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Induction and Assessment of Levodopa-induced Dyskinesias in a Rat Model of Parkinson's Disease

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TITLE:

Induction and Assessment of Levodopa-Induced Dyskinesias in a Rat Model of Parkinson's Disease

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SUMMARY:

This article describes methods to induce and evaluate levodopa-induced dyskinesias in a rat model of Parkinson's disease. The protocol offers detailed information regarding the intensity and frequency of a range of dyskinetic behaviors, both dystonic and hyperkinetic, providing a reliable tool to test treatments targeting this unmet medical need.

ABSTRACT:

Levodopa (L-DOPA) remains the gold-standard therapy used to treat Parkinson's disease (PD) motor symptoms. Unfortunately, L-DOPA-induced dyskinesias (LIDs) are unwanted involuntary movements that develop with prolonged use of this dopamine precursor. It is estimated that the incidence of LIDs escalates to approximately 90% of individuals with PD within 10–15 years of treatment. Understanding the mechanisms of this malady and developing both novel and effective anti-dyskinesia treatments requires consistent and accurate modeling for pre-clinical testing of therapeutic interventions. A detailed method for reliable induction and comprehensive rating of LIDs following 6-OHDA-induced nigral lesioning in a rat model of PD is presented here. Dependable LID assessment in rats provides a powerful tool that can be readily utilized across laboratories to test emerging therapies focused on reducing or eliminating this common treatment-induced burden for individuals with PD.

INTRODUCTION:

Although it has been more than 50 years since levodopa (L-DOPA) was first introduced as a treatment for individuals with PD[1,2], it remarkably remains the most effective therapy for parkinsonian motor symptoms. The clinical motor symptoms associated with PD stem from the

dopamine (DA) neurons in the substantia nigra (SN) pars compacta. L-DOPA effectively restores striatal DA levels, resulting in motor benefit early in the disease[3,4]. Inopportunely, with long-term treatment, most of those afflicted with PD will develop L-DOPA induced dyskinesias (LID), including chorea, dystonia, and athetosis, which often significantly impact activities of daily living[5-7].

While several behavioral models of LID in rodents exist, allowing for pre-clinical research detailing the effects of anti-dyskinetic therapies, differences in modeling and behavioral assessment of LIDs have called into question the reproducibility of results between labs as well as the reliability of these experimental tools for PD research. The current protocol is a straightforward method for LID induction and rating, developed in association with a clinical movement disorder specialist[8] and is appropriate for use in a rat model of PD utilizing 6-hydroxydopamine (6-OHDA)-induced unilateral nigral lesioning[9,10]. The LID rating scale provided here includes scoring for both the intensity and frequency of dyskinetic behavior in various individual body parts. Pertinent information regarding workflow optimization of experiments and the appropriate care and handling of parkinsonian and dyskinetic animals is also provided.

PROTOCOL:

The animals presented here were maintained and handled in compliance with the institutional guidelines. All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC) in compliance with federal and state regulations.

1. Drug-free confirmation of 6-OHDA lesion status

1.1. Postural Tail hang test[11-13]

NOTE: Assess lesion status at least 1 week following unilateral 6-hydroxydopamine (6-OHDA) lesion induction[9,10] in experimental subjects (e.g., male or female, adult Sprague Dawley or Fisher 344 rats). The protocol of the 6-OHDA lesioning begins 1 week after the lesioning of the rats, which must be performed before inducing LIDs. See Reference[9] for details on 6-OHDA lesioning.

1.1.1. Suspend the rat approximately 6 cm above its cage, firmly holding at the base of the tail, for ~5 s.

1.1.2. Record the direction of body contortion as + for a successfully lesioned animal twisting contralateral to the lesioned side and - for lack of twisting or twisting in both directions.

NOTE: These tests are optional but recommended. See[14-17] for additional drug-free testing options/variations.

1.2. Step adjusting drag test (adapted from[16])

NOTE: Assess lesion status for at least 1 week following unilateral 6-OHDA lesion induction in experimental subjects (e.g., male or female, adult Sprague Dawley or Fisher 344 rats).

1.2.1. Hold the rat by the base of its tail, elevating the back feet off the surface by ~6 cm; drag backward across a flat, smooth but not slippery surface, ~75 cm, over 5–10 s.

1.2.2. Observe and record the number of tapping/step adjusting movements of each forepaw over three repeated tests.

1.2.3. Score the subject as + for successful unilateral lesioning when 0–2 forepaw taps are observed contralateral to the lesioned side, together with rapid tapping (~10 taps) from the forepaw ipsilateral to the lesioned side (e.g., animals unilaterally lesioned on the left side show tapping deficit (0–2 taps) with the contralateral right forepaw).

1.2.4. Conversely, score moderate to rapid tapping (5–10 taps) from both forepaws as - to indicate incomplete or unknown lesion status.

NOTE: An anxious animal can show rapid tapping/step adjusting even if successfully lesioned. If this is suspected, place the rat back in their home cage and re-test ≥30 mins later.

2. Preparation of reagents and supplies

2.1. Determine L-3,4-dihydroxyphenylalanine (Levodopa or L-DOPA) methyl ester hydrochloride and benserazide hydrochloride, a peripheral decarboxylase inhibitor (see **Table of Materials**) dose, rating frequency, and experimental timeline that is appropriate for the investigational question[12,18-20] (**Figure 1**).

NOTE: Investigational questions can be posed to seek any number of questions ranging from asking whether a specific therapy might reduce existing LID or prevent induction of LID and whether therapeutic efficacy is dependent on the dose of levodopa; or whether LID expression and/or therapeutic efficacy varies depending on the sex, species, and age of the subject.

[Place **Figure 1** here]

2.2. Weigh rats weekly to calculate the appropriate drug quantity based on the ongoing weight changes during the study.

NOTE: Due to increased activity in LID+ rats, there is potential for weight loss with long-term L-DOPA treatments.

