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Subthalamic nucleus deep brain stimulation induces sustained neurorestoration in the mesolimbic dopaminergic system in a Parkinson's disease model

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ABSTRACT

Background: Deep brain stimulation (DBS) of the subthalamic nucleus (STN) is an established therapeutic principle in Parkinson's disease, but the underlying mechanisms, particularly mediating non-motor actions, remain largely enigmatic.
Objective/hypothesis: The delayed onset of neuropsychiatric actions in conjunction with first experimental evidence that STN-DBS causes disease-modifying effects prompted our investigation on how cellular plasticity in midbrain dopaminergic systems is affected by STN-DBS.
Methods: We applied unilateral or bilateral STN-DBS in two independent cohorts of 6-hydroxydopamine hemiparkinsonian rats four to eight weeks after dopaminergic lesioning to allow for the development of a stable dopaminergic dysfunction prior to DBS electrode implantation.
Results: After 5 weeks of STN-DBS, stimulated animals had significantly more TH^+ dopaminergic neurons and fibres in both the nigrostriatal and the mesolimbic systems compared to sham controls with large effect sizes of $g_{Hedges} = 1.9–3.4$. DBS of the entopeduncular nucleus as the homologue of the human *Globus pallidus internus* did not alter the dopaminergic systems. STN-DBS effects on mesolimbic dopaminergic neurons were largely confirmed in an independent animal cohort with unilateral STN stimulation for 6 weeks or for 3 weeks followed by a 3 weeks washout period. The latter subgroup even demonstrated persistent mesolimbic dopaminergic plasticity after washout. Pilot behavioural testing showed that augmentative dopaminergic effects on the mesolimbic system by STN-DBS might translate into improvement of sensorimotor neglect.
Conclusions: Our data support sustained neurorestorative effects of STN-DBS not only in the nigrostriatal but also in the mesolimbic system as a potential factor mediating long-latency neuropsychiatric effects of STN-DBS in Parkinson's disease.

1. Introduction

Bilateral deep brain stimulation of the subthalamic nucleus (STN-DBS) is a well-established therapeutic principle for mid- to late-stage Parkinson's disease (PD) with long-lasting motor symptom benefits (Deuschl et al., 2013; Krack et al., 2003). Although alleviations of non-motor symptoms have only recently attracted further interest (Deuschl et al., 2013; Deuschl et al., 2006; Volkmann et al., 2010), current data demonstrate that STN-DBS also improves various non-motor symptoms and quality of life (Dafsari et al., 2016; Deuschl et al., 2006; Volkmann et al., 2010). Most motor improvements emerge rapidly within seconds to a few hours related to high-frequency stimulation onset and likely represent correction of abnormal neuronal network activity (Liu et al., 2008). In contrast, non-motor symptom response after DBS onset is

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delayed by minutes or even weeks and is not precisely defined for most non-motor symptoms (Dafsari et al., 2018; Hogg et al., 2017; Johnson et al., 2008; Wolz et al., 2012). This long latency suggests other factors than electrical normalization of network activity or immediate neurochemical effects including synaptic plasticity mechanisms (Jakobs et al., 2019; Liu et al., 2008). Moreover, even though the pathological involvement of the mesolimbic dopaminergic system and its putative relationship to non-motor (neuropsychiatric) aspects are consistently reported in PD (Caminiti et al., 2017; Hirsch et al., 1988; Rinne et al., 1990; Voon et al., 2009; Vriend et al., 2014), the possibility of STN-DBS actions on the mesolimbic dopaminergic deficit is completely neglected so far.

Although there is no convincing clinical evidence for DBS-related disease modification (Hesse et al., 2008; Hilker et al., 2005) putatively due to inappropriate clinical methodologies, numerous preclinical studies conducted in established toxic rodent and nonhuman primate models of PD have demonstrated beneficial effects of unilateral STN lesion or chronic STN-DBS for 1 to 4 weeks on dopaminergic survival within the *Substantia nigra* (SN) (Chen et al., 2000; Fischer et al., 2017b; Maesawa et al., 2004; Musacchio et al., 2017; Nakao et al., 1999; Piallat et al., 1996; Spieles-Engemann et al., 2010; Temel et al., 2006; Wallace et al., 2007). However, in contrast to the clinical situation, STN lesion or DBS had been applied prior to or shortly after the dopaminergic lesion in most reports (Chen et al., 2000; Maesawa et al., 2004; Nakao et al., 1999; Piallat et al., 1996; Spieles-Engemann et al., 2010; Temel et al., 2006). Regarding a more recent approach of nigral α-synculein overexpression in animal models to better mimic the human pathology, the two available studies revealed no evidence for protection against α-synuclein-induced axonopathy by unilateral STN-DBS, but conflicting results on nigral dopaminergic neuron counts, possibly due to the different vectors and types of α-synuclein (AAV2/5-mediated overexpression of human wt α-synuclein compared to AAV1/2-dependent human A53T α-synuclein induction) in the studies. (Fischer et al., 2017b; Musacchio et al., 2017). When comparing parkinsonian animal models either based on chemical neurotoxins such as 6-OHDA or α-synuclein overexpression-mediated neurotoxicity, the discrepant results suggest different neuroprotective mechanisms of STN-DBS during the ongoing degenerative process. Indeed, several potential mechanisms have been proposed by which STN-DBS might provide neuroprotective actions including reduction of overactivity and excitotoxicity of glutamatergic projections from the STN to the SN, suppressed neuroinflammation or increased expression of growth factors such as BDNF (Chen et al., 2020; Fischer et al., 2017a; Nakao et al., 1999; Rodriguez et al., 1998; Spieles-Engemann et al., 2011).

The long-latency onset of non-motor (neuropsychiatric) actions of STN-DBS in clinical settings in conjunction with the promising results of STN-DBS-mediated neuroprotection within the nigrostriatal dopaminergic system prompted our investigation on the effects of STN-DBS on the mesolimbic dopaminergic system projecting from the ventral tegmental area (VTA) to the ventral striatum (*Nucleus accumbens*; NACC). In our study, we used the established hemiparkinsonian model generated by injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle, the only PD model with proven reliable dopaminergic degeneration in the ventral midbrain including the VTA (Lees et al., 1985; Papp and Bal, 1987; Winter et al., 2007). To avoid interference of STN-DBS effects with the ongoing degeneration process as discussed above, we applied STN-DBS eight weeks after dopaminergic lesioning to allow for the development of a stable dopaminergic dysfunction prior to DBS electrode implantation. Specificity of STN-DBS effects was proven by DBS of the entopeduncular nucleus (EPN) as the homologue of the human *Globus pallidus internus* (GPI) in an additional cohort.

2. Materials and methods

2.1. Animals and surgery

For details on surgical procedures, please refer to Supplementary Methods. All procedures were approved by responsible authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern and Landesdirektion Sachsen, Germany) and carried out in line with ARRIVE guidelines and the EU Directive 2010/63/EU for animal experiments. We investigated three independent animal cohorts using adult female Wistar rats for cohorts 1 and 2 (240–280 g) and adult male Wistar-Han rats for the third cohort (~300 g, animal cohort 3 as published by Badstübner and colleagues (Badstuebner et al., 2017); both from Charles River Laboratories, Sulzfeld, Germany). Control animals without electrode placement ($n = 7$) were collected from previous studies ((Muller et al., 2015) and unpublished studies).

