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Implementation of improved postoperative care decreases the mortality rate of operated mice after an abundant 6-hydroxydopamine lesion of nigrostriatal dopaminergic neurons

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Summary

A mouse model of Parkinson's disease with an abundant lesion of nigrostriatal dopaminergic neurons can be achieved by stereotactic injection of 6-hydroxydopamine into the medial forebrain bundle. However, postoperative mortality can be excessively high without intensive postoperative care. Here, we show that improvements in stereotactic operations and postoperative care result in significant benefits for both animal well-being and research efficiency. Adopting a wide combination of mostly previously described improvements resulted in a decrease of postoperative mortality from 71% to 14% and an increase in successful abundant dopaminergic lesions from 46% to 81%. The techniques adopted are described in detail. In addition, we describe a simple protocol for gradual preoperative handling which can be utilized to decrease animal stress, aggressive and aversive behaviors, and to facilitate postoperative care and other subsequent handling. We propose that the implementation of these improvements greatly decreases the risk of animal suffering and that the improvements are worth adopting in any research group utilizing abundant 6-hydroxydopamine-induced dopaminergic lesions in mice. Suggestions for further improvement are also presented.

Introduction

Various ways exist to establish rodent models of Parkinson's disease, a neurodegenerative motor disorder caused by the death of dopaminergic neurons that have their cell bodies in the substantia nigra pars compacta (SNc) and project into the dorsal striatum. Neurodegeneration and motor symptoms of Parkinson's disease can be mimicked in animals by degenerating these nigrostriatal neurons with different neurotoxins, administered either systemically or intracranially, or by genetic manipulations (*Bové & Perier, 2012*). Importantly, different methods can cause different degrees of neurodegeneration, which affects not only the severity of the parkinsonian symptoms but, in the case of intracranial neurotoxin injection models requiring stereotactic surgery, also the required intensity of postoperative care. Particularly in the case of abundant lesions, the time and commitment needed for postoperative care may surprise researchers new to the method.

This article focuses on a mouse model utilizing abundant unilateral nigrostriatal lesions, where the neurotoxin 6-hydroxydopamine (6-OHDA) is inject-

ed into the medial forebrain bundle (MFB) of one brain hemisphere. When successful, this method of lesioning causes a loss of over 90% of dopaminergic neurons in the ipsilateral SNc (*Bové & Perier, 2012*). However the model leads to a transient severe disturbance in motor coordination which can significantly hinder the recovery of operated animals. In order to consistently achieve successful and abundant dopaminergic neurodegeneration, along with low postoperative mortality, we have during the past several years made significant efforts to improve the MFB 6-OHDA lesion procedure as well as the postoperative care.

The multiple adopted improvements can be divided into three main categories which are 1) preoperative handling and care 2) operation parameters and 3) postoperative care. Preoperative handling decreases the experience of stress in mice and facilitates overall handling related to e.g., postoperative care, drug injections and behavioral tests. Improvements in operation parameters during surgery were adopted, based on published methods(*Thiele et al., 2011*), to improve the hit rate to the correct brain area as well as to minimize damage to other brain areas. Most importantly, a broad combination of improvements in postoperative care, adapted from descriptions in various previous studies, greatly increased the proportion of surviving animals.

Here, to provide a collated technical description of the various available improvements, we describe in detail how to successfully conduct a stereotactic 6-OHDA injection into the mouse MFB, how to offer appropriate postoperative care, and how to facilitate handling and increase well-being with preoperative handling. We also show that adopting these improvements resulted in statistically significant benefits for both animal well-being, decreasing average postoperative mortality from 71% to 14%, and research efficiency, with the proportion of successfully lesioned mice increasing from 46% to 81%.

Materials and Methods

Animals

Due to potential bias related to genetic manipulations, only studies conducted with C57BL/6J mice from a commercial breeder (Harlan Netherlands, Horst, Netherlands) or wild type mice of genetically modified strains with a C57BL/6J background (maintained in The Laboratory Animal Centre or Neuroscience Center and Institute of Biomedicine, University of Helsinki, Helsinki, Finland) were included in this study. Both sexes were used, but the use of female mice was preferred to avoid penile prolapse complications. Separate studies had different numbers of animals with different age and body weight distributions. When possible, aged and thus more weighty mice were used to lower the impact of postoperative weight loss. Mice obtained from the commercial breeder were allowed at least several weeks of acclimatization before the initiation of any experimental procedures. Detailed information about the experimental animals is given in Table 1.