2.2.1. Provide rats with nutritionally complete, highly palatable treats (see **Table of Materials**) following L-DOPA injections.

2.3. Calculate the amount of L-DOPA and benserazide required for each weekly concentration, weighing out lyophilized aliquots for each day of injection, and storing in combination for 1–2 weeks at -20 °C in glass amber vials until the day of treatment.

NOTE: Target dose is 12 mg/kg or 12 mg/1000 g. Example of calculations for determining the amount of L-DOPA and saline needed for each day of a week using 12 mg L-DOPA/kg body weight at an injection volume of 1 cc/kg of rat weight is given in **Supplementary File 1**.

3. Room and cage set up

NOTE: Initiate L-DOPA treatment 3–4 weeks following 6-OHDA lesion surgery.

3.1. On the first day of L-DOPA treatment, transfer rats to single housing, including IACUC-approved enrichment.

3.2. Maintain in single housing throughout the study to avoid peer interference with behavioral assessments.

3.3. On LID rating days, place the home cages on a steel wire rack, turned at an approximately 45˚ angle for optimal viewing of the rat (**Figure 2A**). Flip the identification tags (**Figure 2B**) upward and remove water bottles, food racks, and all types of enrichment in the cage (**Figure 2C**) to avoid interference with behavioral assessments.

[Place **Figure 2** here]

4. Levodopa injections and dyskinesia rating

4.1. Subcutaneous injections of L-DOPA[21,22]

4.1.1. Immediately preceding the daily injection of L-DOPA, add the appropriate volume of sterile saline to the pre-weighed lyophilized L-DOPA and benserazide mix in the amber vial and shake well for 10 s (step 2.3).

NOTE: Target injection volume is 1 cc/1000 g body weight (with 12 mg/L-DOPA per cc). The volume of the sterile saline will depend on the number of animals per study.

4.1.2. Fill individual syringes (e.g., 1.0 or 0.5 mL with 26 G needle) with the required volume for each animal (1 mL/kg rat weight) and label each syringe with individual animal identification.

NOTE: Keep the filled syringes protected from light until the time of injection since in an aqueous environment, L-DOPA rapidly oxidizes in the presence of oxygen and light[23-26].

4.1.3. Bring the first cage to the injection bench.

4.1.4. Remove the rat from its cage and place it on the injection surface.

4.1.5. Gently restrain the head and shoulders against the surface on which the rat is resting with the palm of the non-dominant hand.

4.1.6. Gently, scruff the skin on the back overlying the scapulae with the thumb and forefinger of the non-dominant hand, inject L-DOPA volume with the dominant hand into the subcutaneous space between/below the fingers, keeping the needle as parallel to the body as possible to avoid intramuscular injection.

NOTE: The rats were not anesthetized before injection.

4.1.7. Dispose of each used individual syringe in a sharp's container.

4.1.8. Replace the rat into its individual cage and add nutritionally complete treats except on LID rating days to avoid interference with behavioral assessments until after ratings are complete.

4.1.9. Set the timer for 1–2 min depending on the rating time desired and the number of rats in the study on rating days. Retrieve the next cage and inject the next rat when the timer indicates.

4.1.10. Repeat this, injecting one rat every 1–2 min, until all the rats are injected.

4.2. Levodopa-induced dyskinesia rating post-injection

4.2.1. Rate the intensity (**Table 1**) and frequency (**Table 2**) of dystonic and hyperkinetic dyskinesia movements at the desired number of timepoints that should include the initial onset of LID behavior, peak behavior, and the phase of decline (see **Supplementary File 2** for an example of LID rating log sheet).

4.2.2. For male and female adult Sprague Dawley or Fisher 344 rats, and a sample size of N = 40 rats, begin the dyskinesia ratings 20 min after the first L-DOPA injection, and then at 50 min intervals until 220 or 270 min post-injection, depending on when LID behaviors have discontinued in 90%–100% of rats.

4.2.3. If using 1 min rating intervals, set a timer for 1 min. Rate the first rat for that first minute. Move to the next rat and rate it for 1 min and continue through all the rats with a rating for 1 min intervals.

4.2.4. Have a timer positioned next to the cage visibly so that the LID behavior intensity (**Table 1**) can be observed while estimating the frequency of any given behavior (**Table 2**) during the rating period.

4.2.5. After ratings for the first timepoint are completed, start with the first rat 50 min post-injection until all time points are completed.

NOTE: Due to the overlap of L-DOPA injection and LID rating tasks, two persons are needed on the rating days, one for injecting and one for behavioral ratings.

REPRESENTATIVE RESULTS:

LIDs in parkinsonian rats can manifest as a range of abnormal involuntary movements (AIMs), including dystonic, hyperkinetic, and stereotypic behaviors. LID rating criteria for such behaviors are presented here to include both intensity (**Table 1**) and frequency (**Table 2**). This provides an overall LID severity score for each rat that reflects both the quality (intensity) and quantity of time spent engaging (frequency) in these behaviors at each rating timepoint. The final LID severity score is calculated by multiplying the intensity score by the frequency score for each behavioral component. Scoring criteria for individual attributes of LID behaviors are provided here as written descriptions (**Table 1–2**), and examples are shown as still images with detailed reports (**Figure 3**) and as videos (**Animated Videos 1–4**). Additional descriptive information about individual movements and scoring is provided in the figure legends.

A comprehensive assessment of the impact of any treatment on amelioration of LID behaviors over time can be observed using an L-DOPA dose-escalation approach, 12 weeks in total length, with 8 weeks of L-DOPA injections following 6-OHDA lesioning (**Figure 1**). In this scenario, L-DOPA is given 5x per week (Monday–Friday) for 2 weeks at each prescribed L-DOPA dose. Behavioral ratings that take place on days 1, 6, and 10 of each L-DOPA dose provide a robust approach for treatment efficacy assessment (**Figure 1**). However, researchers may find that altered timelines and dosing schemes better answer their experimental questions. Indeed, the dose of L-DOPA and benserazide, the peripheral decarboxylase inhibitor, can vary depending on the investigator's experience with doses and/or the hypotheses to be tested. It is of note that the most common doses of benserazide are between 10–15 mg/kg, which is supported by the report by Tayarani-Binazir et al. (2012)[20] showing that optimal behavioral effects of L-DOPA are found with 10 mg/kg of benserazide, with no additional benefit at 15 mg/kg.