Rats of all three cohorts underwent unilateral 6-OHDA lesioning of the medial forebrain bundle as described previously (Badstuebner et al., 2017; Muller et al., 2015). Successful lesioning was determined by means of amphetamine- or apomorphine-induced rotational behaviour. DBS electrode implantation was performed 8 weeks (cohorts 1 and 2) or 4–5 weeks (cohort 3) after lesioning to allow for the development of a stable functional dopaminergic dysfunction prior to DBS (Supplementary Fig. S1a). Groups of animals within the three cohorts were matched according to pre-implantation rotational behaviour test results (Supplementary Fig. S1b,c). Animals received commercially available unipolar platinum iridium electrodes (Plastics One, US) bilaterally into the STN (cohort 1) or the EPN (cohort 2) combined with a single ground electrode implanted posterior to stimulation electrodes near the midline. Electrodes were connected to a six-channel pedestal (P1 Technologies, Roanoke, VA). Rats were left to recover for at least 5–7 days until stimulation onset. Animals of study cohort 3 were implanted with either uni- or bipolar custom-made platinum iridium electrodes (Pt90/Ir10) insulated with polyesterimide with bare tips (Polyfil, Switzerland; FHC, US) on the ipsilateral side as described previously (Badstuebner et al., 2017). In all cohorts, electrode placement was evaluated by *post-mortem* analyses of Nissl stained sections of the STN/EPN regions to control for correct electrode placement within the target nucleus (see Supplementary Fig. S1d for representative analysis of STN targeting). We used two different external high frequency stimulators carried in a rodent backpack as described earlier (Badstuebner et al., 2017; Ewing et al., 2013) with the following stimulation parameters: 200 μA, 130 Hz, 60 μs (cohorts 1, 2) and 100 μA, 130 Hz; 90 μs (cohort 3) for a duration of 3, 5 or 6 weeks depending on treatment allocation. After active DBS, the integrity of the wiring deployed in each rat was tested and only rats with intact systems were included into the analyses. Sham-stimulated animals were treated identical to active DBS animals.

2.2. Behavioural testing

Study cohort 3 served as a cohort to compare behavioural outcomes with histology measures of the midbrain dopaminergic systems and thus underwent behavioural testing using the corridor task (CT) for assessing the sensorimotor neglect (Dowd et al., 2005) and the open field test (OFT) for spontaneous mobility and anxiety (Seibenhener and Wooten, 2015) prior to perfusion (after 3 and 6 weeks of DBS or sham stimulation) as described previously (Badstuebner et al., 2017) (see Supplementary Methods for details).

2.3. Cell labelling, perfusion and immunohistochemistry

Proliferating cells were labelled twice using two different thymidine analogues in cohorts 1 and 2 (see Supplementary Methods). After 3, 5 or 6 weeks of either DBS or sham stimulation, animals were anaesthetized, transcardially perfused and brains were collected for future

investigations. DAB staining for tyrosine hydroxylase (TH)$^+$ dopaminergic neurons and projections, Nissl staining for total neuron counts and immunohistochemistry for detecting thymidine analogue incorporation or Iba1$^+$ microglia were preformed using standard techniques (Brandt et al., 2017; Hermann et al., 2009) and described in detail in the Supplementary Methods.

2.4. Quantitative histology and statistics

For cell counts, TH$^+$ cells in cohorts 1 and 2 were imaged and quantified throughout the entire SN and VTA per slice using a motorized Axio.Observer.Z1 and ZEN Blue 2.3 software with Tiles and Position Module (all Carl Zeiss, Oberkochen, Germany). In cohort 3, we used the optical fractionator method of the StereoInvestigator 8.0 software (MBF Bioscience, US) combined with a motorized BX51 microscope (Olympus, Tokyo, Japan) as described earlier (Muller et al., 2015). TH$^+$ fibres were quantified in coronal sections using optical densities (OD) in the dorsal striatum (STRd) and the core nucleus accumbens (NACC; see Supplementary Methods). We determined mean differences of morphological parameters between groups using two-sided unpaired *t*-test, one-way ANOVA, one-way ANCOVA or two-way mixed ANOVA for multiple comparisons and *post-hoc t*-tests with Bonferroni adjustment as appropriate (refer to Supplementary Methods). For estimating effect sizes, we used Cohen's *d* with Hedges' correction resulting in the corrected (less biased) effect size g_{Hedges} (Hedges, 1981; Lakens, 2013) with $g_{Hedges} < |0.5|$ considered as small, $|0.5| \leq g_{Hedges} < |0.8|$ as medium and $g_{Hedges} \geq |0.8|$ as large effect sizes as suggested by Cohen (Cohen, 1988). The data met the assumption of normal distribution of data (Shapiro-Wilk test and visual check of box plots). Behavioural test results were correlated using Pearson correlation test and multivariate regression analyses with entering the candidate independent variables TH$^+$ cell counts in the SN and VTA of the lesioned hemisphere and the DBS stimulation protocol (time and type of stimulation). Correlation coefficients of $r > |0.5|$ were considered a relevant correlation. We conducted all statistical analyses using the SPSS software (Version 25.0; IBM, Chicago, IL). Number of animals/conditions and specific statistical analyses used in each experiment are indicated in figure/table legends and/or text. All data are presented as mean of at least three individual experiments ± standard error of the mean (SEM). Statistical significance was set at $P < 0.05$ (Bonferroni adjusted for α-inflation in multiple comparisons).

3. Results

3.1. Bilateral STN-DBS augments both midbrain dopaminergic systems in hemiparkinsonian rats

To assess potential effects of STN-DBS on cellular plasticity within the nigrostriatal and mesolimbic dopaminergic systems, we initially performed a histology study using the well-established 6-OHDA hemiparkinsonian rat model (Badstuebner et al., 2017; Muller et al., 2015) combined with a bilateral long-term DBS system as developed earlier (cohort 1) (Ewing et al., 2013). STN-DBS electrodes were implanted 8 weeks after 6-OHDA lesioning and stimulation was started one additional week later to investigate DBS effects in hemiparkinsonian animals with stable dopaminergic dysfunction (see Fig. 1a for experimental design). The two animal groups of cohort 1 were closely matched concerning their functional dopaminergic dysfunction with 15.0 ± 1.6 rpm in the amphetamine-induced rotation test for the control group (STN_{SHAM}) and 17.3 ± 2.6 rpm for the stimulation group (STN_{STIM}; $P = 0.770$; unpaired two-sided *t*-test). 6-OHDA lesioning, bilateral STN-DBS electrode placement and STN stimulation did not provoke any alterations in Nissl$^+$ neuron counts and their morphology within the STN on both hemispheres (Figs. 1b,c, Supplementary Fig. S1d).

We next analysed the two midbrain dopaminergic systems by quantification of TH$^+$ dopaminergic cell numbers. In STN_{SHAM} rats, we demonstrated a significant decrease in TH$^+$ neurons of the lesioned hemisphere by ~90% (SN) and ~ 65% (VTA) compared to the non-lesioned hemisphere (Fig. 1d-h). Bilateral STN_{STIM} provoked no alterations as compared to STN_{SHAM} in TH$^+$ cell counts in both midbrain regions in the non-lesioned hemisphere. In contrast, animals that received bilateral STN_{STIM} for 5 weeks had significantly more TH$^+$ cells in the lesioned hemisphere in both the SN (increase of ~100%) and the VTA (increase of ~45%) compared to STN_{SHAM} (Fig. 1d,f,h, Supplementary Figs. S3a,b). The effect sizes with g_{Hedges} of 3.4 for SN and g_{Hedges} of 1.9 for VTA were found to exceed Cohen's convention (Cohen, 1988) for large effects (d = 0.80). Of note, placement of electrodes without electrical stimulation (STN_{SHAM}) did not alter TH$^+$ neuron counts within the SN when compared to matched controls without electrode placement (Supplementary Fig. S2a,b).

The increase in TH$^+$ cells in both midbrain dopaminergic regions by STN_{STIM} translates into a significant increase of dopaminergic innervation (TH$^+$ fibre intensity) in the lesioned hemisphere of the STRd as the target area of nigral dopaminergic neurons and the NACC as the target region of mesolimbic dopaminergic neurons (Fig. 2a,c,e). The effect sizes g_{Hedges} were 1.9 for STRd and 2.0 for NACC demonstrating large effects. No changes of TH$^+$ fibre intensity in the striatum between STN_{SHAM} and STN_{STIM} animals were observed in the non-lesioned hemisphere (Fig. 2a,b,d).

