individuals throughout all the experiment and all groups were run in parallel. Extreme care was taken to consistently maintain the timing of the various methods and conditions during the lesion procedure.

**Weight and motor behavioral assessment.** In the post-lesion period, rats had access to water soaked food pellets and fruit loop cereals and their body weight was recorded daily. 15 rats that did not display severe motor symptoms and lost less than 20% of initial weight at day 7 post-lesion were discarded from the experiment. Rats with severe weight loss (>20% body weight) were retained in their original groups and subjected to the entire experiment (N = 18, 10 in 6-OHDA lesion + DCS group, and 8 in 6-OHDA lesion group). These were labeled as "strongly lesioned rats". Rats that lost more than 25% of initial weight were additionally hand-fed with peanut butter daily, until their weight reached 75%.

A few days after lesioning, strongly lesioned rats exhibited several motor impairments, including postural and gait instability, and reduced forelimb dexterity[39]. These symptoms manifested into an inability to grasp or chew food, which was clearly observed while the rats tried to eat. Motor behavior was assessed twice a week, on days 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, and 39 post lesion. Rats were placed in an elliptical open field (85 cm × 70 cm axes, 60 cm tall) for 30 min and motor behavior was recorded from a bottom view camera.

The posture and position of the rats in the open-field was extracted from digitized video recordings with custom designed algorithms implemented in Matlab (MathWorks, USA). Image processing of single frames from the video was used to extract the rat shape. An ellipsoid was fitted to the rat shape, and the length of the major axis of the ellipse (expressed in pixels) was used as a measure of posture. Digital videos of the ellipse superimposed on rat shape were saved and later used to confirm the accuracy of the algorithm. We observed that rats with crouched posture had shorter major axis length, while normal rats had longer lengths. For posture analysis only those frames during which the rats were mobile were used. Instantaneous speed and instantaneous acceleration vectors were calculated from the distance between the

X,Y location of the rat every 1/30 seconds in the open field. Lesioned rats showed jerky movement that lacked smoothness and fluidity. To quantify this abnormal behavior, we calculated the derivative of the instantaneous speed, thus obtaining a measurement of instantaneous acceleration. A spectrogram with a window of 4 seconds, sliding every 0.033 seconds was constructed with the 'mtspecgramc' function (Chronux toolbox) from the acceleration signal. The power spectra between 0.5–4.75 Hz of the time bins where locomotion was greater than 5 cm/s were averaged. For measuring stride length of rats, videos from top view camera were used. The front most part of the camera view was selected for better visualization of hind legs. A locomotion bout with at least 3 strides in the selected part of the open field was randomly selected from entire session and stride length was measured using 'implay' function in Matlab and averaged for the 3 strides. Stride was defined as the pixel distance between hind leg (right/left) take off point and subsequent landing point of same leg.

**Chronic electrical stimulation of the dorsal column of the spinal cord.** After the 30 min behavioral session, rats of the 6-OHDA + DCS group were allowed to rest in their home cage for 30 min. Following this period, they were reintroduced in the open field and continuous DCS was applied for 30 min. Before each stimulation session, stimulation current intensities were determined for each rat, as described in previous study[5]. DCS consisted of biphasic square pulses of 1 ms duration, delivered at 333 Hz at a current intensity ~1.2 times the sensory threshold (mean ± sd, intensity at 333 Hz was 167 ± 52 uA). These intensities ensured that DCS did not cause an arousal effect[5].

**Tyrosine hydroxylase staining and quantification.** Forty-two days after lesion, animals were perfused with 4% paraformaldehyde and the brains were kept in 30% sucrose until sectioning. During tissue sectioning, 30ìm free-floating sections were obtained from the striatum (AP: 2 to −1) and substantia nigra (SN) (AP: −4.2 to −6.6), defined according to the rat brain Atlas[40]. Tyrosine hydroxylase (TH) immunohistochemistry was used to study the extent and position of striatal lesions and quantify the depletion of dopaminergic neurons in the nigrostriatal pathway as described elsewhere[41].