All mice were maintained in pathogen-free conditions according to FELASA 2014 recommendations (*Mähler et al., 2014*) and housed in individually ventilated plastic cages (GM500; cage dimensions,W x D x H, 391 x 199 x 160 mm; Tecniplast, Buguggiate, VA, Italy) with half of the cage covered by a wire bar food hopper. For enrichment, bedding (aspen chips, 5 x 5 x 1 mm, 4HP, Tapvei, Paekna, Harjumaa, Estonia), nesting material (aspen strips, PM90L, Tapvei, Paekna, Harjumaa, Estonia) and a brick (aspen brick, 100 x 20 x 20 mm, Tapvei, Paekna, Harjumaa, Estonia) were placed in the cages. Mice were

Table 1. Detailed information on the mice subjected to intra-MFB 6-OHDA injections.

Study group	1	2	3	4	5	6	7	8
N (total)	28	17	6	15	10	20	12	14
Age (weeks)	13–26	15–17	15	28	20–23	30	10–20	17–21
Weight (grams)	20–33	22–29	18–26	23–31	20–25	21–36	21–24	20–28
Gender	Both	Females	Both	Females	Females	Females	Females	Females
Strain	C57BL/6J	C57BL/6J	C57BL/6J	C57BL/6J	C57BL/6J	C57BL/6J	C57BL/6J	C57BL/6J
Genotype	α5 +/+	WT	α5 +/+	WT	α5 +/+	WT	α5 +/+	HDC +/+
Source	LAC	HA	LAC	HA	LAC	HA	LAC	NC

α5 = alfa5 nicotinic acetylcholine receptor subunit
HDC = histidine decarboxylase
HA = Harlan Netherlands, Horst, Netherland
LAC = Laboratory Animal Centre, University of Helsinki, Helsinki, Finland
NC = Neuroscience Center and Institute of Biomedicine, University of Helsinki, Helsinki, Finland

kept in groups of three to six (with the exception of keeping males singly or in pairs when unavoidable due to fighting) under a 12/12h light-dark cycle with lights off at 18:00. The mice had free access to standard food pellets (Harlan Teklad 2916C; Harlan, Indianapolis, IN, USA) and filtered, UV-irradiated water. The ambient temperature was held at +23±2 °C and the relative humidity at 50±15%. Animal experiments were conducted according the 3R principles of the EU directive 2010/63/EU governing the care and use of animals used for scientific purposes, and subsequent local laws and regulations [Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013, Government Decree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013)]. Study protocols were authorized by the national Animal Experiment Board of Finland (licence numbers ESAVI/198/04.10.07/2014, ESAVI/431/04.10.07/2015 and ESAVI/441/04.10.07/2016).

Preoperative handling and care

Introduction of preoperative handling was initiated with study group 2 and subsequently conducted with all animals. Handling of mice, aimed at reducing the stress of animals and at facilitating postoperative care and future handling, was initiated two to three weeks before surgery and performed gradually within three to four days. The researcher used the same protective overalls on each day to familiarize the animals to the researcher's odor.

On the first day of the preoperative handling the aim was to introduce the researcher to the mice and to apply tail marks with a marker pen. The mice were allowed to sniff the researcher's gloves, first while remaining under the nesting material and subsequently with the nesting material removed. Concurrently, the researcher talked quietly to familiarize the mice with the researcher's voice. The mice were lifted by their tail one at a time onto the researcher's hand, which remained in the cage to allow the mice to freely jump away.

On the second day, the actions described above were repeated, and additionally the animals' weights were measured and a new handling protocol was introduced. Up to five mice were lifted onto the researcher's arm while standing close to the home cage; the mice had the freedom to return to the home cage or sniff and explore the researcher's arm.

On the third day, the same protocol was performed as the day before, but before lifting the animals from the tail to the researcher's hand, the researcher tried to move the mice by lifting from the body: the researcher put his/her hand gently under the mouse, so that the mouse was against the wall of the home cage, and the mouse was lifted gently with the hand underneath the body.

On the last day, the same protocol was performed as described before, but the mice were no longer lifted from the tail. Additionally, petting (gently stroking the head and sides with a finger) was initiated if the mice allowed it. In subsequent study phases, after the mouse was habituated to non-tail lifting, it was always handled and transferred by lifting from underneath the body, unless impossible due to circumstances of the experiment being performed.