We next tested whether the cellular effects of STN-DBS are specific for TH$^+$ dopaminergic neurons by investigating the total neuron counts using Nissl staining (Fig. 2f-j). STN_{STIM} did not change nigral Nissl$^+$ neuron counts as compared to STN_{SHAM} in both hemispheres (Fig. 2f-h). Consistently, the proportion of TH$^+$ dopaminergic neurons relative to total Nissl$^+$ neurons revealed an increase in the proportion of nigral TH$^+$ neurons by STN_{STIM} compared to STN_{SHAM} (Fig. 2i,j). Labelling of proliferating cells with thymidine (BrdU) analogues revealed very rare TH$^+$/BrdU$^+$ neurons (<0.1%) in all conditions in both dopaminergic systems (SN and VTA) without any differences between DBS conditions (data not shown). This BrdU labelling most likely represents apoptotic cell death of TH$^+$ neurons (Bauer and Patterson, 2005; Kruman et al., 2004) and thus confirms the stability of the dopaminergic degeneration in our animal model.

6-OHDA-induced dopaminergic neurodegeneration is accompanied by neuroinflammatory processes such as microgliosis during the active degenerative process which seems to plateau two weeks after the initial lesion (Farrand et al., 2017; Marinova-Mutafchieva et al., 2009; Walsh et al., 2011). We thus analysed the effects of both the 6-OHDA lesion itself and STN-DBS on microgliosis as measured by Iba1$^+$ cell numbers (Fig. 3). In agreement with the stable nature of dopaminergic neurodegeneration in our long-term 6-OHDA model, we did not observe significantly increased nigral microgliosis on the lesioned as compared to the non-lesioned side. Consistently, STN_{STIM} did not alter Iba1$^+$ microglial cell numbers within the SN as compared to STN_{SHAM} (Fig. 3b).

3.2. Bilateral EPN-DBS shows no effects on midbrain dopaminergic systems in hemiparkinsonian rats

We used the same experimental setup as for STN-DBS to test whether DBS effects on dopaminergic cellular plasticity are specific for STN-DBS or can be reproduced by EPN-DBS (cohort 2; Fig. 4a). The two groups of animals were closely matched concerning their dopaminergic dysfunction with 13.5 ± 1.9 rpm in the amphetamine-induced rotation test for the EPN_{SHAM} group and 16.2 ± 1.2 rpm for the EPN_{STIM} group ($P = 0.249$; unpaired two-sided *t*-test; Supplementary Fig. 1c). 6-OHDA lesioning, bilateral EPN-DBS electrode placement and EPN stimulation did not provoke any alterations in Nissl$^+$ neuron counts and their morphology within the EPN in both hemispheres (two-way mixed ANOVA showed no significant main effect of 6-OHDA lesion on Nissl$^+$ neuron numbers within the EPN overall [$F(1,12) = 0.25$, $P = 0.627$], no significant interaction between 6-OHDA lesion and EPN electrode placement/stimulation in terms of Nissl$^+$ neuron counts [$F(1,12) = 0.293$, $P = 0.751$] and no significant main effect of EPN electrode