There are multiple ways to present and analyze LID data when determining whether a given treatment has a meaningful impact in ameliorating this aberrant behavior. **Figure 4A** provides example of data presented as peak dose LID over the entire experimental time line and doseage schedule. Peak dose LID can be defined as the time point at which the group average LID score is the greatest. Alternatively, one can report absolute or maximal peak LID scores for each animal during a rating period regardless of when it occurs. Additionally, LID rating data can be examined over each daily rating time course at different L-DOPA concentrations with the groups combined and/or plotted as individual animals (**Figure 4B**). This latter approach allows assessment of whether some treatments can reduce, for example, the total amount of time a subject displays LID, but not necessarily impact peak dose severity. Finally, one can quantify total LID severity scores over the entire rating session. For a given subject, the LID severity score for each time point is summed to produce a total score for that rating period. This approach emulates the area under the curve estimations, which also can be calculated[19].

It is essential to appreciate that LID/AIMs scoring data, created using rating scales for assigning values to dyskinesia severity and/or duration (**Tables 1 and 2**), are ordinal data. Thus the most

appropriate statistical tests are non-parametric. While there is no direct equivalent non-parametric test for two-way analysis of variance[27], LID data can be analyzed with non-parametric alternatives to the one-way ANOVA. Specifically, the Kruskal-Wallis test is used for between-subject comparisons of two or more independent groups. Friedman tests are a non-parametric alternative to a one-way ANOVA with repeated measures used for comparisons within subjects. Both are used with post-hoc tests (i.e., Dunn or Dunn-Bonferroni) following a significant Kruskal-Wallis or Friedman's test. For examining whether there are substantial differences between two independent groups, the Mann-Whitney U test is considered the non-parametric equivalent of the independent *t*-test[28,29].

FIGURE AND TABLE LEGENDS:

Figure 1: Example of treatment timeline. Example L-DOPA dose-escalation timeline of 12 weeks in total length, with 8 weeks of L-DOPA injections beginning 3 weeks after 6-OHDA lesioning and 4 weeks following experimental treatment. In this example, L-DOPA is subcutaneously injected 5x per week (Monday–Friday) at approximately the same time each day, for 2 weeks at each prescribed L-DOPA dose (3, 6, 9, and 12 mg/kg). Behavioral LID ratings take place on days 1, 6, and 10 of each L-DOPA dosage level.

Figure 2: Example of the cage set up for LID ratings of large-scale rat experiments. (**A**) Multiple cages can be set up for LID rating using large metal racks that allow for optimal viewing of each animal. Cages should be spread apart at a 45° angle with ID cards flipped upward (**B**), food, water bottles, nesting materials, and other enrichment removed to limit visual obscureness of the rat and distractions to the rat while examining dyskinetic behaviors (**C**). The metal racks need to be a few feet away from any wall to allow the rater to examine the rat at the front or back of the cage as needed. It is essential to label enrichment apparatuses (e.g., C- red rat retreat houses) with individual animal IDs to replace them into the same cage from which they came. This is particularly important when using animals of different sexes not to increase stress to the experimental subjects.

Figure 3: Freeze-frame images showing examples of LID intensity scoring in parkinsonian rats. (**A**) Representative image of a non-dyskinetic parkinsonian rat displaying the often-typical hunched parkinsonian posture in the absence of dystonia or hyperkinesia. (**B**) Parkinsonian rat with overall mild LID severity, indicating mild-to-moderate right forelimb dystonia and a lack of neck, trunk, or hindlimb dystonia; in the accompanying video (**Animated Video 1**), small-amplitude right forepaw dyskinesia (RFPD) and orolingual movements can be seen. (**C**) Parkinsonian rat with moderate neck and trunk dystonia (bold text corresponds with directional arrows indicating trunk twisted at 90° angle). (**D**) Dyskinetic parkinsonian rat with mild neck and moderate trunk dystonia, moderate-to-severe right forelimb dystonia; in the accompanying video (**Animated Video 3**), mixed amplitude RFPD can be observed. (**E**) Parkinsonian rat with moderate-to-severe neck and severe trunk dystonia, severe right hindlimb, and moderate right forelimb dystonia (bold text corresponds with directional arrows indicating the trunk is twisted ~180°; this is especially notable in example #2).

Figure 4: Example LID data complied over 8 weeks of LID ratings with escalating L-DOPA dosage. (**A**) Peak dose LID severity (80 min post-L-DOPA) across time and doses. (**B**) Daily time course (20–170/200 min post-L-DOPA). For each dose and day, the top graphs reflect mean ± SEM; the bottom graphs show individual subject responses over time. Statistics were calculated using Kruskal-Wallis with Dunn's multiple comparison tests (for between-subjects tests) and Friedman tests with Dunn's multiple comparison tests (for within-subjects tests). *Abbreviations*: Control (Ctr) (n = 7); Treatment (Tx) (n = 10). This figure is reprinted and adapted with permission from Reference[19].

Table 1: LID rating criteria for Intensity of dystonic or hyperkinetic behaviors in parkinsonian rats. These rating criteria provide a range of intensity measures related to the quality/severity of the rat's abnormal involuntary postures and/or movements. Specific attributes described for postures and behaviors are generally classified as mild, moderate, or severe, with specific descriptors provided here. A final severity score is determined as the product of Intensity x Frequency (**Table 2**).

Table 2: LID Rating criteria for Frequency of dystonic or hyperkinetic behaviors. These criteria provide a quantification measure for the rate at which any given behavior occurs or is repeated during the observation period. This is important in assessing the overall severity, given that infrequent behavior warrants a less severe score than a persistent one. A final severity score is determined as the product of Intensity (**Table 1**) x Frequency.

