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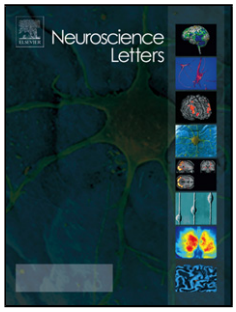

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Title: Effects of antidyskinetic nicotine treatment on dopamine release in dorsal and ventral striatum

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Highlights

- Nicotine treatment inhibited development of levodopa-induced dyskinesia in mice
- Antidyskinetic nicotine treatment reduced α6β2*-mediated dopamine release
- α4β2*-mediated dopamine release was unaffected by antidyskinetic nicotine treatment
- Nicotine treatment restored basal dopamine release in lesioned ventral striatum

Abstract

The treatment of Parkinson's disease is often complicated by levodopa-induced dyskinesia (LID), and antidyskinetic treatment options are currently sparse. Nicotinic acetylcholine receptors have been suggested as potential targets for treatment of LID, as nicotinic agonists have been reported to alleviate LID in animal models. We aimed at the first independent replication of an antidyskinetic effect by nicotine using a mouse model of LID, and at investigation of its mechanisms by studying the release of [^{3}H]dopamine from synaptosomes prepared from the dorsal and ventral striatum. Chronic nicotine treatment in drinking water inhibited the development of LID in mice lesioned unilaterally with 6-hydroxydopamine and treated chronically with levodopa and benserazide. The antidyskinetic nicotine treatment had no effect on [^{3}H]dopamine release mediated by α4β2* nicotinic receptors, but decreased α6β2*-mediated [^{3}H]dopamine release in the lesioned dorsal striatum and the ventral striatum. In addition, nicotine treatment restored [^{3}H]dopamine release in the lesioned ventral striatum to intact levels. The results support a role for nicotinic receptors as drug targets for treatment of LID, and suggest that

striatal presynaptic α6β2* receptors are important mediators of nicotine's antidyskinetic effect.

Keywords: Parkinson's disease; levodopa; dyskinesia; nicotine; dopamine

Introduction

Parkinson's disease is a neurodegenerative disorder characterized by the death of dopaminergic neurons in the substantia nigra pars compacta (SNc) and a deficit of dopamine in the striatum [1]. Dopamine replacement therapy with levodopa (L-3,4-dihydroxyphenylalanine) is effective but often complicated by levodopa-induced dyskinesia (LID) [2]. While the pathophysiology of LID involves numerous mechanisms, brain areas and neurotransmitter systems, irregularities in striatal dopaminergic signaling are thought to have a major role, including abnormal presynaptic handling of levodopa and hypersensitization of postsynaptic dopamine receptors [2]. As treatment options for LID remain sparse, a need for new treatments exists [2].

Neuronal nicotinic acetylcholine receptors are ion channel receptors composed of five subunits, with the homomeric α7 receptor and the heteromeric α4β2* receptor (the asterisk denoting the possible presence of other subunits) being the most widely expressed in the mammalian brain [3]. Through nicotinic receptors, the brain cholinergic system modulates the activity of numerous other neurotransmitter systems, including the nigrostriatal dopaminergic pathway, where both α4β2* and α6β2* presynaptic nicotinic receptors modulate striatal dopamine release [4–6]. Several paths of evidence point to a role for nicotinic receptors in the pathophysiology of Parkinson's disease and LID and as potential drug targets, with both neuroprotective and antidyskinetic effects by nicotinic receptor ligands reported in animal models [7]. In particular, robust beneficial effects by chronic treatment with the prototypical non-selective nicotinic receptor agonist nicotine have been described in rodent and primate models of LID by Quik and colleagues [7].

In this study, we aimed at an independent replication of antidyskinetic effects by nicotine using a mouse model of Parkinson's disease and LID. In addition, to further investigate the mechanisms of nicotinic receptor-mediated antidyskinetic effects, we studied in synaptosomes the effects of dopaminergic denervation and chronic nicotine treatment on basal and nicotinic receptor-mediated striatal dopamine release. Previous studies have described distinct populations of α4β2* and α6β2* nicotinic receptors

differentially regulating dopaminergic neurotransmission in the dorsal and ventral striatum [8]. Furthermore, differential effects on these two receptor populations by nigrostriatal damage [9] and long-term nicotine treatment [10] have been described. Therefore, we studied separately dopamine release mediated by α4β2* and α6β2* nicotinic receptors in both the dorsal and ventral striatum.