Additional preoperative actions were performed in preparation for the operation itself and postoperative care. During preoperative handling (2–3 weeks before surgery) a high calorie dietary supplement (Bacon softies; Bio-Serv, Flemington, NJ, USA) was introduced, the standard nesting material (aspen strips) replaced with soft cotton pads (Nestlets, Article ref. 14010, Plexx, Elst, Netherlands), and a small plastic house (Mouse House, Tecniplast, Buguggiate, VA, Italy) added to every cage for additional enrichment. During each study there was one responsible researcher assigned who took care of conducting the experiments but also monitoring the animals, changing the animal cages (once a week) and confirming that sufficient food was available. When necessary, another researcher assisted the responsible researcher but never replaced that person.

Stereotactic operation

The 6-OHDA MFB lesion procedure described below was developed on the basis of previously published methods (*Lundblad et al., 2004; Thiele et al., 2011*). A premedication of desipramine (25 mg/kg, i.p.) was administered in certain studies (for more details, see Table 4) 30 minutes prior to injection of 6-OHDA to decrease 6-OHDA-induced damage to noradrenaline and serotonin neurons. Buprenorphine (0.1 mg/kg, i.p) was administered for pain relief 5 minutes prior isoflurane anesthesia (4% induction, 0.5–2% maintenance, individually adjusted). The mouse was then positioned into a stereotactic frame (Stoelting Co, Wood Dale, IL, USA), the head was shaved, and an incision was made after applying lidocaine local anesthesia. When the skull was exposed, a 10 µl syringe (NanoFil, World Precision Instruments Inc., Sarasota, FL, USA) with a 33 G needle was filled with fresh 6-OHDA-solution (15 µg/µl, except in the first trial the concentration was 3 µg/µl) and covered with aluminum foil. The needle was placed at the Bregma,

the Bregma was marked, and the needle moved to the Lambda. If the D/V difference between the Bregma and Lambda was greater than ± 0.2, the position of the animal's head was adjusted and the Bregma and Lambda checked again. The needle was then placed at the following coordinates from Bregma: A/P -1.2 and M/L -1.1. A hole was made through the skull using a drill (Foredom, Stoelting Co, Wood Dale, IL, USA) and the needle inserted into the medial forebrain bundle at D/V -5.0. The injection volume was 0.2 µl with a speed of 0.1 µl/min (except in the first trial the volume was 1 µl with a speed of 0.5 µl/min), resulting in administration of 3 µg 6-OHDA in total. After the injection, the needle was left in place for 5 minutes and then slowly retracted during 2 minutes. The wound was closed by two to three stitches and 0.5 ml of sterile and warm saline (NaCl 0.9%) was delivered subcutaneously. Carprofen (5 mg/kg, s.c.) was administered for pain relief after the operation, and the mouse was taken off the stereotactic frame and placed in a warm recovery cage until regaining consciousness. For further pain relief, buprenorphine was re-administered 6 h after surgery and carprofen re-administered 20–24 h after surgery.

Postoperative care

Following surgery, the mice received 14 days of daily intensive postoperative care (for a summary, see Table 2). If the mice showed signs of hypothermia (shaking and still), the cage was placed on a heating pad and kept there 4 to 6 hours, taking care to use a low level of heating to provide a warmer cage while avoiding hyperthermia. Small plastic houses and soft nesting material (cotton pads), already added preoperatively, were also used to mitigate hypothermia. Mice received 1 ml injections (s.c.) of sterile and warm saline twice daily (maximum 10 days) to mitigate dehydration, and carprofen injections if they showed signs of pain (e.g., vocalization, piloerection, ungroomed appearance, aggression, lack of group behavior, abnormal posture or shaking, immobilization, sunken eyes; *National Research Council Committee on Recognition and Alleviation of Pain in Laboratory Animals, 2009*). High calorie dietary supplements, Bacon softies (Bio-Serv, Flemington, NJ, USA), Nutrigel (Virbac, Carros, France) and Nutriplus Gel (ClearH2O®, Portland, ME, USA) were provided to compensate for difficulties in eating and weight decrease. Body weight and behavior (signs of dehydration, activity, eating, drinking) were systematically monitored using a specific welfare scoring table (Appendix 1).

If needed, the mice were fed by hand twice daily with water provided directly into the mouth via a 1 ml syringe (for video material, see Appendices 2 and 3). In some cases a more active and forceful feeding was necessary. This was achieved by holding the mouse in an intraperitoneal injection position (grasping from the neck scruff and lifting the belly towards the researcher) and approaching the mouth with a tiny spoon filled with Nutri-plus Gel. The mouth was touched with the spoon and eating was monitored. When feeding in this way, particular attention should be paid to ensure that the angle of the spoon is optimal with respect to the tongue and jaw movements of the mouse.