Animated Video 1: Mild LID with small amplitude RFPD and chewing of the left forepaw. Dyskinetic parkinsonian rat with no appreciable neck or trunk dystonia, but moderate right forepaw dystonia, small amplitude RFPD noted as rapid repeated flickering movement of the right forepaw. This rat also displays orolingual behavior involving vacuous chewing directed at the left forepaw. L-DOPA dose: 12 mg/kg; video recorded ~70 min post-injection.

Animated Video 2: Moderate trunk and neck dystonia. Dyskinetic parkinsonian rat with moderate trunk and neck dystonia exemplified by the 90° difference in the position of the forelimbs and hindlimbs (see diagram in **Figure 2C**). This rat also displays prominent stereotypic sniffing and occasional vacuous biting at the litter. L-DOPA dose: 12 mg/kg; video recorded ~70 min post-injection.

Animated Video 3: Moderate-to-severe right forepaw dystonia with mixed amplitude RFPD. Dyskinetic parkinsonian rat with mild neck and moderate trunk dystonia, moderate-to-severe right forepaw dystonia, mixed amplitude RFPD seen as smaller wiping movements near the mouth mixed with larger amplitude downward pulling of the right forepaw. This rat also has frequent rotational behavior that can be quantified in addition to LID profiles. L-DOPA dose: 12 mg/kg; video recorded ~70 min post-injection.

Animated Video 4: Head bob and orolingual movements with tongue protrusions. Dyskinetic parkinsonian rat with severe trunk and forepaw dystonia (hindlimb dystonia not visible), moderate-to-severe neck dystonia, and a constant head bob with ongoing tongue protrusions.

The head bob resembles the 4 Hz tremor seen in PD; however, in rats, it is only noted in dyskinetic parkinsonian rats following L-DOPA administration. This rat also has mixed amplitude RFPD, seen as more significant amplitude movements early in the video and small wiping-like motions near the face, characteristic of small amplitude RFPD seen later. Slight movement was observed on the severely dystonic/extended forelimb early in the video; however, this is related to the head and neck tremor activity and would not be classified as small-amplitude RFPD. When the observer taps strongly on the cage, these LID behaviors are not interruptible, suggesting that this is a severe LID that cannot be overcome with a startle.

Supplementary File 1: Example for calculating the amount of L-DOPA. Example of calculations for determining the amount of L-DOPA and saline needed for each day of a week using 12 mg L-DOPA/kg body weight at an injection volume of 1 cc/kg of rat weight.

Supplementary File 2: Example LID rating log sheet. This sheet can be used or adapted to log LID intensity and frequency scores during rating sessions.The final severity score is determined as the product of Intensity (**Table 1**) x Frequency (**Table 2**). These rating scales can also be used to quantify LID in mice[30].

DISCUSSION:

Presented here are details for the reproducible induction and rating of LIDs in a parkinsonian rat model following unilateral 6-OHDA lesioning of the nigrostriatal DA system. While it was once thought that rodents did not develop LID and that rotational asymmetry may be the analog of LID in rats[31], rat and mouse models have been well characterized over the past two decades and are a well-accepted tool for LID research[15,32-34]. The protocol presented here is specifically helpful for larger-scale experimental designs. It provides details for rating the intensity and frequency of a range of dyskinetic behaviors, both dystonic and hyperkinetic. Notably, images and videos with corresponding detailed notes and scoring of dystonic and hyperkinetic LID behaviors will assist the experimenter's assessment following L-DOPA administration. While an example of every possible abnormal involuntary movement (AIM) is not provided, a range of behaviors varying from mild to moderate to severe is offered as a framework for working. This protocol has been documented to help evaluate various therapies in parkinsonian rats[19,35-37]. It augments other published rodent rating scales that focus primarily on the duration of prescribed LID behaviors[33,38,39]. Further, the outlined procedure seeks to align scoring methods in a rat PD model with current practices assessing LIDs in non-human primates (NHPs), the benchmark PD model[40,41].

Commonly found in the parkinsonian rodent literature are LID, or AIM rating scales that involve examining the occurrence or frequency of (1) limb dyskinesias (e.g., rhythmic jerks of the forelimb contralateral to the lesion[33], or rapid, purposeless movement of the forelimb controlled by the lesioned hemisphere[39]); (2) axial dyskinesias (e.g., torsion of axial muscles affecting the neck, trunk and tail[33], or dystonic twisting of the neck and torso contralateral to the lesioned hemisphere[39]); and (3) orolingual dyskinesias (e.g., masticatory movements of the empty mouth with tongue protrusions[33], or repetitive mastication or tongue protrusions when the rat's mouth was empty and not in contact with any object[39]). These three behavioral categories are given a

severity score based on their frequency during the observation period. The severity score representing the amount of time an animal spent exhibiting these behaviors, frequently without characterization of the quality of behavior, is then combined into the final ALO (axial, limb, and orolingual) score.

In contrast, the current protocol provides a more detailed scale that allows not only the evaluation of global AIMs, but also enables examination of AIMs in independent body parts (i.e., neck, trunk, forelimb, hindlimb, and mouth), the intensity/quality of the AIM being assessed, differentiation of dystonia from hyperkinetic behaviors, and, as with other scales, incorporates the frequency of any given AIM's occurrence. The overall goal is to allow a comprehensive assessment of the LID behavior expressed in each subject. For example, for the single behavioral readout of trunk dystonia, a rat exhibiting mild trunk dystonia (i.e., 45° difference between upper and lower torso, not predictive of turning behavior) for an entire observation period is very different than a rat exhibiting severe trunk dystonia (i.e., constant severe dystonia/twisting, corkscrew-like posturing, approaching 180°; head and feet in opposite directions, unable to ambulate) for the entire observation. Further, given the topographic representation of the rat's body in the striatum, differentiating individual attributes of LIDs can be informative[36].