Materials and methods

Animals

Adult (9–12 weeks) C57BL/6J mice (Envigo, Netherlands) were group housed in a temperature- and humidity-controlled environment under a 12 h light/dark cycle. Experiments were conducted according to the 3R principles of the EU directive 2010/63/EU governing the care and use of experimental animals and to local laws and regulations, and authorized by the Animal Experiment Board of Finland**.**

Mouse model of Parkinson's disease and levodopa-induced dyskinesia

Unilateral dopaminergic denervation was induced by stereotactic injection of 6-hydroxydopamine (6-OHDA; Sigma-Aldrich, St. Louis, MO) into two sites within the left dorsal striatum, essentially following the model of Lundblad et al. [11] with the following modifications: isoflurane anesthesia was used; 6 µg 6-OHDA per site was injected at a concentration of 6 µg/µl; desipramine (25 mg/kg, i.p.; Sigma-Aldrich) was administered 30 min prior to inhibit damage to noradrenergic neurons. Topical lidocaine (Orion Pharma, Finland) and systemic buprenorphine (0.1 mg/kg, s.c.; RB Pharmaceuticals, UK) were used for pain relief. Postoperative care included warm saline injections and food pellets softened by soaking. 11 out of 39 operated mice (28 %) were euthanized due to unacceptable weight loss as determined by the animal experiment authorization. Of the remaining mice, 5 (all female) were used for immunohistochemistry, 7 (all female) for assessment of the effects of the lesion on [^{3}H]dopamine release, and 16 (both sexes) for chronic drug treatments followed by [^{3}H]dopamine release experiments.

The extent of dopaminergic denervation was assessed using tyrosine hydroxylase immunohistochemistry in 30 µm free-floating coronal brain sections and performed as previously described [12]. Stained sections were imaged using a 3DHISTECH slide scanner (3DHISTECH, Budapest, Hungary), and optical densities across the dorsal striatum and the SNc were measured from images converted to grayscale using ImageJ 1.50 (NIH, Bethesda, MD). Optical density of background staining was subtracted from raw data

values, and results were expressed as percent of intact side. Lesion extent was calculated as the mean of three consecutive sections (every 6th section) between 1.2 and 0.6 mm (striatum) or -2.9 and -3.4 mm (SNc) from the Bregma.

Eight weeks after surgery, 16 lesioned mice were randomly assigned to levodopa-nicotine or levodopa-water groups. Five animals that did not develop LID were later removed from all analyses, resulting in six animals (four females) in the nicotine group and five animals (three females) in the control group. Dyskinesia-inducing doses [11] of levodopa (30 mg/kg; Sigma-Aldrich) and benserazide (12 mg/kg; Sigma-Aldrich) were administered to all mice once (s.c.) every weekday (Mon-Fri). Nicotine administration was initiated simultaneously with levodopa treatment. Nicotine (gradually increased to 300 µg/ml; free base, Sigma-Aldrich) was administered to one treatment group in drinking water as previously described [13], with the control group receiving normal drinking water. The concentration of 300 µg/ml was chosen as it is the concentration used in previous studies in 6-OHDA-lesioned mice [14,15] and as higher concentrations do not result in higher nicotine intake [13]. Previous studies in our lab have shown that this method of administration results in mean brain concentrations of nicotine ranging from 243 to 329 ng/g [16]. Drug treatments were continued for 7–8 weeks, after which the animals were killed 1 hour after the last levodopa injection and the brains used in synaptosomal dopamine release assays.

Dyskinesia severity was assessed weekly. Following the levodopa injection, the mouse was individually placed for 150 min in a transparent cylinder flanked by two vertical mirrors and video-recorded every 30 min for 1 min. Dyskinesia severity was quantified from the recordings by a researcher blinded to the treatment condition. Dyskinetic behaviors were divided into three subtypes (axial, orolingual, and forelimb dyskinesia), and each subtype was scored on a scale of 0–4 according to criteria detailed in Table 1. A weekly score was calculated for each dyskinesia subtype as the area under the curve of the animal's time point scores, giving the weekly score a theoretical range from 0 to 16.