Genitals of male mice were checked every day in order to detect any signs of developing penile prolapse (redness and swelling of the penis). If penile prolapse was observed, it was immediately treated by rinsing the genital area with sterile warm water, applying honey-based wound care ointment (Vetramil, FaunaPharma, Espoo, Finland) and gentle massage of the bladder area. Despite intensive postoperative care, some individual mice did not recover and needed to be sacrificed based on humane endpoint criteria described in Table 3 and Appendix 1.

Data analysis

Mice that underwent the 6-OHDA lesion surgery were divided into the following categories: Alive (mice that were successfully lesioned and survived the postoperative period), Dead (mice that died during postoperative care), Dead in Surgery (mice that died during the operation), Unlesioned (mice that were unsuccessfully lesioned). Lesion success was determined *post mortem* on the basis of tyrosine hydroxylase (dopaminergic neuron marker) immunostaining of the substantia nigra pars compacta, performed after a variable time from surgery (ranging from 1.5 to 6 months) depending on the specific study in question.

The postoperative mortality rate (%) was calculated as the ratio of mice that died during postoperative care vs. all successfully lesioned animals (Dead / Alive + Dead). Mice that died during surgery and unsuccessfully lesioned mice were not included. The rate of successful lesioning (%) was calculated as the ratio of successfully lesioned mice vs. all mice that survived (Alive / Alive + Unlesioned).

The statistical significance of the differences in the postoperative mortality rate and the rate of successful lesioning before vs. after the introduction of improvements was investigated with Pearson's Chi-Square tests.

Table 2. Improvements in utilization of the medial forebrain bundle 6-hydroxydopamine mouse model.
Actions during different experimental phases before and after the introduction of the improvements.

Phase	Before improvements	After improvements
Whole study		
Participants	Several researchers conduct the experiments Animal caretakers perform cage changes and feeding	Designated researcher responsible for carrying out the whole experiment The responsible researcher performs cage changes and feeding
Preoperative		
Mice - Age - Sex	 Young mice preferred Both sexes	 Aged and weighty (bodyweight not less than 20 g) mice preferred Female mice preferred
Nesting material	Woody nesting material	Woody nesting material replaced with soft nesting material One plastic house placed into the home cage
Housing	Housed randomly	Group housed whenever possible
Handling	No handling by the researcher before operation	Gradually proceeding handling protocol to habituate the mice to the researcher
During surgery		
Isoflurane anesthesia	Isoflurane kept at 1.5 – 2% as regularly advised	Isoflurane kept as low as possible (0.5 – 2%) without reappearance of reflexes
6-OHDA infusion volume 6-OHDA infusion speed Needle in place	2 µl 0.5 µl/min Needle left in place for 2 min after the infusion	Decreased infusion volume: 0.2 µl Slower infusion speed: 0.1 µl/min Needle left in place longer: 5 min
Postoperative care		
- Duration	Care provided for 1–2 weeks during weekdays only	Care provided for 14 successive days, also during weekends
- Welfare checks	Welfare not assessed or recorded systematically	Welfare scored daily with a specific table
- Nutrition	Softened laboratory standard food placed on the bottom of the cage	Softened standard food covered with Nutri-plus gel and placed in a cup on the bottom of the cage High-calorie supplement (Bacon Softies) on the bottom of the cage Hand-feeding with Nutri-plus gel twice a day Drinking water offered via 1 ml syringe twice a day
- Body temperature	No action	Hypothermic mice kept in a warmed cage 4–6 h daily
- Rehydration	Warm and sterile saline and/or glucose delivered s.c. once a day when necessary	Warm and sterile saline (1 ml) delivered s.c. 1–2 times a day for max. 10 days
- Penile prolapse	No proactive actions to avoid penile prolapse	Genitals of male mice checked daily and signs of penile prolapse treated immediately

Table 3. Determination of humane endpoints for MFB-lesioned mice. The mice were monitored daily for 14 successive days after the operation and offered intensive postoperative care. In addition to an immediate endpoint of over 25% loss of weight, systematic scoring of well-being was performed using a welfare scoring table to assess eating, drinking and activity, with mice euthanized if a set score was exceeded (see Appendix 1).