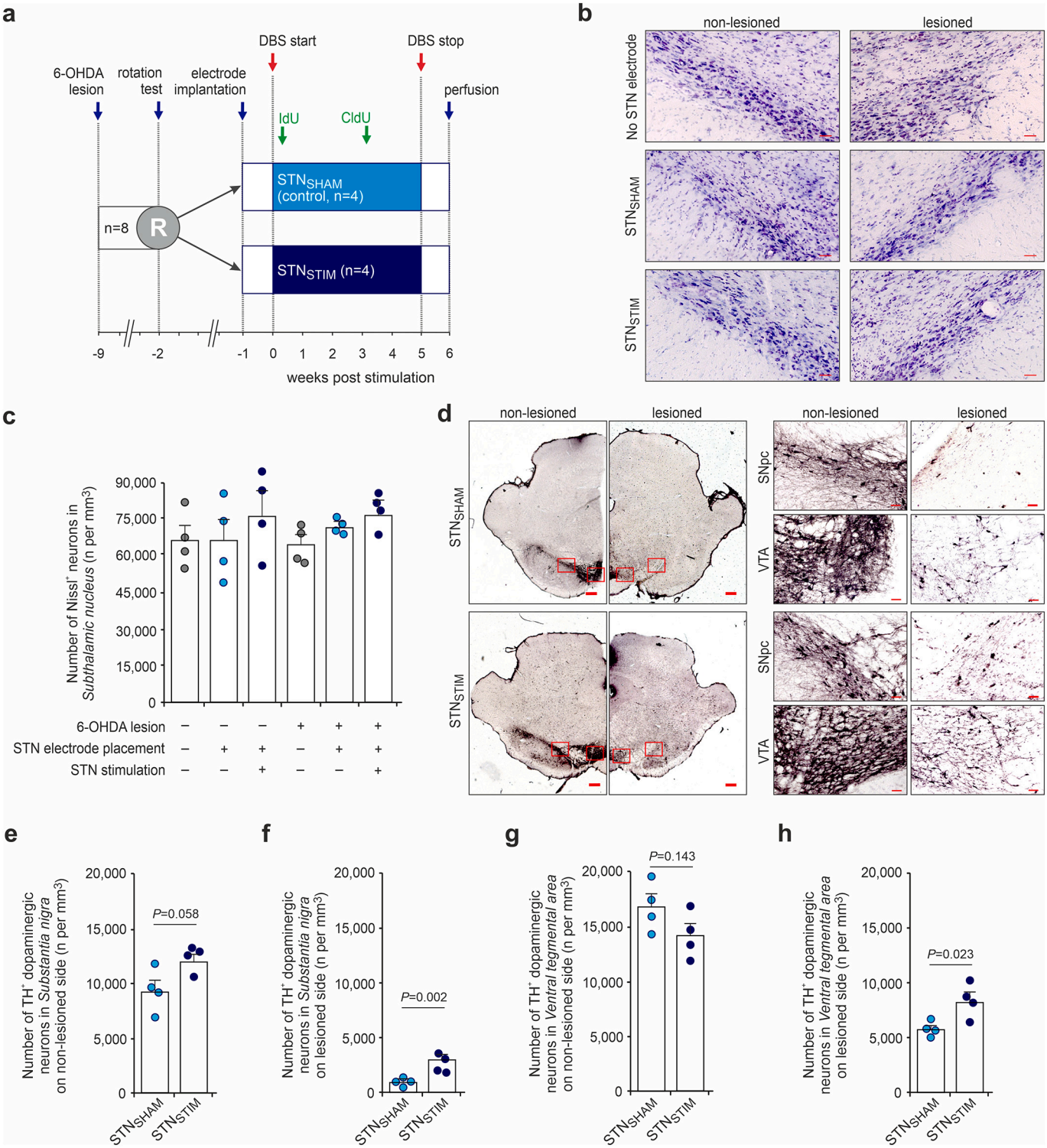

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Fig. 1. Effects of long-term bilateral subthalamic nucleus deep brain stimulation (STN-DBS) on cellular plasticity of the midbrain dopaminergic systems in hemiparkinsonian rats (cohort 1).
(a) Schematic representation of experimental paradigm to investigate the effects of STN-DBS on dopaminergic cell counts within the *Substantia nigra* (SN) and the *Ventral tegmental area* (VTA) in 6-hydroxydopamine (6-OHDA) hemiparkinsonian rats. To secure a stable dopaminergic dysfunction, bilateral STN-DBS electrodes were implanted 8 weeks after 6-OHDA lesion followed by high frequency stimulation for 5 weeks with perfusion of animals 1 week after termination of stimulation (STN_{STIM} group). STN_{SHAM} (electrode implantation without active stimulation) served as control. IdU and CldU were applied as proliferation markers 2 days after initiation of DBS and 3 weeks later. (b) Representative immunohistological analyses of $Nissl^+$ neurons within the STN of hemiparkinsonian rats with no STN electrode placement (EPN_{SHAM} group), bilateral STN-DBS electrode placement only (STN_{SHAM}) and with bilateral stimulation (STN_{STIM}). Scale bars, 50 μm. (c) Quantitative analysis of $Nissl^+$ neurons within the STN showed no effects of 6-OHDA lesion, electrode placement (EPN_{SHAM} animals were used as controls for STN electrode placement) and STN_{STIM}: Two-way mixed ANOVA showed no significant main effect of 6-OHDA lesion on overall nigral $Nissl^+$ neuron numbers ($F(1,12) = 0.714$, $P = 0.415$). There was no significant interaction between 6-OHDA lesion and STN electrode placement/stimulation in terms of $Nissl^+$ neuron counts ($F(1,12) = 0.382$, $P = 0.768$). There was no significant main effect of STN electrode placement/stimulation on $Nissl^+$ neuron counts ($F(1,12) = 1.32$, $P = 0.314$). (d) Representative immunohistological analyses of tyrosine hydroxylase (TH) staining in the *Substantia nigra* (SN) and *Ventral tegmental area* (VTA) in the midbrains of hemiparkinsonian rats with bilateral STN-DBS. Scale bars, 500 μm or 50 μm. (e,f) Quantitative analysis of nigral TH^+ neurons showed no effects of STN-DBS on the non-lesioned hemisphere (e), but a significant increase of nigral TH^+ cells in the lesioned hemisphere as compared to sham controls (f). (g,h) Quantitative analysis of TH^+ neurons within the VTA revealed no effects of STN-DBS in the non-lesioned hemisphere (g), but a significant increase of TH^+ cells within the VTA of the lesioned hemisphere as compared to sham controls (h). *P*-values are from unpaired two-sided *t*-tests (for number of animals, see a).

placement/stimulation on $Nissl^+$ neuron counts [$F(1,12) = 2.29$, $P = 0.144$]).

As shown in Fig. 4, EPN_{STIM} did not change TH^+ cell counts within the SN and the VTA in hemiparkinsonian animals compared to EPN_{SHAM} in both hemispheres. Consistently, we did not observe any differences in striatal TH^+ fibre densities between EPN_{SHAM} and EPN_{STIM} animals (data no shown). Notably, placement of electrodes without electrical stimulation (EPN_{SHAM}) did not alter nigral TH^+ neuron counts when compared to matched controls without electrode placement (Supplemental Fig. S2c,d). These results are in agreement with previous data – though on unilateral EPN-DBS – (Fischer et al., 2015) by showing that in contrast to STN-DBS EPN-DBS does neither provide morphological neuroprotection nor neurorestoration in both midbrain dopaminergic systems in the 6-OHDA hemiparkinsonian model.

3.3. Augmentative effects of STN-DBS on dopaminergic VTA neurons in hemiparkinsonian rats outlast the stimulation period

To confirm the results of STN-DBS in cohort 1, we performed a completely independent study using the same 6-OHDA hemiparkinsonian rat model but an unilateral STN-DBS experimental paradigm over an observation period of 6 weeks (cohort 3 (Badstuebner et al., 2017); Fig. 5a). As a significant advantage, this DBS system allows for behavioural testing of freely moving animals (Badstuebner et al., 2017). The four groups of animals included in the present analyses were perfectly matched concerning their dopaminergic dysfunction with 6.2 ± 1.5 rpm in the apomorphine-induced rotation test for the control group (STN_{SHAM}), 6.1 ± 1.9 rpm for the STN_{STIM} 3 weeks group, 6.6 ± 1.5 rpm for the STN_{STIM} 3 weeks (+ 3 weeks washout) group and 6.3 ± 1.2 rpm for the STN_{STIM} 6 weeks group ($F = 0.02$; $P = 0.995$; one-way ANOVA).

Quantitative analysis of TH^+ neurons within the non-lesioned (non-stimulated) midbrain showed only minor effects of STN-DBS in both midbrain dopaminergic regions (Fig. 5b-d; SN: $F = 4.33$; $P = 0.025$ and VTA: $F = 7.56$; $P = 0.004$; one-way ANOVA). TH^+ neuron counts within the lesioned midbrain revealed no effects of STN-DBS within the SN (Fig. 5c; $F = 1.78$; $P = 0.201$; one-way ANOVA; Supplementary Fig. S3c), but significant differences of TH^+ cell counts within the VTA of the lesioned hemisphere (Fig. 5d; $F = 10.09$; $P = 0.001$; one-way ANOVA; Supplementary Fig. S3d). The effect sizes showed large effects with g_{Hedges} of 3.6 for STN_{SHAM} versus STN_{STIM} for 3 weeks +3 weeks washout and of 2.8 for STN_{SHAM} versus STN_{STIM} for 6 weeks. *Post-hoc* analyses using unpaired two-sided *t*-test with Bonferroni adjustment revealed a significant increase of TH^+ VTA neuron counts after 6 weeks of STN-DBS by ~55% when compared to STN_{SHAM} (Fig. 5d). Notably, similar effects were observed after 3 weeks of STN_{STIM} followed by 3 weeks washout prior to histological analyses (increase of ~80%; Fig. 5d). Together, unilateral STN-DBS exerts augmentative effects in the degenerated VTA but not SN in the 6-OHDA hemiparkinsonian rat model, which outlast the stimulation period for at least 3 weeks.

3.4. Behavioural testing suggests that STN-DBS effects on dopaminergic VTA neurons might translate into behavioural changes

Although the reported experiments were primarily planned as a histology and not as a behavioural study, we determined potential associations of TH^+ neuron counts in both midbrain regions with pilot behavioural outcome measures as an outlook for future studies (Fig. 5e; Supplementary Table S1; Supplementary Fig. S4). The open field test (OFT) was used for assessment of spontaneous mobility and anxiety (Seibenhener and Wooten, 2015) and the corridor task (CT) for the assessment of sensorimotor neglect (Dowd et al., 2005). Pearson correlation tests revealed no correlation with a magnitude greater than |0.5| of OFT results with TH^+ cell counts in both midbrain dopaminergic regions. However, multivariate regression analyses with entering the candidate independent variables TH^+ cell counts in SN and VTA of the lesioned hemisphere and DBS treatment group revealed that OFT parameters indicative of motor function (velocity, total distance moved) but not anxiety (centre-to-total distance ratio) were associated with DBS treatment group allocation and nigral dopaminergic neuron counts (Supplementary Table S1; Supplementary Fig. S4). In contrast, there was a significant correlation of mesolimbic dopaminergic neuron counts and behavioural test results from CTs (Pearson correlation coefficient $r = 0.533$; $P = 0.028$), which survived multivariate regression analysis (corrected $R^2 = 0.357$, F-value = 4.40, $P = 0.011$; association with mesolimbic TH^+ neuron counts: $\beta = 0.603$, $P = 0.025$; Fig. 5e; Supplementary Table S1). Although these results should be considered as pilot data and need confirmation in adequately powered behavioural studies, they indicate that augmentative effects in the mesolimbic system by STN-DBS might translate into behavioural changes.