Another component of this protocol to consider carefully is the experimental design of escalating L-DOPA dosing over an extended (i.e., 8-week) treatment time course (**Figure 1**). It is cautioned that while designing a study to employ only low doses of L-DOPA *(*e.g., 3–6 mg/kg) and/or administering the dose of choice for a short period (e.g., 2–4 weeks) may show evidence of LID amelioration with therapeutic intervention in parkinsonian rats. However, the relevance to individuals with PD, where chronic administration and dose escalation are often necessitated, is questionable. Such divergent treatment protocols between pre-clinical and clinical investigations could be suggested to underlie the lack of efficacy seen in clinical trials undertaken with drugs that showed promise in pre-clinical rodent studies.

While the protocol presented here provides instructions on a comprehensive approach for rating LID in parkinsonian rats and the rat model of LID has been established as a reliable model of clinical LID[3,15,38,42,43], there are inherent limitations to any model. Animal models are tools that are useful in emulating particular attributes of human disease and making predictions about, for example, the impact that therapeutic intervention might have on a set of disease features. One feature of pre-clinical LID models commonly debated as a potential limitation is that rats require significant DA depletion. LID develops more rapidly in rodent and NHP models than in patients; these models do not reflect clinical practice LID. However, it is often underappreciated that regardless of species (i.e., human, non-human primate, or rodent), near-complete loss of striatal DA innervation is generally required for LID to manifest. Once L-DOPA is administered to subjects with severe striatal DA depletion, LID is induced[1,19,38,44-46]. A second feature that has fostered skepticism toward rodent models of LID comes from the expectation that the neurological signs of LID should resemble the physical manifestations of LID seen in primates (i.e., human and non-human). Indeed, the appearance of the abnormal movements classified as LIDs in rats varies in features from that seen in primates (e.g., choreoathetosis in primates *vs.* stereotypy/hyperkinesia in rodents). While it is beyond this article to comprehensively address

this topic[46], briefly, such differences in signs between species are based on the fact that humans are habitual bipedal, non-primates frequent bipedal, and rodents quadrupedal. Thus, various species have specific repertoires of behaviors manifest by specific osteoarticular and muscular structures and modulated by species-specific neural systems. Accordingly, animal modeling of human-like symptoms should be based primarily on an expectation of functional similarity rather than on physical identity. Interpretation of findings from animal models with due prudence has, and will continue to provide valuable predictions for disease treatment.

While there is currently one FDA-approved drug for the treatment of LID in PD, amantadine, and the surgical intervention of DBS can ameliorate LID, the efficacy and tolerability are not optimal, and not all patients will qualify for DBS surgery. As eloquently reviewed by Cenci and colleagues[47], there is a notion expressed by some that individuals afflicted with PD would rather be ON (i.e., experiencing the motor benefit of L-DOPA) with dyskinesias than OFF. As these authors poignantly state: The deeper truth is that patients would very much prefer to be ON without dyskinesia. As researchers and clinicians, we should aspire to make that goal a reality. To this end, translational research on LID is to be encouraged and persistently pursued. In pursuing this goal, we present, here, our method of induction and rating of LID. Our rating scale is designed to combine a range of intensity measures related to the quality/severity of the AIMs and indicates the amount of time a variety of attributes of dyskinesia are displayed. The overall intention of this model is to allow the determination of an objective, a numerical value that accurately reflects the level of LID severity, both the intensity and frequency, of not only global LID but also specific body parts, in a particular subject so that experimental therapeutics can be rigorously tested for their ability to modify this behavioral disorder.

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DISCLOSURES:

No conflicts of interest were declared.

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Figure 1

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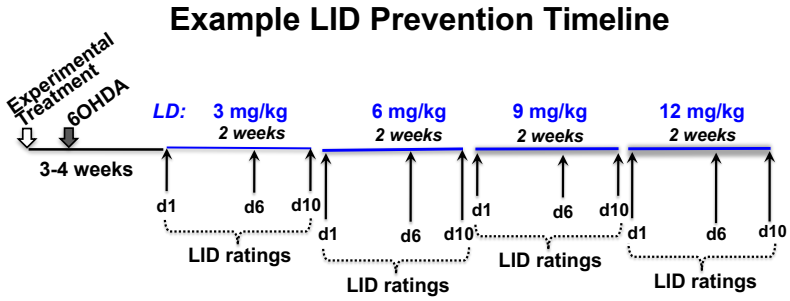