Weight monitoring (immediate endpoint)
Over 25% loss of weight
Eating and drinking (included in welfare scoring)
Mouse is not eating when hand-fed
Mouse is not drinking when watered via syringe
Mouse is dehydrated: skin is not retracted following skin pinch and eyes are sunk in the head
Activity (included in welfare scoring)
Mouse is not moving spontaneously, frozen and/or shaking

Results

Effects of preoperative handling and care

The effects of the gradual handling protocol described above were not systematically investigated, but the introduction of the preoperative handling led to what appeared to the researchers to be obvious and significant reductions in - or even complete abolishment of - aggressive and escape behaviors such as biting, jumping, vocalization and general aversion towards the researcher, as well as to greatly facilitated subsequent handling due to voluntarily approaching the researcher and accepting physical restraint. See Appendix 4 for video material (mice previously handled with the above protocol) supporting the above observations. Furthermore, urination and defecation when handled, a measure of stress and anxiety in mice (*Hurst & West, 2010*), were markedly decreased or even abolished.

Preoperative handling proved particularly useful in facilitating the postoperative care procedures requiring fine coordination such as injection administration and hand feeding and watering (see Appendices 2 and 3 for video of hand feeding and watering). In addition, preoperative handling facilitated all later stages of the individual studies, allowing easy performance of procedures such as drug administration and behavioral experiments as well as quick and stress-free euthanasia by cervical dislocation.

Postoperative mortality and lesion success

A timeline for the introduction of the different improvements is shown in Figure 1. Table 4 lists the postoperative mortality and the proportion of successful abundant dopaminergic lesions within the different study groups as well as the average before and after improvement introduction. Average postoperative mortality was greatly reduced after the introduction of the systematic welfare scoring along with the other improvements (between studies 1 and 2), decreasing from 71% to 14%. The difference in postoperative mortality was statistically highly significant ($\chi^2(1) = 27.8$, $P = 1.3E\text{-}7$, Pearson Chi-Square test). Euthanized mice accounted for most of the mortality. Before the implementation of the improvements, a few spontaneously dying (i.e., not euthanized) mice were observed during the period of postoperative care; after the implementation, spontaneously dying mice were very rare (one animal in total). Concurrently, improvements in surgical procedures increased the average rate of successful abundant lesions from 46% to 81%. The difference in successful lesions was statistically significant ($\chi^2(1) = 7.45$, $P = 0.006$).

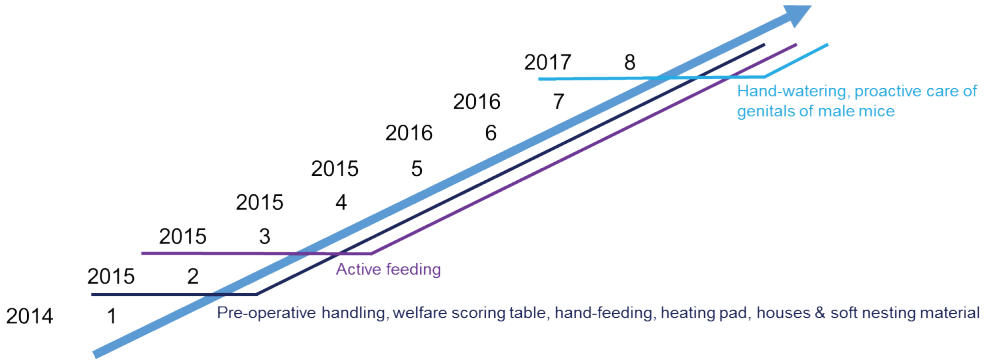

Figure 1. Timeline of the different improvements introduced. Individual studies are listed in chronological order on the left side of the arrow and improvements are listed on the right side of the arrow.

Table 4. Significant decrease in postoperative mortality after the introduction of welfare scoring and other improvements. Alive = only successfully lesioned mice included, Dead = died during postoperative care, †Surgery = died during operation, Unlesioned = based on tyrosine hydroxylase immunostaining of the substantia nigra pars compacta. Postoperative mortality% = Dead / Alive + Dead, Successfully lesioned% = Alive / Alive + Unlesioned

Study group	Alive	Dead	†Surgery	Unlesioned	n (total)	Postoperative mortality (%)	Successfully lesioned (%)	Exceptions in surgery protocol
1A	2	11	0	3	16	85	40	Desipramine, infusion volume and speed
1B	4	4	0	4	12	50	50	Desipramine
Total - Before improvements	6	15	0	7	28	71	46	
2	9	2	2	4	17	18	69	
3	3	1	0	2	6	25	60	Desipramine
4	12	1	0	2	15	8	86	
5	5	2	1	2	10	29	71	
6	17	1	0	2	20	6	89	
7	9	2	1	0	12	18	100	Desipramine
8	9	1	1	3	14	10	75	
Total - After improvements	64	10	5	15	94	14	81	