Synaptosomal [^{3}H]dopamine release assay

The mice were killed by cervical dislocation and the dorsal and ventral striatum dissected as described previously [17]. Preparation of P1 synaptosome pellets, uptake of [^{3}H]dopamine (PerkinElmer, Waltham,

MA), superfusion of synaptosomes and measurement of released radioactivity were performed as previously described [5] with the exception that the synaptosome pellets were resuspended in 0.8 ml of uptake buffer. Nicotinic receptor-mediated release was determined by superfusion for 20 s with superfusion buffer containing 10 µM nicotine (tartrate salt, Sigma-Aldrich). To determine α4β2*-mediated release, 50 nM α6β2* antagonist α-conotoxin MII (gift from Dr. J. M. McIntosh, University of Utah, UT) was added to the superfusion buffer for 3 min before stimulation. α6β2*-mediated release was calculated as the difference between total and α4β2*-mediated release. Each mouse and brain area were assayed with 2–4 parallel replicates. Assays from one mouse (lesion only group) failed due to technical difficulties.

[^{3}H]dopamine release data were analyzed using R 2.15.2 (R Core Team). Nicotine-stimulated release was normalized to basal release to enable assessment of receptor function independently of the release-reducing effects of the lesion. Data were plotted as counts per minute (CPM) versus fraction number, and basal release was calculated for each 10-second fraction by single exponential decay from fractions collected before and after the stimulated release peak. Fractions exceeding basal release by 15 % or more were summed to give the amount of nicotine-stimulated release above baseline. Basal release calculated for the first fraction was converted to CPM/s and used as an overall measure of basal release.

Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed with IBM SPSS Statistics 24 (IBM, Armonk, NY) with the level of significance set at 0.05. Dyskinesia data were analyzed with two-way repeated measures analyses of variance (ANOVA) with Bonferroni's multiple comparisons tests and Greenhouse-Geisser correction for violations of the assumption of sphericity. Dopamine release data were analyzed with unpaired two-tailed Student's t-tests or with two-way analyses of variance with Bonferroni's multiple comparisons tests.

Results

Chronic nicotine treatment inhibited levodopa-induced dyskinesia

The present lesioning method resulted in a loss of 65±1% of tyrosine hydroxylase-positive immunostaining in the dorsal striatum and 60±10 % in the SNc (n = 5; see Fig. 1). The daily intake of

nicotine (calculated from average consumption per cage) at the highest concentration of 300 µg/ml was 36.0 ± 9.6 mg/kg. As shown in Fig. 2, chronic treatment with nicotine in drinking water inhibited the development of all subtypes of LID. At the last measuring time point (week 7 of nicotine treatment), mean dyskinesia scores of the nicotine-treated animals were lower than those of vehicle-treated animals (–50 % for axial dyskinesia, –80 % for orolingual dyskinesia, –70 % for forelimb dyskinesia). The effect of nicotine was statistically significant in the case of axial dyskinesia (treatment X time interaction, P = 0.026) and forelimb dyskinesia (main effect of treatment, P = 0.020), with a tendency in the case of orolingual dyskinesia (treatment X time interaction, P = 0.073).

Effects of the dopaminergic lesion and nicotine treatment on synaptosomal [^{3}H]dopamine release

Both basal (Fig. 3A) and nicotinic receptor-mediated (Fig. 3B–C) [^{3}H]dopamine release was measured from synaptosomes prepared from the dorsal and ventral striatum of the lesioned and intact hemispheres. Basal [^{3}H]dopamine release from samples of the lesioned dorsal striatum was significantly reduced 4–5 weeks after the 6-OHDA injections, to 37±5 % of the intact side (t-test, $P < 0.001$). In animals assayed 15–16 weeks after the 6-OHDA injections, including 7–8 weeks of chronic treatment with levodopa and either nicotine or water, basal release from samples of the lesioned dorsal striatum was also significantly reduced (74±10 % of the intact side; main effect of hemisphere, P = 0.035). Basal [^{3}H]dopamine release from dorsal striatum samples of nicotine-treated animals was slightly but not statistically significantly increased (main effect of treatment, P = 0.22).

In samples of the lesioned ventral striatum, 4–5 weeks after the lesion basal [^{3}H]dopamine release was reduced to 61±9 % of the intact side (t-test, P = 0.009). In animals assayed after the chronic drug treatments (15–16 weeks after the lesion), basal release from the lesioned ventral striatum was not significantly reduced (86±10 % of the intact side, main effect of hemisphere, P = 0.12). In particular, in nicotine-treated animals basal release from the lesioned ventral striatum was comparable to the intact hemisphere (main effect of treatment, P = 0.011; treatment X hemisphere interaction, P = 0.074).