Figure 2

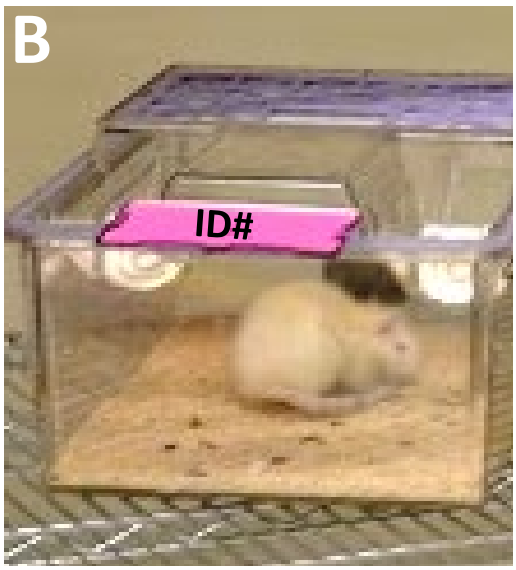

Figure 3

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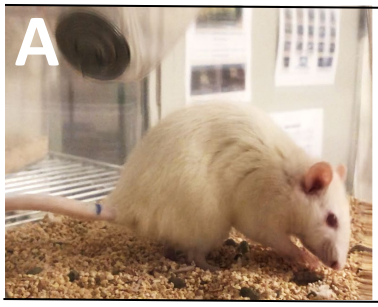
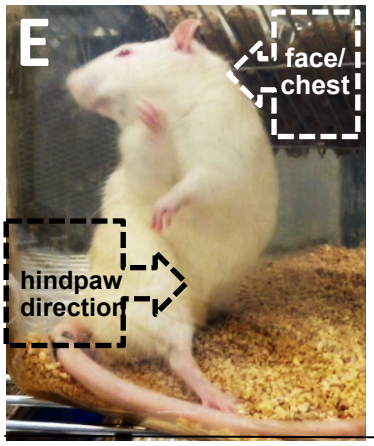
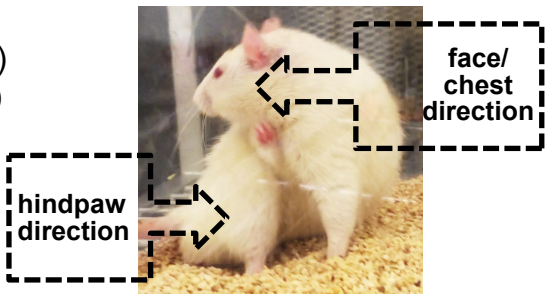
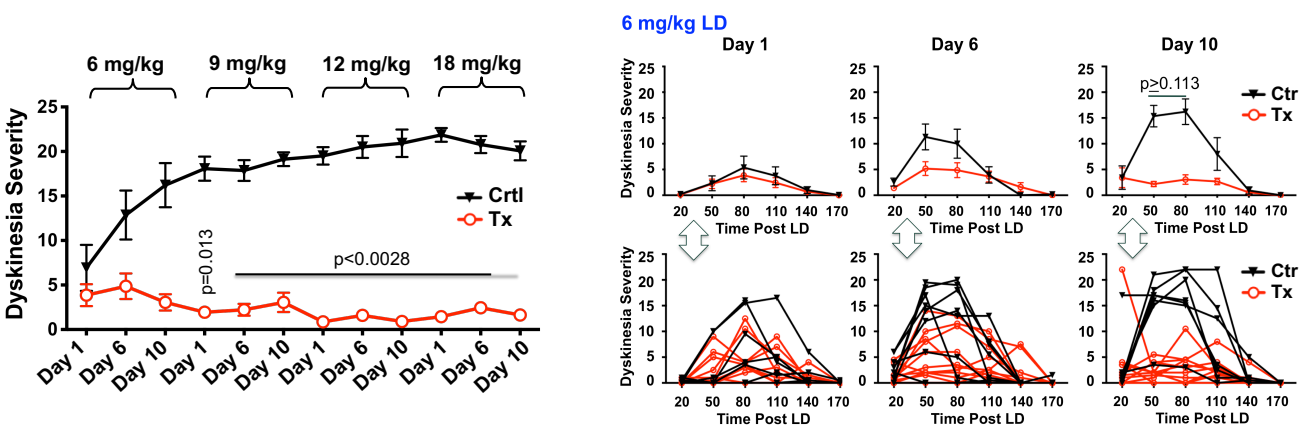
	LID Rating	Other behaviors/Notes
	None	Non-dyskinetic 6-OHDA lesioned rat displaying a typical hunched parkinsonian posture
	Neck Dystonia (0) Trunk Dystonia (0) Rt Hindlimb Dystonia (0) Rt Forelimb Dystonia (1.5) Small amplitude RFPD (1.0)$^{+}$ Orolingual (1.5) $^{\infty}$	Vacuous chewing at left forepaw* *See Animated Video 1 for details**$^{+\infty}$
	Neck Dystonia (2) **Trunk Dystonia (2)** Rt Hindlimb Dystonia (0) Rt Forelimb dystonia (0) Small amplitude RFPD (0)$^{+}$ Orolingual (1.5) $^{\infty}$	Straub tail Apparent tip toeing of hindlimbs Sniffing/biting at the litter* *See Animated Video 2 for details **$^{+\infty}$
	Neck Dystonia (1) Trunk Dystonia (2) Rt Hindlimb Dystonia (0) Rt Forelimb dystonia (2.5) Mixed amplitude RFPD (1.5)$^{+}$ Orolingual (0.5) $^{\infty}$	*See Animated Video 3 for details* $^{+\infty}$
	Neck Dystonia (2.5) **Trunk Dystonia (3)** Rt Hindlimb Dystonia (3) Rt Forelimb dystonia (2) Orolingual (0)	*Example 2*: Neck Dystonia (3) **Trunk Dystonia (3)** 

Figure 4



Animated Video 1

Click here to access/download

Video or Animated Figure

AV1. Mild LID, RFPD, and chewing of left forepaw..mp4

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Video or Animated Figure

AV2. Moderate trunk and neck dystonia..mp4

Animated Video 3

Click here to access/download

Video or Animated Figure

AV3. Right forepaw dystonia with RFPD..mp4

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Video or Animated Figure

AV4. Head bob with tongue protrusions..mp4

Table 1

	Score
Neck	0
	1
	1.5
	2
	2.5
	3
Trunk	0
	1
	1.5
	2
	2.5
	3
Forelimb dystonia	0
	1
	1.5
	2
	2.5
	3
Hindlimb dystonia	0
	1
	1.5
	2
	2.5
	3

Right forepaw dyskinesia (RFPD)	0
	1
	1.5
	2
Orolingual	0
	1
	1.5
	2
Head bobbing/tremor	0
	1
Constant chewing of litter (CCL)	0
	1