Discussion

Modeling late-stage Parkinson's disease in mice can be challenging, as an abundant toxin-induced nigrostriatal lesion often leads to transient but severe motor disturbances, even in the case of the typically utilized unilateral lesions that affect only one brain hemisphere. Reaching acceptable levels of postoperative survival can thus require quite intensive care. Here, we have described the technical details and impact of improvements in postoperative care and other techniques related to the MFB 6-OHDA mouse model that have been adopted in our research group during the past several years. The improvements consisted of a combination of mostly previously described techniques (see Table 5), and as shown have markedly increased the proportion of surviving animals. The postoperative care and other methodology described here thus comprises a stable and reliable combination of methods to achieve significant improvements in the proportion of surviving animals as well as the well-being of the animals. However, it must be stressed that refinement of animal experiments is a continuing process, and many possibilities for further improvement remain.

The achievement of these results is likely to be due to the sum of all improvements made rather than any specific improvement. While direct comparison of survival rates with previously reported MFB model mouse studies is difficult, as a number of studies have not described in detail the methods used for postoperative care or humane endpoints (e.g., the maximum loss of weight allowed) or the specifics of how reported survival and/or mortality rates were calculated, the achieved average survival rate of 86% closely resembles the survival rate reported recently by a number of other groups utilizing multifaceted postoperative care (Table 5). However, as 100% survival rates after an abundant dopaminergic lesion have been reported by at least one research group (*Francardo et al., 2011; Sebastianutto et al., 2016*), further improvement remains possible. Note also that while postoperative survival is obviously critical for research purposes and easy to quantify, other measures of animal well-being could allow for the evaluation of further and possibly more subtle refinement. Each of the different improvements described here, as well as some opportunities for further improvement, will next be discussed.

Table 5. Descriptions of postoperative care and survival rate after MFB 6-OHDA lesions in mice from selected methodological articles. ND = not described

Reference	Hand-feeding	Supplemental nutrition	Rehydration	Monitoring well-being, criteria for sacrifice	Treatment of penile prolapse	Ensuring sufficient body temperature	Duration of postoperative care	Group housing	Survival rate (6-OHDA dose)
Lundblad et al., 2004	ND	ND	ND	ND	ND	ND	ND	ND	18% (3 µg)
Cenci & Lundblad 2007	Yes	Food soaked in sugar/water solution	Glucose-saline solution (s.c.) daily	Weak mice separated from healthy mice. Mice that remain weak and drowsy sacrificed.	ND	ND	2–3 weeks, 1st week daily	ND	ND
Francardo et al., 2011	Yes	Food soaked in sugar/water solution	Glucose-saline solution (s.c.) 1st week daily, continuation as necessary	Weaker mice separated from healthy mice.	ND	ND	1–3 weeks, 1st week daily	ND	80–100% (3.2 µg)
Thiele et al., 2011	ND	Nutrigel, kitten milk replacement, sugared water	Lactated Ringer's solution (s.c.) 1 week or until improvement	Weight, movement, food and water intake and presence and consistency of fecal matter monitored.	Lubricating jelly to the penis, palpitation of the bladder and rehydration.	ND	2 weeks daily	Yes	82% (3 µg)
Glajch et al., 2012	ND	Pediasure	Saline (s.c.) daily	Grooming and overall appearance monitored.	ND	Continuously on a heating pad (35 °C)	ND	ND	85% (2.5 µg)
Heuer et al., 2012	ND	Wet food	Glucose-saline solution (s.c.) 2 weeks	Weight monitored, if below 85 % need for euthanasia evaluated	ND	ND	2 weeks	Yes	83% (6 µg)
Heuer et al., 2013	ND	Wet food	Glucose-saline solution (s.c.) 3 times daily	Body weight monitored	ND	ND	2 weeks	Unclear	93% (6 µg)
Boix et al., 2015	Yes	Food soaked in sugar/water solution, DietGel Boost	Glucose-saline solution (s.c.)	ND	ND	ND	2 weeks	ND	80% (3.6 µg)
Sebastianutto et al., 2016	ND	DietGel Boost	Glucose/ Ringer acetate solution (s.c.) 2–3 weeks	ND	ND	Overnight in warm ventilated cabinet (30 °C) for 1 week	2–3 weeks	ND	100% (3.2 µg)

Selection of animals for the study should naturally be primarily based on scientific justifications. However, in our experience as old and weighty mice as possible should be chosen when justified, because the higher starting weight confers partial protection from the transient but marked weight decrease typically associated with an abundant nigrostriatal lesion. In addition, it may be preferable to select female mice, as they are not affected by the penile prolapse complications which can affect male mice after a severe dopaminergic lesion (*Thiele et al., 2011*) and can also be more easily group housed due to less fighting. It should naturally be kept in mind that the study design may prevent the inclusion of only female mice due to possible gender-specific confounds such as the estrus cycle.