4. Discussion

The present study indicates that bilateral long-term STN-DBS augments TH^+ dopaminergic neurons and their axonal projections not only in the nigrostriatal, but also in the mesolimbic dopaminergic systems of 6-OHDA hemiparkinsonian rats with stable dopaminergic dysfunction. These plasticity effects on the two midbrain dopaminergic systems are specific for STN-DBS and not observed in bilateral EPN-DBS. Since we compared active with sham DBS (electrodes in place without electrical stimulation) in our cohorts, potential lesion effects by electrode placement do not explain the STN-DBS actions on dopaminergic plasticity. In addition, quantification of $Nissl^+$ neurons showed no changes of neuron counts and morphology within the target nucleus by STN-DBS which also excludes a permanent subthalamotomy-like lesion induced by the DBS itself. The chronic STN-DBS effects on the mesolimbic system are

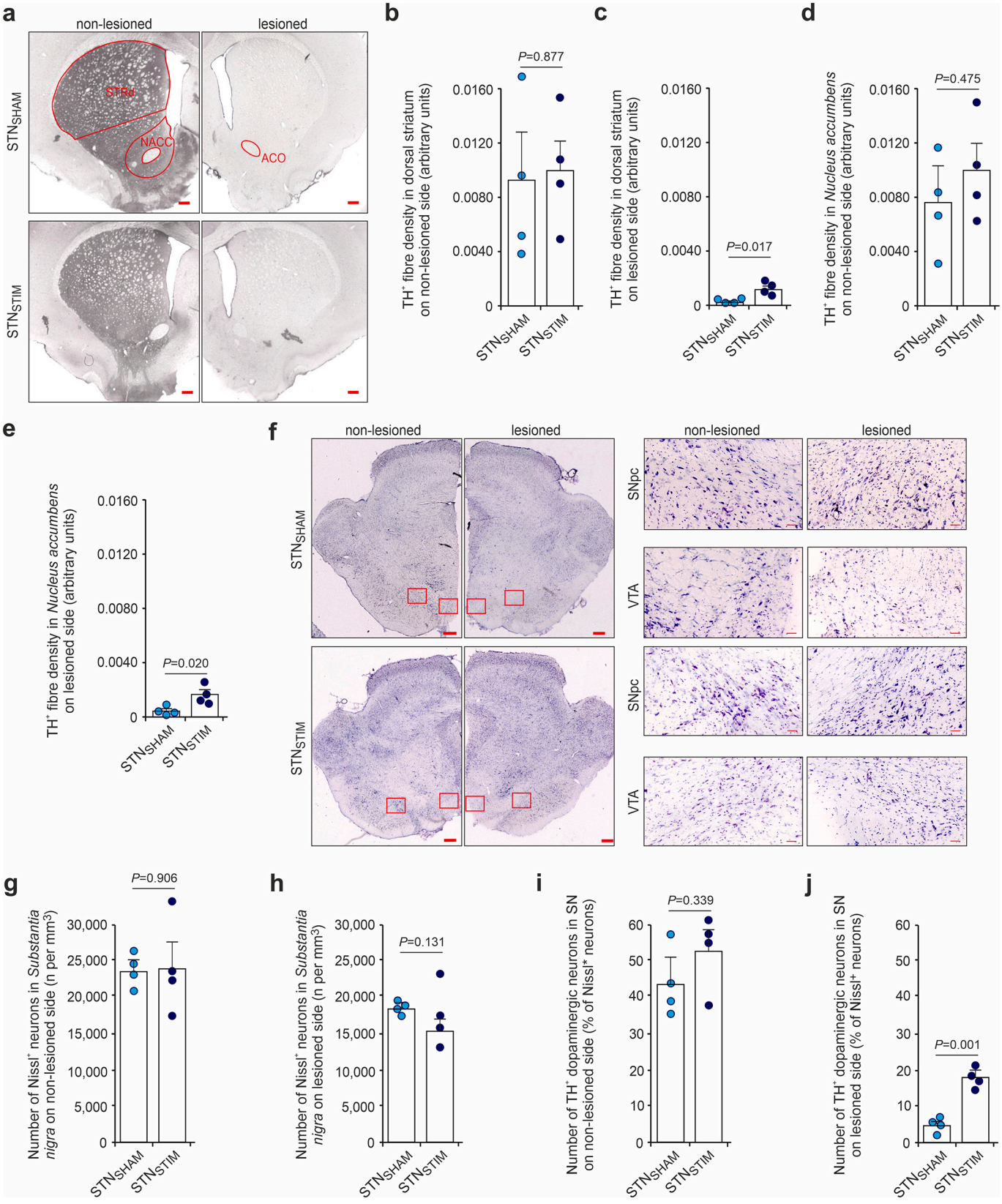

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Fig. 2. Effects of long-term bilateral subthalamic nucleus deep brain stimulation (STN-DBS) on TH^+ fibre density within the striatum of hemiparkinsonian rats (cohort 1).
(a) Representative immunohistological analyses of tyrosine hydroxylase (TH) stainings in the striatum of hemiparkinsonian rats with bilateral STN-DBS (study design as in Fig. 1a). Marked regions are the dorsal striatum (STRd) and the *Nucleus accumbens core region* (NACC) as the main target areas of the midbrain dopaminergic systems and the anterior commissure (ACO) as the reference region (white matter). Scale bars, 500 μm. (b,c) Densiometric analysis of TH^+ fibre densities within the STRd as the main target region of *Substantia nigra* neurons showed no effects of STN-DBS in the non-lesioned hemisphere (b), but a significant increase in the lesioned hemisphere (c). (d,e) Densiometric analysis of TH^+ fibre densities within the NACC revealed no effects of STN-DBS in the non-lesioned hemisphere (d), but a significant increase in the lesioned hemisphere (e). (f) Representative immunohistological analyses of $Nissl^+$ neurons in the *Substantia nigra* (SN) and *Ventral tegmental area* (VTA) in the midbrains of hemiparkinsonian rats with bilateral STN-DBS. Scale bars, 500 μm or 50 μm. (g,h) Quantitative analysis of nigral $Nissl^+$ neurons showed no effects of STN-DBS in the non-lesioned (g), and lesioned hemispheres (h). (i,j) Quantitative analysis of nigral TH^+ dopaminergic neurons relative to nigral $Nissl^+$ neuron counts revealed no effects of STN-DBS in the non-lesioned hemisphere (h), but a significant increase of TH^+ neurons relative to $Nissl^+$ neuron counts within the SN of the lesioned hemisphere as compared to sham controls (i). *P*-values are from unpaired two-sided t-tests (for number of animals, see Fig. 1a).