Description

none
mild displacement of head posture; head returns to neutral (straight ahead) position
mix of mild and moderate posture
head with more notable displacement (approx 90° angle to body); head remains pulled in the direction of the dystonic movement
mix of moderate and severe posture
constant severe torsion of neck musculature (>100° angle between head and shoulders)

none
mild dystonia/twisting of the trunk; 45° difference between upper and lower torso; held for appreciable time (10-20 sec) and not predictive of rotations
mix of mild and moderate dystonia
moderate dystonia/twisting of the trunk; 90° difference between upper and lower torso
mix of moderate and severe dystonia
constant severe dystonia/twisting, "corkscrew"-like posturing (approaching 180°; head and feet in opposite directions), unable to ambulate and may go into barrel roll rotation

none
Mild; abnormal/sustained posturing of wrist or digits; either clenched fist or rigid forearm but not usually both simultaneously
mix of mild and moderate dystonia
Moderate; clenched fist and rigid forelimb with no downward holding in hyperextended position
mix of moderate and severe dystonia

Severe; clenched fist and rigid forearm with downward hyperextension

none
hindlimb mildly held in abnormal posture; flexion or extension.
mix of mild and moderate dystonia

moderate rigid extension or flexion of hindlimb; w/o splaying of the digits

mix of moderate and severe dystonia
severe rigid posturing of limb with abnormal hyperextension +/- splaying of digits

absent

small amplitude movements, simple side to side, or up and down wiping in the region of the face or mouth; or tapping at the cage wall or litter

mix of small and large amplitude movements; most common
large amplitude movements involving severe extension of the right forelimb, pulls downward and opposite of neck

none
principally closed mouth chewing, teeth grinding
vacuous chewing, there may be some tongue protrusion, repeated biting at the litter
prominent repeated tongue protrusion with prominent open mouth chewing

none
present (repetitive and rhythmic bobbing of the head (approx 4hz))

none
present (constant chewing of litter; goal directed obsessive biting or chewing of litter, repeatedly picking up, chewing, dropping, picking up, chewing, dropping of litter)

Score	Description
0	Absent
1	Intermittent, < 50% of observation period
2	Intermittent, ≥ 50% of observation period
3	Persistent throughout entire observation period

Kathy Steece-Collier, Ph.D.
Professor
Margaret E. Caulfield, PhD
Research Associate
Department of Translational Neuroscience

MICHIGAN STATE UNIVERSITY

8/3/2021

Nilanjana Saha, PhD
Review Editor
JoVE

Dear Dr. Nilanjana Saha,

We are pleased to submit a revised version of our manuscript entitled “Induction and Assessment of Levodopa-induced Dyskinesias in a Rat Model of Parkinson’s Disease”, from authors Margaret E. Caulfield, Jennifer A. Stancati, and Kathy Steece-Collier, for publication as a methods article in JoVE.

We sincerely thank the editorial staff for their constructive comments and are grateful that our manuscript was reviewed so carefully. We have made every effort to address the comments that could feasibly be addressed in the revised manuscript. This includes careful proofreading and responses to all of the editor’s comments. All changes were tracked and comments retained.

Sincerely,

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Grand Rapids, MI 49503
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Kathy Steece-Collier, Ph.D.
Professor

Margaret E Caulfield, Ph.D.
Research Associate

Response to editorial and peer review comments:

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Careful proofreading has been completed.

2. Please revise the following lines to avoid previously published work: 27-28, 48-50, 54-55, 253-255, 297-299. These lines have been removed from the text.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). All personal pronouns have been replaced.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials (including reagents, instruments, software, etc.). Accordingly, in Line 177/188, please include the reagent catalog numbers in the Table of Materials. Please sort the Materials Table alphabetically by the name of the material. Commercial language has been removed and the materials list updated accordingly.

5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.
8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

The protocol section has been extensively revised to incorporate comments 5-8.

9. Please add more details to your protocol steps:
Step 1: Please specify the age/gender/strain of the animal used. This revision has been completed. See lines 68, 77-78, and 177.

Line 170: What treats are provided? What is meant by the appropriate condition? The term “appropriate condition” has been removed and generic terms are used for the nutritionally complete treats as instructed in comment 4 above. See lines 103-104, 147, and 160-161. The specific BioServ treats we purchase are referenced in the Materials Table.

10. Please check whether the Figures and the Tables are in sequential order. The figures have been modified and ordered to align with the flow of the text and reference order.

11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

The discussion section has been extensively revised to incorporate comments 11a-e.

a) Critical steps within the protocol.
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

12. Please designate the Animated Figures/videos as Animated Figures/videos 1, 2 etc in the text. Please don't represent them as Figures in the text. There are 3 Figures (currently, Fig 1, 2 & 7) . The rest are animated videos. Kindly represent accordingly. These revisions have been completed. Please see revised figure legends.

13. Please spell out the journal titles in the References. These revisions have been completed. Please see the revised bibliography.

Reviewers' comments:

Reviewer #1:
The manuscript is well written. We thank this reviewer for their laudatory comment.

Reviewer #2:
Manuscript Summary:
The authors attempt to present a detailed method in a rat model of PD for LID induction and rating as well as provide information to help optimize the workflow, appropriate care and handling of parkinsonian and dyskinetic animals in order to provide standardization of tools across laboratories.

Major Concerns:
It falls short on this goal in many ways including the lack of some critical details and numerous mistakes. This would create confusion rather than clarifying and setting a standard for the field.
The manuscript has been revised to increase attention to detail and specificity, particularly with regards to the protocol section. This section has been streamlined to include mainly action steps that can be demonstrated on video and recapitulated by JoVE readers.

Throughout the manuscript, the side of the lesion in the brain and the deficit of body parts are mistakenly noted. One example: "A successfully unilaterally lesioned rat will principally drag the forepaw on the same side of the lesion." It should be contralateral side.
We apologize for this confusion and have changed the text to make sure the sides of the animal being referenced are appropriate. Please see lines 84-93.

LID rating scores presented does not make sense mathematically. For example, lines 273 to 275 on p 9. This calculation has been removed from the manuscript since it is not shown as representative data in this manuscript. Instead, a generic reference to the calculations using example data has been provided.

The authors should provide video clips covering all subtypes of LID and their corresponding severity scale based on their own rating system. Otherwise, it is not clear if this adds to the existing literature with some published papers with video examples.