Animals were group housed whenever possible, not only due to stress caused by social isolation, but also to mitigate hypothermia. Nesting material was changed from woody material to soft material, and plastic houses provided, in an effort to mitigate hypothermia. The soft nesting material is also easier for the mice to manipulate even in a weakened state. It should also be mentioned that the regular aspen strip nesting material in use at our facilities has a tendency to be become coated with the gel-like dietary supplement materials used in postoperative care and get stuck to the mice, sometimes even causing constrictive injury. All changes to the housing conditions were performed 2–3 weeks before surgery to allow habituation. Further improvement could be achieved by introducing the housing changes even earlier, ensuring full habituation.

Related to the entire study process, we have aimed at assigning one primary researcher to be responsible for the entire experiment, with assistance from other researchers when necessary. In particular, during the experiment (beginning from preoperative handling) the primary researcher is responsible for all handling of the animals e.g., during cage changes. While we have not systematically studied the effects of handling by one vs. several persons, we suggest that this is likely to increase the animals' habituation to the researcher's smell, voice and handling practices and thus to reduce the stress the mice experience.

The introduction of the gradual handling protocol appeared to be very effective in reducing aggressive and anxiety-related behaviors in the mice and facilitating subsequent handling as well as postoperative care. Nevertheless, the handling protocol could surely be further improved. One possibility for further improvement could be to begin non-aversive (non-tail) handling immediately on day one, instead of beginning with tail handling. Another improvement would be to avoid the disruption of nests during handling.

A limitation of the present study is that the effects of preoperative handling were not systematically studied. However, for a demonstration of the effects of the handling protocol see the included video material (Appendix 4), where a distinct lack of aversive behaviors directed towards the researcher as well as markedly easy handling can be observed. See also Appendices 2 and 3 demonstrating that the pre-handled mice accept hand-feeding and watering after surgery. Also note that very similar pre-experimental handling has been previously described by Fridgeirsdottir *et al.* (*2014*), who observed improved performance and less variability in handled mice in the Morris water maze task, likely due to reduced stress and anxiety. Different handling methods have also been previously studied in mice and found to affect the voluntary interaction with the researcher even with brief exposure. The common method of picking up and restraining the mouse by its tail was found to induce aversion, high anxiety and no habituation even after many handling sessions, while non-aversive handling methods such as lifting the mouse with cupped hands (similar to our method of handling) or using a tunnel resulted in low anxiety, voluntary approach and acceptance of restraint (*Ghosal et al., 2015; Gouveia & Hurst 2017; Hurst & West 2010*). Our observations of greatly reduced anxiety-like and aversive behaviors are thus in full concordance with previous literature. In addition, utilizing a combination of "massage" (resembling our petting technique) and non-aversive handling was found to reduce stress-related plasma corticosterone increases in response to a novel environment (*Ghosal et al., 2015*). This finding suggests that the reductions in aversive behaviors observed – in the literature as well as in the present study – are mirrored by a reduced physiological stress response.

Our improvements to the surgical protocol included lower isoflurane maintenance anesthesia (0.5 – 2%) as well as decreased 6-OHDA infusion volume and speed. The use of a lower isoflurane concentration was adopted to promote faster recovery from anesthesia. It is critically important, however, that deep enough anesthesia is maintained to ensure no experience of pain. The depth of anesthesia (e.g., absence of reflexes) must therefore be carefully monitored at all times. The reduced 6-OHDA infusion volume and speed were adopted to minimize damage to structures close to the MFB, such as the hypothalamus which regulates feeding and drinking behavior (*Thiele et al., 2011*). These improvements were also likely to contribute to the significantly increased pro-

portion of successful lesions, which in turn allows for a reduction in the total number of animals needed.