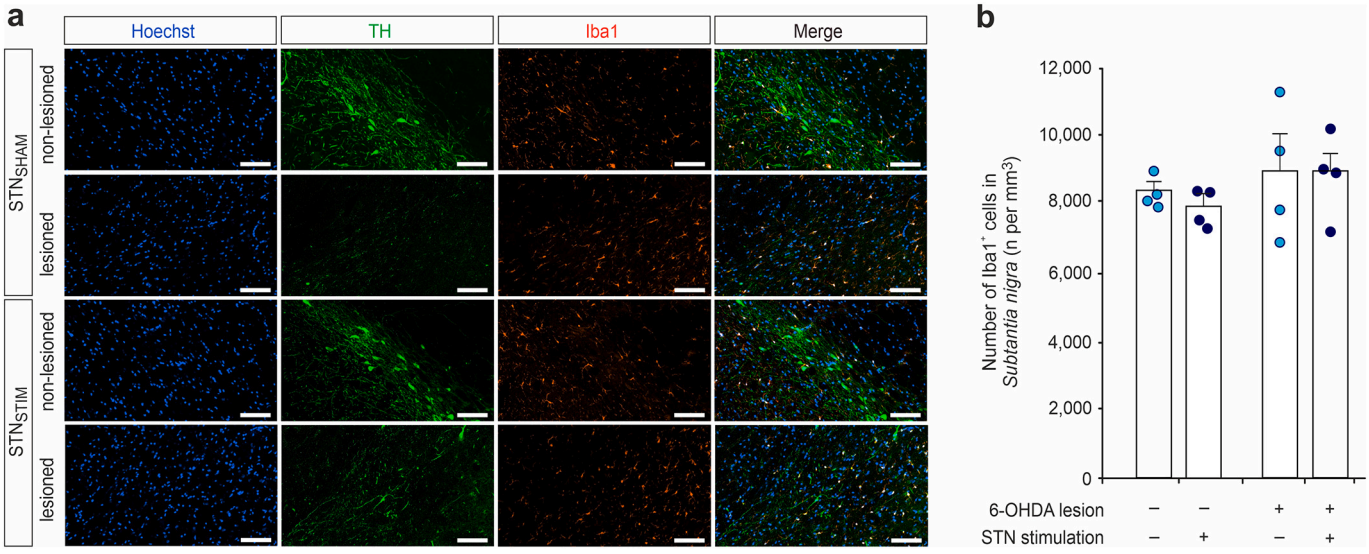

Fig. 3. Effects of 6-OHDA lesion and long-term bilateral subthalamic nucleus deep brain stimulation (STN-DBS) on microglia activation within the *Substantia nigra* in hemiparkinsonian rats (cohort 1).
(a) Representative immunohistological analyses of $Iba1^+$ neurons within the *Substantia nigra* (SN) of hemiparkinsonian rats with bilateral STN-DBS electrode placement only (STN_{SHAM}) and with bilateral stimulation (STN_{STIM}). Scale bars, 100 μm. (b) Quantitative analysis of $Iba1^+$ cells within the *Substantia nigra* (SN) showed no effects of 6-OHDA lesion or STN_{STIM}: Two-way mixed ANOVA showed no significant main effect of 6-OHDA lesion on overall nigral $Iba1^+$ cell numbers ($F(1,6) = 1.71$, $P = 0.238$). There was no significant interaction between 6-OHDA lesion and STN stimulation in terms of $Iba1^+$ cell counts ($F(1,6) = 0.09$, $P = 0.776$). There was no significant main effect of STN stimulation on $Iba1^+$ cell counts ($F(1,12) = 0.17$, $P = 0.697$; $n = 4$).

confirmed in an independent cohort of hemiparkinsonian rats treated with unilateral STN-DBS. Notably, these effects outlast the stimulation period by up to 3 weeks and putatively translate into behavioural changes. Translationally relevant, similar to the clinical setting we applied STN-DBS during the stable phase of dopaminergic dysfunction long-time after lesioning. Since there was no indication of an ongoing dopaminergic degenerative process, these effects are most likely to be interpreted as sustained neurorestorative actions of STN-DBS.

Numerous preclinical studies conducted in established toxic rodent and nonhuman primate PD models have demonstrated beneficial effects of unilateral STN lesion or STN-DBS for 1 to 4 weeks on nigral dopaminergic survival (Chen et al., 2000; Fischer et al., 2017b; Maesawa et al., 2004; Musacchio et al., 2017; Nakao et al., 1999; Piallat et al., 1996; Spieles-Engemann et al., 2010; Temel et al., 2006; Wallace et al., 2007). Though, these studies applied the STN lesion prior to the application of the dopaminergic toxin 6-OHDA and thus investigated effects of STN lesion on acute dopaminergic toxicity (Chen et al., 2000; Nakao et al., 1999; Piallat et al., 1996), which does not reflect the common clinical situation in PD. Available STN-DBS studies investigated neuroprotective effects shortly after lesion induction (0–4 weeks) and thus during the time course of ongoing toxin-induced dopaminergic degeneration (Fischer et al., 2017b; Maesawa et al., 2004; Musacchio et al., 2017; Spieles-Engemann et al., 2010; Temel et al., 2006). Indeed, Spieles-Engemann and colleagues showed by a detailed time-course experiment that STN-DBS was applied during the ongoing degeneration process (Spieles-Engemann et al., 2010). Recent studies using a rat model with vector-based overexpression of α-synuclein within the SN revealed conflicting results, most likely due to different levels of overexpression of α-synuclein protein. However, in both studies no evidence for protective effects against α-synuclein-induced axonopathy by STN-DBS was provided (Fischer et al., 2017b; Musacchio et al., 2017). Our results on unilateral STN-DBS (cohort 3) are in agreement with the latter study by Fischer and colleagues showing no effects of STN-DBS for 3 to 6 weeks on nigral dopaminergic neuron counts (Fischer et al., 2017b). The conflicting results are likely due to the latency between lesioning and STN-DBS application: Studies showing neuroprotective effects applied unilateral STN lesion or DBS prior to or shortly after the induction of the dopaminergic lesion (0–3 weeks). STN inhibition thus interferes with the actively ongoing degeneration process in the sense of a neuroprotective action against the acute insult (Maesawa et al., 2004; Musacchio et al., 2017; Spieles-Engemann et al., 2010; Temel et al., 2006). The two studies showing no neuroprotective effects have used a more chronic condition (4–5 weeks latency) more comparable to the clinical situation in which STN-DBS does not interfere with the degenerative process itself but only with the diseased brain systems. In contrast, we provide first evidence that bilateral – as opposed to unilateral STN-DBS – induces neurorestorative actions in the diseased nigrostriatal dopaminergic system under the same conditions. The factors mediating this