For quantification of TH-staining, the tissue samples were mounted and pictures were taken using a microscope with the same camera configuration and light intensity for each slice. TH-reactivity in both striatum and substantia nigra pars compacta (SNc) in all groups (sham control, 6-OHDA lesion and 6-OHDA lesion + DCS) was assessed by computer densitometry using digital images captured with the camera attached to the microscope. Average densitometric values were obtained by using the ImageJ software (http://rsb.info.nih.gov/ij/) from 2 images where both structures could be unequivocally defined (see Figure 3). The measurements were obtained inside a 0.04 $mm^2$ square window positioned across the structures of interest. In order to evaluate the general TH-reactivity throughout the striatum and SNc we obtained three samples per structure. To minimize the effects of within-group variability, we adopted a normalized scale based on the non-reactive cortical white matter (averaged over measurements of 3 different sites using the same window). For every animal, the average optical density (OD) for the striatum or SNc was designated S, for the cortical white matter W and a contrast index C was calculated according to the equation: $C = (S - W)/(S + W)$[42].

Using a high-magnification microscope (NIS-element, Nikon, Japan) equipped with a software package, a total of 6 SNc slices between AP: 5.8–6.33 mm of bregma were used to bilaterally count immunoreactive neurons and an average cell count was calculated for every animal. To obtain an unbiased estimate of cell numbers, we applied the Abercrombie's correction factor[43], which compensates for the over counting of sectioned profiles, using the equation $P = A(M/M + L)$, where P is the corrected value, A is the raw density measure, M is the section's thickness (in micrometers) and L is the average diameter of cell bodies (n = 40 by group) along the axis perpendicular to the plane of section. The mean value of TH-IR nigral neurons for each animal was expressed as a percentage of cell loss, compared to the mean cell count for sham control rats.

**Statistical analysis.** All results are expressed as mean ± sem. Statistical analysis was performed using a computer program (Graphpad Prism 5.0, Graphpad Software, USA). Weight and motor behavior data was subjected to two-way analysis of variance (ANOVA) with repeated measures, followed by post-hoc multiple comparison tests. Whenever distributions failed the normality test, non-parametric tests such as Mann-Whitney (t-test) were used. Spearman's Rank Correlation test was used to study the correlation between different parameters.