Perhaps the most critical steps taken with respect to the increased postoperative survival rate were improvements in postoperative care. Intensive postoperative care is crucial and should be applied daily for at least two weeks. Care should naturally be continued for longer if needed – however, we suggest that a cut-off time be considered if mice continue to require intensive care for longer than two weeks. Note also that even more frequent (e.g., twice daily) observation and care may be necessary, particularly if spontaneously dying animals are encountered.

Unilateral lesioning of the MFB leads to severe but transient motor coordination problems due to the massive dopaminergic cell death affecting one hemisphere. Thus, obvious problems in motor control of tongue, chin and forepaws can often be observed, and in practice it appears that the animals need to relearn how to use one side of their body after an abundant lesion. It should be stressed, however, that despite the near-total nigrostriatal lesion of one brain hemisphere, surviving animals regain their gross motor coordination abilities after two or more weeks of intensive care and are able to move, eat and drink as normal, with remaining parkinsonian symptoms typically revealed only by specific behavioral tests or under pharmacological stimulation.

Postoperative supplementary nutrition is essential to ensure adequate food intake, and survival, in the most severely affected mice. We have used several types of commercial high-energy palatable food pellets and gels, with a number of other commercial products used successfully by others (see Table 5). The supplementary foods were introduced before surgery to habituate the animals. During postoperative care, supplementary food gels were also hand-fed to animals showing severe difficulties in eating and drinking or, often, to all animals. Drinking water was also hand-fed, and warm saline injected daily to further mitigate dehydration. Note that others have also successfully used saline/glucose or Ringer solution injections for hydration (see Table 5). Further essential improvements in postoperative care included careful monitoring for symptoms of hypothermia and keeping hypothermic mice in a warmed cage, and careful monitoring of genitalia of male mice to enable treatment of any developing penile prolapse before it reached a severe stage. Further improvement could be achieved by keeping all operated mice at constant thermoneutrality (30 °C for mice; *Fischer et al., 2018*) using more advanced thermostat-regulated heating devices, instead of utilizing non-regulated heating pads and only for limited amounts of time per day.

It is necessary to emphasize that every mouse used is an individual and it is highly important to observe them individually. A successful abundant nigrostriatal lesion leads most notably to transient weight loss. However, while weight loss is an indicator of well-being, and often utilized as an objective humane endpoint criterion, the entirety of the behavior and appearance of the animal should be taken into account when assessing well-being. At times, it can be very clear that the mouse exhibiting the most severe weight loss (as % of initial weight) is not the mouse suffering the most. For instance, while in our studies an animal that reaches a weight loss of more than 25% of initial weight is sacrificed, as stipulated by our animal use permit, in the same cage there might be an animal that remains immobile and is obviously suffering despite having lost only 15–20% of its weight. To facilitate the monitoring of animal well-being we have therefore developed a systematic method using a specific welfare scoring table (Appendix 1). This is used to record animal weight and behavior and to determine whether a humane endpoint has been reached for each individual animal. It should also be noted that opportunities for further improvements in humane endpoints and the monitoring and assessment of individual animal condition (including the welfare scoring system) undoubtedly exist. Additional improvements could include systematic consideration of gradual vs. precipitous weight loss, measurements of food and water intake, monitoring of breathing, or following body temperatures with methods such as subcutaneously implanted RFID (radio-frequency identification) chips or infrared thermometry (*Mei et al., 2018*). Note, however, that changes in motor activity after surgery should in general not be considered as humane endpoints (excluding dramatic changes such as being frozen or shaking), given that motor dysfunction is an essential feature of the parkinsonian animal model and can be particularly but temporarily pronounced during the postoperative period, including in animals that will fully recover. On the other hand, re-establishment of normal motor activity such as nest building could be considered a sign of recovery.

In conclusion, in this article we have provided a collated technical description of a number of mostly previously described improvements related to the utilization of the MFB 6-OHDA mouse model of Parkinson's disease. We show that the implementation of these improvements resulted in significant increases

in both postoperative survival and successful abundant nigrostriatal dopaminergic lesions. We suggest that this combination of improvements also greatly decreases the risk of animal suffering and is worth adopting in any research group utilizing or planning to utilize the MFB 6-OHDA model. Finally, we stress that refinement of animal experiments is a continuing process, and there remain many opportunities for further improvement – both those few suggested above as well as probably many others.

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Appendices

Appendix 1. PDF: Welfare scoring table

Appendix 2. Video material: Delivery of supplemental food by hand

Appendix 3. Video material: Delivery of drinking water by hand

Appendix 4. Video material: Behavior of mice handled previously with the gradual handling protocol