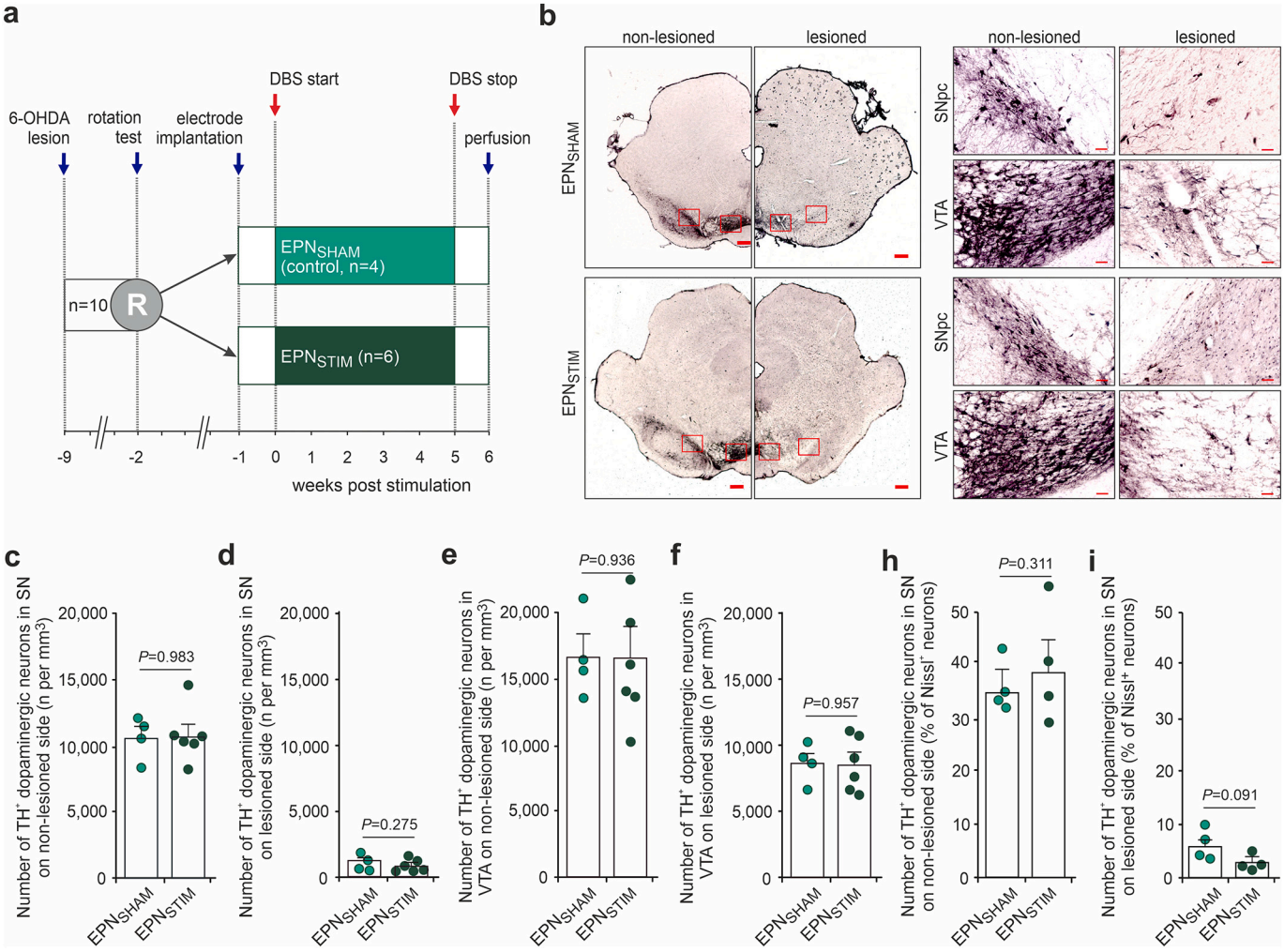

Fig. 4. Effects of long-term bilateral deep brain stimulation in the entopeduncular nucleus (EPN-DBS) on cellular plasticity of the midbrain dopaminergic systems in hemiparkinsonian rats (cohort 2).
(a) Schematic representation of experimental paradigm to investigate the effects of DBS on dopaminergic cells counts within the *Substantia nigra* (SN) and the *Ventral tegmental area* (VTA) in 6-hydroxydopamine (6-OHDA) hemiparkinsonian rats. To secure a stable dopaminergic dysfunction we performed bilateral EPN electrode implantation 8 weeks after 6-OHDA lesion followed by high-frequency stimulation for 5 weeks with perfusion of animals 1 week after termination of stimulation (EPN_{STIM}). EPN_{SHAM} (electrode implantation without stimulation) served as control. (b) Representative immunohistological analyses of tyrosine hydroxylase (TH) stainings in the *Substantia nigra* (SN) and *Ventral tegmental area* (VTA) in the midbrains of hemiparkinsonian rats with bilateral EPN-DBS. Scale bars, 1 mm or 100 µm. (c-i) Quantitative analysis of TH^+ neurons (absolute numbers or in relation to $Nissl^+$ neurons) within the non-lesioned and lesioned midbrain showed no effects of EPN-DBS in both midbrain dopaminergic regions (SN and VTA). *P*-values are from two-sided unpaired t-tests (for number of animals, see a; Nissl histology was performed in 4 animals for each group).

discrepancy remain unclear, particularly because there are no relevant projections from contralateral STN into the SN (Schmitt and Eipert, 2012; Schmitt et al., 2016). Lastly, our data on EPN-DBS effects on nigral dopaminergic neurons are in close agreement with the other available study reporting no effects of EPN-DBS – though unilateral – on dopaminergic morphology in the nigrostriatal system (Fischer et al., 2015). Of note, cohorts 1 and 2 were female animals which allows us to provide very rare data of DBS in non-male animals. Despite half of PD patients are female there is a dramatic lack of studies regarding female animals most likely due to eventually interfering sex hormones (Casas et al., 2013; Ferraz et al., 2008). Nevertheless, sex, strain and slight protocol differences have to be considered in our data interpretation and may interfere with STN-DBS effects on the SN.

To the best of our knowledge, we are the first to provide evidence from two independent – even different in sex and strain – animal cohorts that STN-DBS specifically induces neurorestorative effects in the mesolimbic dopaminergic system with both higher VTA dopaminergic cell counts and increased density of axonal projections towards the ventral striatum. In contrast to the results in the nigrostriatal system, these effects are independent of unilateral versus bilateral stimulation and outlast DBS washout for at least 3 weeks. The biological or behavioural relevance of these histological findings remains unclear. However, as an outlook for future studies, STN-DBS effects on the mesolimbic system might translate into behavioural changes with reduction of the sensorimotor neglect, which was previously shown to be partly related to VTA pathology (Lees et al., 1985). Future studies with more specific assessments of mesolimbic pathology such as reward behavioural tests (Der-Avakian et al., 2016) are urgently needed to confirm these results. However, even though clinical translation from our model system should be performed with sufficient caution and no data on the mesolimbic dopaminergic system after STN-DBS are available for the human disease, STN-DBS effects on the mesolimbic system might be involved in its non-motor (neuropsychiatric) benefits such as effects on mood or impulsivity (Caminiti et al., 2017; Hirsch et al., 1988; Rinne et al., 1990; Voon et al., 2009; Vriend et al., 2014). Indeed, the differences between STN- and EPN-DBS in this respect might in part explain the differences in