1. Carlsson, A. Biochemical and pharmacological aspects of Parkinsonism. *Acta Neurol. Scand. Suppl.* **51**, 11–42 (1972).
2. Nagatsua, T. & Sawadab, M. L-dopa therapy for Parkinson's disease: past, present, and future. *Parkinsonism Relat Disord* **15 Suppl 1**, S3–8, doi:10.1016/S1353-8020(09)70004-5 (2009).
3. Benabid, A. L. Deep brain stimulation for Parkinson's disease. *Curr. Opin. Neurobiol.* **13**, 696–706 (2003).
4. Morgante, L. *et al.* How many parkinsonian patients are suitable candidates for deep brain stimulation of subthalamic nucleus? Results of a questionnaire. *Parkinsonism Relat Disord* **13**, 528–531, doi:10.1016/j.parkreldis.2006.12.013 (2007).
5. Fuentes, R., Petersson, P., Siesser, W. B., Caron, M. G. & Nicolelis, M. A. Spinal cord stimulation restores locomotion in animal models of Parkinson's disease. *Science* **323**, 1578–1582, doi:10.1126/science.1164901 (2009).
6. Thevathasan, W. *et al.* Spinal cord stimulation failed to relieve akinesia or restore locomotion in Parkinson disease. *Neurology* **74**, 1325–1327, doi:10.1212/WNL.0b013e3181d9ed58 (2010).
7. Nicolelis, M. A., Fuentes, R., Petersson, P., Thevathasan, W. & Brown, P. Spinal cord stimulation failed to relieve akinesia or restore locomotion in Parkinson disease. *Neurology* **75**, 1484; author reply 1484–1485, doi:10.1212/WNL.0b013e3181f46f10 (2010).
8. Fenelon, G. *et al.* Spinal cord stimulation for chronic pain improved motor function in a patient with Parkinson's disease. *Parkinsonism Relat Disord* **18**, 213–214, doi:10.1016/j.parkreldis.2011.07.015 (2012).
9. Agari, T. & Date, I. Spinal cord stimulation for the treatment of abnormal posture and gait disorder in patients with Parkinson's disease. *Neurol. Med. Chir. (Tokyo).* **52**, 470–474 (2012).
10. Landi, A. *et al.* Spinal Cord Stimulation for the Treatment of Sensory Symptoms in Advanced Parkinson's Disease. *Neuromodulation: journal of the International Neuromodulation Society*, doi:10.1111/ner.12005 (2012).
11. Hassan, S., Amer, S., Alwaki, A. & Elborno, A. A patient with Parkinson's disease benefits from spinal cord stimulation. *Journal of clinical neuroscience: official journal of the Neurosurgical Society of Australasia*, doi:10.1016/j.jocn.2012.08.018 (2013).
12. Aguilar, J. *et al.* Spinal direct current stimulation modulates the activity of gracile nucleus and primary somatosensory cortex in anaesthetized rats. *The Journal of physiology* **589**, 4981–4996, doi:10.1113/jphysiol.2011.214189 (2011).
13. Stancak, A. *et al.* Functional magnetic resonance imaging of cerebral activation during spinal cord stimulation in failed back surgery syndrome patients. *Eur J Pain* **12**, 137–148, doi:10.1016/j.ejpain.2007.03.003 (2008).
14. Dejongste, M. J., Hautvast, R. W., Ruiters, M. H. & Ter Horst, G. J. Spinal Cord Stimulation and the Induction of c-fos and Heat Shock Protein 72 in the Central Nervous System of Rats. *Neuromodulation: journal of the International Neuromodulation Society* **1**, 73–84, doi:10.1111/j.1525-1403.1998.tb00020.x (1998).
15. Maeda, Y., Ikeuchi, M., Wacnik, P. & Sluka, K. A. Increased c-fos immunoreactivity in the spinal cord and brain following spinal cord stimulation is frequency-dependent. *Brain Res.* **1259**, 40–50, doi:10.1016/j.brainres.2008.12.060 (2009).
16. Lenard, L. *et al.* Feeding and body weight regulation after 6-OHDA application into the preoptic area. *Brain Res. Bull.* **27**, 359–365 (1991).
17. Ferro, M. M. *et al.* Comparison of bilaterally 6-OHDA- and MPTP-lesioned rats as models of the early phase of Parkinson's disease: histological, neurochemical, motor and memory alterations. *J. Neurosci. Methods* **148**, 78–87, doi:10.1016/j.jneumeth.2005.04.005 (2005).
18. Roedter, A. *et al.* Comparison of unilateral and bilateral intrastriatal 6-hydroxydopamine-induced axon terminal lesions: evidence for interhemispheric functional coupling of the two nigrostriatal pathways. *J. Comp. Neurol.* **432**, 217–229 (2001).
19. Muller, J. *et al.* Progression of dysarthria and dysphagia in postmortem-confirmed parkinsonian disorders. *Arch. Neurol.* **58**, 259–264 (2001).
20. Ondo, W. G. *et al.* Weight gain following unilateral pallidotomy in Parkinson's disease. *Acta Neurol. Scand.* **101**, 79–84 (2000).
21. Bachmann, C. G. & Trenkwalder, C. Body weight in patients with Parkinson's disease. *Mov. Disord.* **21**, 1824–1830, doi:10.1002/mds.21068 (2006).
22. Moro, E. *et al.* Chronic subthalamic nucleus stimulation reduces medication requirements in Parkinson's disease. *Neurology* **53**, 85–90 (1999).
23. Macia, F. *et al.* Parkinson's disease patients with bilateral subthalamic deep brain stimulation gain weight. *Mov. Disord.* **19**, 206–212, doi:10.1002/mds.10630 (2004).
24. Gironell, A., Pascual-Sedano, B., Otermin, P. & Kulisevsky, J. [Weight gain after functional surgery for Parkinsons disease]. *Neurologia* **17**, 310–316 (2002).
25. Pirke, K. M., Broocks, A., Wilckens, T., Marquard, R. & Schweiger, U. Starvation-induced hyperactivity in the rat: the role of endocrine and neurotransmitter changes. *Neurosci. Biobehav. Rev.* **17**, 287–294 (1993).
26. Holsheimer, J. Which Neuronal Elements are Activated Directly by Spinal Cord Stimulation. *Neuromodulation: journal of the International Neuromodulation Society* **5**, 25–31, doi:10.1046/j.1525-1403.2002._2005.x (2002).
27. Dimitrijevic, M. R., Faganel, J., Sharkey, P. C. & Sherwood, A. M. Study of sensation and muscle twitch responses to spinal cord stimulation. *Int. Rehabil. Med.* **2**, 76–81 (1980).
28. Nashold, B. S. Jr., Somjen, G. & Friedman, H. The effects of stimulating the dorsal columns of man. *Med. Prog. Technol.* **1**, 89–91 (1972).
29. Kaas, J. H. *et al.* Cortical and subcortical plasticity in the brains of humans, primates, and rats after damage to sensory afferents in the dorsal columns of the spinal cord. *Exp. Neurol.* **209**, 407–416, doi:10.1016/j.expneurol.2007.06.014 (2008).
30. Kish, S. J., Shannak, K. & Hornykiewicz, O. Uneven pattern of dopamine loss in the striatum of patients with idiopathic Parkinson's disease. Pathophysiologic and clinical implications. *N. Engl. J. Med.* **318**, 876–880, doi:10.1056/NEJM198804073181402 (1988).
31. Shults, C. W., Kimber, T. & Altar, C. A. BDNF attenuates the effects of intrastriatal injection of 6-hydroxydopamine. *Neuroreport* **6**, 1109–1112 (1995).