Neither α4β2* nor α6β2* nicotinic receptor-mediated [^{3}H]dopamine release, stimulated by 10 µM nicotine and normalized to basal release, was significantly affected by the dopaminergic lesion 4–5 weeks after surgery in either the dorsal or ventral striatum (t-tests; α4β2* dorsal, P = 0.68; α6β2* dorsal, P = 0.76; α4β2* ventral, P = 0.78; α6β2* ventral, P = 0.46). Chronic nicotine treatment had no statistically

significant effect on α4β2*-mediated release in either brain area (main effects of treatment: dorsal, P = 0.66; ventral, P = 0.93). However, nicotine treatment significantly decreased α6β2*-mediated [^{3}H]dopamine release in the lesioned dorsal striatum (main effect of treatment, P = 0.020; treatment X hemisphere interaction, P = 0.033) and in both the intact and lesioned ventral striatum (main effect of treatment, P = 0.028). When non-normalized nicotinic receptor-mediated [^{3}H]dopamine release was analyzed, nicotine treatment had no statistically significant effects (data not shown).

Discussion

Here, we show that chronic treatment with nicotine in drinking water inhibits the development of levodopa-induced dyskinesia in mice suffering from unilateral dopaminergic denervation. Our observation is in line with those of Quik and colleagues [7] and, to the best of our knowledge, represents the first published independent replication of the antidyskinetic potential of the non-selective nicotinic receptor agonist nicotine. Our finding gives additional support to the hypothesis that long-term nicotine treatment can have beneficial effects on dyskinesia associated with long-treatment of human Parkinson's disease with levodopa. It should be noted that as our research focus was solely on nicotine's effects on LID, we did not study the effects of the nicotine treatment on parkinsonism. However, Quik and colleagues have reported no change in parkinsonism or the antiparkinsonian effects of levodopa by concurrent nicotinic agonist treatment in multiple animal models [7]. This suggests that the LID alleviation seen in nicotine-treated animals is not the result of reduced levodopa efficacy.

To study the mechanisms of nicotine's antidyskinetic effect, we investigated basal and nicotinic receptor-mediated release of [^{3}H]dopamine from striatal synaptosomes. Chronic nicotine treatment tended to increase basal [^{3}H]dopamine release in the dorsal striatum, a finding which although not statistically significant would be in line with a microdialysis study reporting increased basal dopamine release in the dorsal striatum after a similar nicotine treatment [18]. Intriguingly, nicotine treatment appeared to restore basal [^{3}H]dopamine release in the lesioned ventral striatum up to intact levels. Although nicotine has neuroprotective properties [7], neurorestoration after nigrostriatal damage was not observed in rats or primates [19], suggesting that the present result is more likely to be explained by functional changes than regrowth of dopamine terminals. This putative ability by nicotine to normalize basal dopamine release in conditions of partial denervation is worth further study, but being observed only in the ventral striatum

might not be directly relevant to motor disorders such as Parkinson's disease and LID. Finally, a smaller reduction in basal [^{3}H]dopamine release in the lesioned dorsal striatum was observed in drug-treated animals when compared to animals assayed at the earlier timepoint. This could represent spontaneous recovery [20,21], however the different gender distribution of the two experimental groups limits the conclusiveness of the comparison.

The current study found no difference between the dorsal and ventral striatum in the proportions of [^{3}H]dopamine release mediated by α4β2* versus α6β2* nicotinic receptors, in contrast to brain slice voltammetry studies [8] but agreeing with previous synaptosomal release studies [5,22]. The dopaminergic lesion had no effect on nicotinic receptor-mediated [^{3}H]dopamine release, when normalized to basal release, suggesting that despite the reduction in dopamine terminals the remaining presynaptic nicotinic receptors functioned with normal efficiency. While the antidyskinetic nicotine treatment had no effect on α4β2*-mediated [^{3}H]dopamine release, α6β2*-mediated release was significantly reduced in nicotine-treated animals in the lesioned dorsal striatum and in the ventral striatum in general. Of the nicotinic receptors expressed on striatal dopamine terminals, at least in mice α6β2* receptors may thus be the primary mediators of nicotine's antidyskinetic effects. This result is mostly in line with previous studies in rodents, which have suggested selective downregulation of α6β2* receptors by chronic nicotine treatment [10], an essential role for α6β2* receptors in LID [23], and that dampening of striatal nicotinic receptor-mediated dopaminergic activity is involved [24,25].