We respectfully submit that it is not reasonable to present video clips of all degrees of all behavioral phenotypes that can be rated. That said, most of the phenotypes will indeed be presented between the animated videos that are provided, the videos with narration that the Journal will come to record in our lab, and the highly descriptive rating scale provided, thus allowing the JoVE reader to successfully use the rating system detailed in this manuscript.

Minor Concerns:
For confirmation of the lesion, videos of the tail hang and step adjusting drag test will be helpful since tail hang has not been widely used by other labs and step adjusting test has been typically done sideways for one paw at a time. We are happy to provide these videos in the upcoming set of rats that will be used for the narrated rating videos that the Journal will record when they come to our lab. In addition, brief explanations for these tests have been included together with multiple references explaining other drug-free lesion confirmatory test and variations of the tail hang and step adjusting drag test. It is important to note that these tests are included in the protocol as recommendations to the user and are not the primary focus of the protocol.

Line 155: The authors claimed that "It is important to prepare the solution for injections no more than 1 hour prior to injection" without reference or supporting data. We have now provided references for the caution statement that levodopa in an aqueous environment rapidly oxidizes in the presence of oxygen and light. See line 144-145, references 21-24.

Reviewer #3:
Minor Concerns:
This is a well written protocol that will undoubtedly be useful to researchers in the dyskinesia field. We also thank this reviewer for their laudatory comment.

I have a few concerns however, and this pertains to the high variability encountered in the rat literature. Thus, some groups commonly use a dose of benserazide of 15 mg/kg, which is not mentioned by the authors, who state that it is usually between 10 and 12. Please mention that some groups use different doses. Thank you for bringing this to our attention. References at line 97 now provide examples for other dosing regimens.

Further, the original figure 7 was broken up into two figures to allow for a stand-alone example of experimental timeline and dosing in the new Figure 1. While this timeline and dosing scheme is appropriate for many of our specific experimental questions, we point out that the LID rating approach described here can be utilized with a variety of experimental designs to allow investigators to explore their own specific questions. This protocol intentionally describes LID ratings that can be utilized under varied experimental designs to allow investigators to proceed appropriately within their specific hypotheses.

Regarding the drug-free tests to assess lesion severity, please mention that there are other available tests as well, for instance the forepaw adjusting stepping test or the cylinder test. Thank you for this suggestion. We have now added a comment to this nature, and references have been provided at line 65.

Lastly, please also mention that dyskinesia assessment is limited to "AIMs duration", which is standard in the literature, but nevertheless does not encompass the "amplitude" rating. Thank you for this astute comment. We have revised the discussion to include the AIMs terminology and outlined

specifically, with references, how this protocol relates to previously published materials. Please see lines 260-284.

Perhaps the title should be revised as well, and the word "standardized" dropped, as it is possible that this assessment of dyskinesia will be standardized within the authors' lab, but it is hard to conceive how other labs would use this approach, if they have been using for instance 10/15 or 12/15 l-dopa/benserazide for years. To incorporate this comment, the title has been changed to “Induction and Assessment of Levodopa-induced Dyskinesias in a Rat Model of Parkinson’s Disease”. We want to clarify though, that we agree that other labs will likely not want to change their dosing and are not trying to suggest that they would need to do so. Indeed, the approach to induce LID and the rating scale presented here can be employed using a variety of levodopa and benserazide doses.

X mg L-DOPA/1 kg = X mg L-DOPA/(sum body weight (BW) of all rats in study: *e.g.*, 30 rats @ 400 g each = *12,000 g)

Target dose: 12 mg/kg or 12 mg/1000 g

12 mg L-DOPA/1000 g BW = X mg L-DOPA/*12,000 g BW

X = (12 mg L-DOPA)(12,000 g BW)/1000 g BW

X = (12 mg L-DOPA)(12,000 g BW)/1000 g BW

X = 144 mg L-DOPA

Target injection volume: 1 cc/1000 g BW (with 12 mg/L-DOPA per cc)

12 mg L-DOPA/ 1cc = 144 mg L-DOPA/ X cc sterile saline

(X cc) (12 mg L-DOPA/ 1 cc) = 144 mg L-DOPA

X = 144 mg L-DOPA/12 mg L-DOPA

X = 12 cc sterile saline

Daily stock solution: 144 mg L-DOPA/12cc saline

Supplementary File 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);210802 KSC JoVE Supp Figure 1.pdf

DATE: TIME:	Experiment Title:								Rating Timepoint:			
LD DOSE:												
ANIMAL ID:	A		B		C		D		E		F	
	I	F	I	F	I	F	I	F	I	F	I	F
DYSTONIA												
NECK	Neck		Neck		Neck		Neck		Neck		Neck	
TRUNK	Trunk		Trunk		Trunk		Trunk		Trunk		Trunk	
R FOREPAW	RFP		RFP		RFP		RFP		RFP		RFP	
R HINDPAW	RH		RHP		RHP		RHP		RHP		RHP	
HYPERKINESIA												
OROLINGUAL	Oroling		Oroling		Oroling		Oroling		Oroling		Oroling	
RFPD	RFPD		RFPD		RFPD		RFPD		RFPD		RFPD	
HEAD BOB	HB		HB		HB		HB		HB		HB	
CCL	CCL		CCL		CCL		CCL		CCL		CCL	
	LID=		LID=		LID=		LID=		LID=		LID=	
MISC Behaviors												
SNIFFING	Sniff		Sniff		Sniff		Sniff		Sniff		Sniff	
EXPLORING	Explore		Explore		Explore		Explore		Explore		Explore	
REARING	Rear		Rear		Rear		Rear		Rear		Rear	
REST/SLEEP	R		R		R		R		R		R	
GROOM	Groom		Groom		Groom		Groom		Groom		Groom	
EAT/DRINK	Eat		Eat		Eat		Eat		Eat		Eat	
REARS												
ROTATION	ccw	cw	ccw	cw	ccw	cw	ccw	cw	ccw	cw	ccw	cw
NOTES												

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