32. Klein, R. L., Lewis, M. H., Muzyczka, N. & Meyer, E. M. Prevention of 6-hydroxydopamine-induced rotational behavior by BDNF somatic gene transfer. *Brain Res.* **847**, 314–320 (1999).
33. Spieles-Engemann, A. L., Collier, T. J. & Sortwell, C. E. A functionally relevant and long-term model of deep brain stimulation of the rat subthalamic nucleus: advantages and considerations. *Eur. J. Neurosci.* **32**, 1092–1099, doi:10.1111/j.1460-9568.2010.07416.x (2010).
34. Spieles-Engemann, A. L. *et al.* Subthalamic Nucleus Stimulation Increases Brain Derived Neurotrophic Factor in the Nigrostriatal System and Primary Motor Cortex. *J Parkinsons Dis* **1**, 123–136 (2011).
35. Hilker, R. *et al.* Disease progression continues in patients with advanced Parkinson's disease and effective subthalamic nucleus stimulation. *J. Neurol. Neurosurg. Psychiatry* **76**, 1217–1221, doi:10.1136/jnnp.2004.057893 (2005).
36. Fuentes, R., Petersson, P. & Nicolelis, M. A. Restoration of locomotive function in Parkinson's disease by spinal cord stimulation: mechanistic approach. *Eur. J. Neurosci.* **32**, 1100–1108, doi:10.1111/j.1460-9568.2010.07417.x (2010).
37. Winkler, C., Kirik, D., Bjorklund, A. & Cenci, M. A. L-DOPA-induced dyskinesia in the intrastriatal 6-hydroxydopamine model of parkinson's disease: relation to motor and cellular parameters of nigrostriatal function. *Neurobiol. Dis.* **10**, 165–186 (2002).
38. Hrdina, P. D. & Dubas, T. C. Brain distribution and kinetics of desipramine in the rat. *Can. J. Physiol. Pharmacol.* **59**, 163–167 (1981).
39. Deumens, R., Blokland, A. & Prickaerts, J. Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. *Exp. Neurol.* **175**, 303–317, doi:10.1006/exnr.2002.7891 (2002).
40. Paxinos, G. & Watson, C. *The rat brain, in stereotaxic coordinates.* Compact 3rd edn, (Academic Press, 1997).
41. Tischler, A. S. Triple immunohistochemical staining for bromodeoxyuridine and catecholamine biosynthetic enzymes using microwave antigen retrieval. *J. Histochem. Cytochem.* **43**, 1–4 (1995).
42. Freire, M. A., Oliveira, R. B., Picanco-Diniz, C. W. & Pereira, A. Jr. Differential effects of methylmercury intoxication in the rat's barrel field as evidenced by NADPH diaphorase histochemistry. *Neurotoxicology* **28**, 175–181, doi:10.1016/j.neuro.2006.06.007 (2007).
43. Abercrombie, M. Estimation of nuclear population from microtome sections. *Anat. Rec.* **94**, 239–247 (1946).


## Acknowledgments

We thank J. Meloy for help with building stimulation electrodes; L. Oliveira and S. Halkiotis for technical support and Miguel Pais-Vieira for thoughtful comments on the manuscript. This research was supported by NIH Transformative award (R01-NS073125-03), NIH Director's Pioneer award (DP1-OD006798) and the grant "Plano de Acao Brasil Suica CNPq 590006/2010-0" awarded to M.A.L.N. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Office of the NIH Director or the NIH.

## Author contributions

A.P.Y., R.F., H.Z. and M.A.L.N. designed experiments; A.P.Y., R.F. and M.A.L.N. wrote the paper; A.P.Y., H.Z., R.F., M.A.M.F. and M.A.L.N. analyzed the data; A.P.Y., H.Z. and C.W. performed the surgeries; A.P.Y. and T.V. conducted experiments; T.V. performed immunohistochemistry.

## Additional information

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Yadav, A.P. *et al.* Chronic Spinal Cord Electrical Stimulation Protects Against 6-hydroxydopamine Lesions. *Sci. Rep.* **4**, 3839; DOI:10.1038/srep03839 (2014).