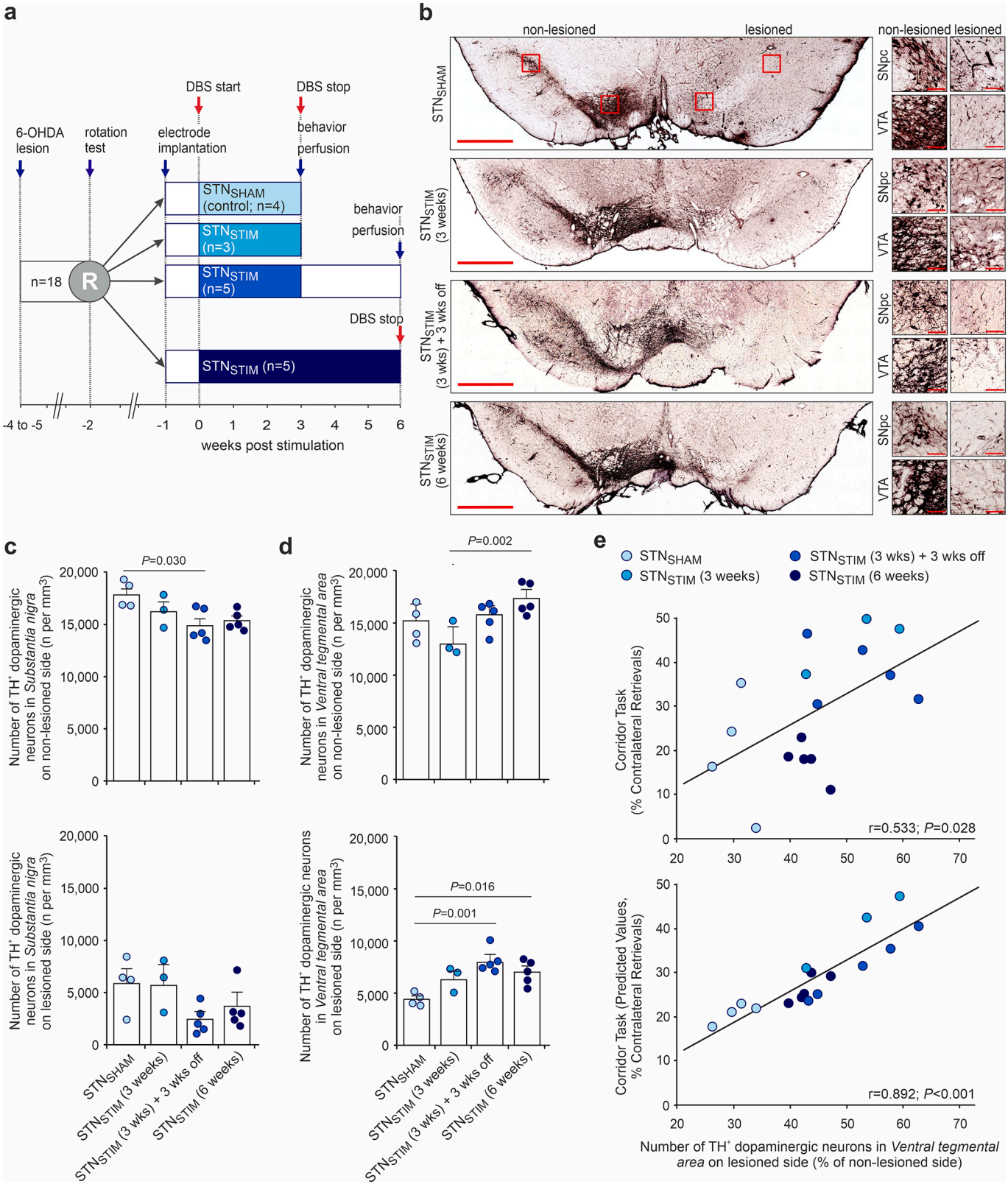

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Fig. 5. Effects of long-term deep brain stimulation in the subthalamic nucleus (STN-DBS) on cellular plasticity of midbrain dopaminergic systems and its functional impact in hemiparkinsonian rats (cohort 3).
(a) Schematic representation of experimental paradigm to investigate the effects of STN-DBS on dopaminergic cell counts within the *Substantia nigra* (SN) and the *Ventral tegmental area* (VTA) in 6-hydroxydopamine (6-OHDA) hemiparkinsonian rats. To secure a stable dopaminergic dysfunction, unilateral STN-DBS electrodes were implanted in the lesioned (parkinsonian) hemisphere 4–5 weeks after 6-OHDA lesion. The STN was stimulated unilaterally for 3 or 6 weeks with immediate perfusion of the animal or for 3 weeks plus 3 weeks washout period without stimulation to investigate the sustainability of DBS effects. Sham STN-DBS (electrode implantation without stimulation) served as control. Motor behaviour was assessed at the end of the observation period (1–5 days prior to perfusion of the animals). (b) Representative immunohistological analyses of tyrosine hydroxylase (TH) staining in the *Substantia nigra* (SN) and *Ventral tegmental area* (VTA) in the midbrains of hemiparkinsonian rats with STN-DBS. Scale bars, 1 mm or 200 μm. (c) Quantitative analysis of TH^+ neurons within the non-lesioned SN showed only minor effects of STN-DBS (F-value = 4.33; $P = 0.025$; one-way ANOVA). Quantitative analysis of TH^+ neurons within the lesioned SN revealed no effects of STN-DBS (F-value: 1.78; $P = 0.201$). Mean total amount of TH^+ neurons per SN in control condition (no lesion, no stimulation) was 7987 ± 791 (n = 4). (d) Quantitative analysis of TH^+ neurons within the non-lesioned VTA showed only minor effects of STN-DBS (VTA: F-value = 7.56; $P = 0.004$). Quantitative analysis of TH^+ neurons within the lesioned VTA showed significant differences of TH^+ cell counts by STN-DBS (F-value: 10.09; $P = 0.001$). *P*-values are from Bonferroni *post-hoc* t-tests. Mean total amount of TH^+ neuron per VTA in controls condition was 6503 ±?425 (n = 4). (e) Correlation of the behavioural outcome measure (% contralateral retrievals) from corridor task (raw data in upper panel, predicted values from multivariate regression analyses in lower panel) and numbers of TH^+ neuron in the lesioned VTA in the various groups of hemiparkinsonian rats. Displayed are the test results from behavioural testing 1–5 days prior to perfusion for histology studies to allow for a direct comparison of animal behaviour and histology. Predicted values in lower panels resulted from multivariate regression analyses with entering the candidate independent variables TH^+ cell counts in the SN and VTA of the lesioned hemisphere and the DBS stimulation protocol (corrected $R^2 = 0.357$, F-value = 4.40, $P = 0.011$; association with VTA TH^+ neuron counts: $\beta = 0.603$, $P = 0.025$; for number of animals, see a). Values in the lower right corner of the diagrams represent test results from Pearson correlation test.

neuropsychiatric actions between STN- and GPI-DBS in PD (Deuschl et al., 2013).

Although we cannot draw final conclusions on the cellular mechanisms underlying the restorative action of STN-DBS from our studies, the effects in our model of completed dopaminergic degeneration are likely not mediated through an interference with the pathophysiology of the neurodegenerative process itself. Dopaminergic neurodegeneration in PD and its animal models is accompanied and putatively enhanced by neuroinflammatory processes such as microgliosis during the active degenerative process (Braak et al., 2007; Esposito et al., 2007). Similar inflammatory processes such as microgliosis were also reported in the early active phase of neurodegeneration in the 6-OHDA rat model with a plateau two weeks after the initial lesion lesion (Farrand et al., 2017; Marinova-Mutafchieva et al., 2009; Walsh et al., 2011). Indeed, not only anti-inflammatory interventions but also STN-DBS starting early within the degenerative process (in some studies prior to toxin application) have shown to suppress neuroinflammation and subsequently provide neuroprotection in PD animal models including the 6-OHDA rat model (Chen et al., 2020; Esposito et al., 2007; Sanchez-Pernaute et al., 2004). In agreement with the stable nature of the dopaminergic neurodegeneration in our long-term 6-OHDA model, we did not detect $Iba1^+$ microgliosis within the degenerated SN. Consistently, STN-DBS did not change microglial cell amounts within the SN independent of the degenerative process.

We together consider our toxic 6-OHDA hemiparkinsonian model a reliable mimic of stable dopaminergic dysfunction with secondary adaptive processes (e.g. changes in network activities) as it can be observed in human late stage PD, though with a potentially different pathophysiology of degeneration. Several studies suggest that a reduction of STN-mediated damage to the midbrain dopaminergic systems through inhibition of the increased glutamatergic exitotoxic input from the STN into the SN/VTA might be one protective mechanism of STN-DBS/lesioning (Chen et al., 2000; Maesawa et al., 2004; Nakao et al., 1999; Piallat et al., 1996; Wallace et al., 2007). The putative need for bilateral STN-DBS for its neurorestorative actions within the nigrostriatal system in conjunction with the fact that there are no relevant projections from contralateral STN into the SN (Schmitt and Eipert, 2012; Schmitt et al., 2016) additionally points to more indirect or network-independent mechanisms. Thus, reduction of metabolic demand of the dopaminergic neurons or increased release of neurotrophic factors might also be involved in STN-DBS-medicated neurorestoration (Fischer et al., 2017a; Meissner et al., 2003; Meissner et al., 2001; Spieles-Engemann et al., 2011). On the cellular level, our BrdU labelling data largely rule out that the increase in dopaminergic neurons by STN-DBS is mediated through dopaminergic neurogenesis. The observed dissociation between TH^+ dopaminergic and total nigral neuron counts indeed points to a rescue of the dopaminergic phenotype of remaining midbrain neurons. Such a rescue of TH expression alone or in combination with total nigral neuron numbers has been described in PD models after STN-DBS/lesioning (Musacchio et al., 2017; Nakao et al., 1999; Paul et al., 2004; Temel et al., 2006; Wallace et al., 2007). The impairment of dopamine synthesis by the loss of TH enzymatic activity is considered an important characteristic of PD (Tabrez et al., 2012) underlining the translational significance of our findings.

Although the final sizes of our animal groups are in the range of most similar studies and are thus considered the current standard in experimental DBS studies in small animal models (Fischer et al., 2015; Fischer et al., 2017a; Fischer et al., 2017b; Musacchio et al., 2017), the rather small sizes of our animal cohorts are a relevant limitation of our studies. This limitation is essentially attributed to the very high experimental efforts of DBS studies in small laboratory animals. We – in part – overcome this limitation by closely matching the different animal groups in each cohort and by confirming major results (sustained STN-DBS effects on dopaminergic plasticity) from cohort 1 in a second completely independent animal cohort (cohort 3). However, future studies need to confirm our results under more stringent preclinical conditions.

5. Conclusions

Our study provides first evidence of sustained neurorestorative effects of bilateral STN-DBS in the nigrostriatal and mesolimbic dopaminergic systems in the 6-OHDA hemiparkinsonian rat model independent of toxin-induced neurodegenerative processes. Future studies to determine the behavioural consequences of these neurorestorative effects in animal models as well as longitudinal clinical studies on morphological and functional outcomes in relation to the mesolimbic system in PD subjects after STN-DBS are needed to further support the translational importance of our findings.

Declaration of Competing Interest

The authors indicated no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2021.105404.

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