Studies in knock-out mice show that nicotine's antidyskinetic effects are mediated by α4β2* receptors as well [15], and a previous study in rats found that an antidyskinetic effect by nicotine was associated with decreases in both α4β2*- and α6β2*-mediated [^{3}H]dopamine release from striatal synaptosomes [25]. However, as α4β2* receptors are the most widely expressed nicotinic receptor subtype [3], and nicotinic agonists can alleviate LID also in rodents with less than 1 % of striatal dopaminergic innervation remaining [14,26], it is possible that some other α4β2* population than that in the striatal dopamine terminals is responsible for the α4β2*-mediated antidyskinetic effects of nicotine. The partial discrepancy between our result and the previous study in rats could be due to a different lesion model or a species difference. Finally, other mechanisms than alterations in dopamine release could also be involved, as release-modulating presynaptic nicotinic receptors are expressed also on striatal glutamate and serotonin

terminals [27,28], both of which are implicated in LID pathophysiology [2]. In conclusion, the present results support a role for nicotinic receptors as drug targets for alleviating dyskinesia associated with the treatment of Parkinson's disease with levodopa and, although more mechanistic studies are still needed, suggest that striatal presynaptic α6β2* nicotinic receptors may have a crucial role in mediating nicotine's antidyskinetic effects.

Author contributions

SL planned and performed the research, analyzed the data and wrote the manuscript.

SKK developed the LID quantification method and reviewed the manuscript.

SR planned the research and reviewed the manuscript.

OS managed the research project, planned the research and reviewed the manuscript.

All authors have approved the final manuscript.

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Conflicts of interest

The authors declare no conflicts of interest.

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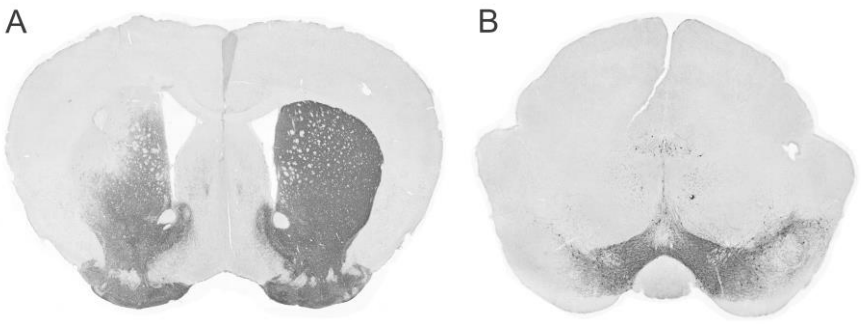
Figures captions

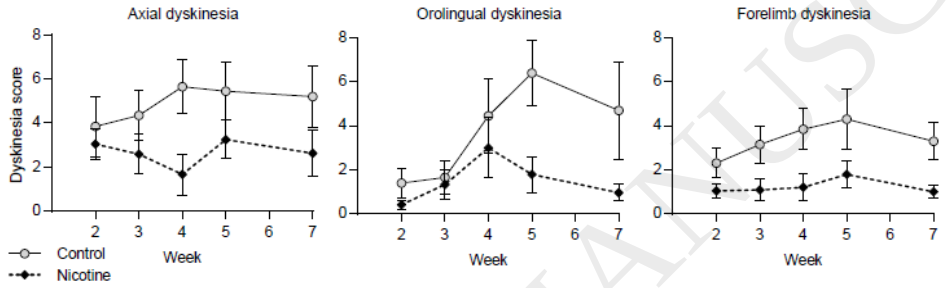
Fig. 1. Representative sections, immunostained for tyrosine hydroxylase, showing the striatum (A) and the substantia nigra (B) of a mouse lesioned with intrastriatal 6-OHDA injections.

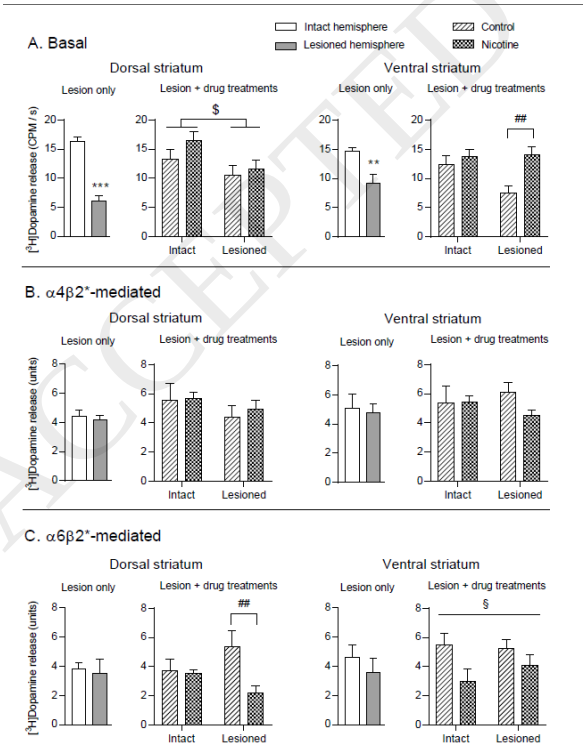
Fig. 2. Nicotine treatment inhibited the development of levodopa-induced dyskinesia. Mice lesioned with unilateral 6-OHDA injections were treated chronically with levodopa and benserazide injections and nicotine in drinking water. Dyskinesia severity was assessed from video recordings. Two-way repeated measures ANOVA: Axial dyskinesia, interaction $P < 0.05$; Orolingual dyskinesia, interaction $P = 0.07$; Forelimb dyskinesia, main effect of nicotine $P < 0.05$. n = 5–6 mice, mean ± SEM shown.

Fig. 3. Effects of dopaminergic denervation and chronic nicotine treatment on [^{3}H]dopamine release from striatal synaptosomes. **A**: Basal [^{3}H]dopamine release from both dorsal and ventral lesioned striatum was significantly reduced compared to the intact hemisphere. Further, in animals treated chronically with nicotine basal [^{3}H]dopamine release from the lesioned ventral striatum was comparable to the intact hemisphere. **B**: Neither the lesion nor nicotine treatment had statistically significant effects on normalized α4β2*-mediated [^{3}H]dopamine release. **C**: Normalized α6β2*-mediated [^{3}H]dopamine release was unaffected by the lesion, but nicotine treatment significantly reduced α6β2*-mediated release in the lesioned dorsal striatum and generally in the ventral striatum. ** $P < 0.01$, *** $P < 0.001$, difference to intact hemisphere; ## $P < 0.01$, difference to control; $ $P < 0.05$, main effect of hemisphere; § $P < 0.05$, main effect of nicotine. n = 5–6 mice, mean + SEM shown.

Figures:







Tables:

Table 1. Criteria used for scoring dyskinetic behaviors in mice.

Subtype	Score	Description
Axial dyskinesia	0	Normal physiological motor repertoire.
	1	Contralateral deviation of the head and trunk with one or both forelimbs contacting the ground. Mouse is steady or moving.
	2	Contralateral deviation of the head and trunk in a bipedal sitting position.
	3	Contralateral deviation of the head and trunk in a bipedal sitting position, causing a **loss of balance**.
	4	In a bipedal sitting position, head and trunk **fixed and in a severely twisted position** followed by a loss of balance.
Orolingual dyskinesia	0	Normal physiological motor repertoire.
	1	Vacuous chewing movements OR **occasional** (≤10 times per minute) biting of the contralateral side/limbs.
	2	Vacuous chewing movements, including tongue protrusions, OR **repeated** (11–19 times) biting of the contralateral side/limbs.
	3	**Sustained** (3–5 s) vacuous chewing movements, including tongue protrusions, OR **frequent** (≥20 times) or **sustained** (3–5 s) biting of the contralateral side/limbs.
	4	**Prolonged** (>5 s) vacuous chewing movements, including tongue protrusions, OR **prolonged** (>5 s) biting of the contralateral side/limbs.
Forelimb dyskinesia	0	Normal physiological motor repertoire.
	1	Isolated jerky movements of the contralateral distal forelimb.
	2	Repetitive, **small** movements (vertical or horizontal) of the contralateral forelimb involving the distal and proximal forelimb; <50 % of time.
	3	Repetitive, small movements of the contralateral forelimb as in 2; 50–100 % of time.
	4	Repetitive, **large** movements (vertical or horizontal) of the contralateral forelimb involving the distal and proximal forelimb.