Protocol for the MPTP mouse model of Parkinson's disease

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This protocol describes our method of producing a reliable mouse model of Parkinson's disease (PD) using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). We discuss the particulars of the model, provide key references and outline what investigators need to know to develop the MPTP mouse model of PD safely and successfully. Completion of this protocol depends on the regimen of MPTP used and on the actual planned studies, which often range from 7 to 30 d. This protocol calls for implementation of safety measures and for the acquisition of several pieces of equipment, which are a one-time investment worth making if one elects to use this model on a regular basis.

INTRODUCTION

Parkinson's disease (PD) is the second most frequent degenerative disorder after Alzheimer's disease. Clinically, its cardinal features include tremor, muscle rigidity, slowness of voluntary movement and postural instability[1]. Although PD neuropathology encompasses a number of different neurotransmitter pathways, the disabling manifestations cited above are attributed primarily to a deficit in brain dopamine[2]. Among the different dopaminergic systems of the brain, the ascending nigrostriatal pathway has been consistently identified as the most severely damaged in PD[2]. In most instances of PD, however, neither the cause nor the mechanisms underlying the death of the nigrostriatal dopaminergic neurons are known[2]. Thus, the outstanding questions with respect to the dopaminergic degeneration are why and how these specific neurons die in PD. Although investigations of post-mortem PD tissues have their place in the quest to answer these questions, they typically provide a fixed image of end-stage PD, which is often not suitable to unravel early or dynamic neurodegenerative events. To this end, PD investigators rely heavily on model systems to explore various aspects of the disease. Over the years, a variety of toxins of uncertain relevance to the cause of PD have been used as agents to destroy dopaminergic neurons[3]. This strategy is quite popular among PD researchers and is based on the premise that dopaminergic neurons may have a stereotyped death cascade that can be activated by a range of insults, including neurotoxins. These toxins have generated a large body of information regarding the molecular basis of the demise of dopaminergic neurons. So far, however, none of the validated toxic models of PD is a homolog of the disease, even though these models replicate many, but never all, of the features of PD. Thus, no single model is expected to be suitable for all studies.

Having stated this limitation, it is fair to say that among the various toxic models of PD, the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) model has become the most commonly used, for at least three reasons. First, MPTP is the only known dopaminergic neurotoxin capable of causing a clinical picture in both humans and monkeys[4] indistinguishable from PD. Second, although handling MPTP requires a series of precautions (see below), its use is not otherwise technically challenging: it does not require any particular equipment such as a stereotaxic frame, nor does it require surgery on live animals as for 6-hydroxydopamine[5] or rotenone[6]. And third, MPTP produces a reliable and reproducible lesion of the nigrostriatal dopaminergic pathway after its systemic administration, which is often not the case for other documented poisons[7]. Although MPTP monkeys remain the gold standard for the preclinical testing of new therapies for PD, most of the studies geared toward unraveling the mechanisms underlying the demise of dopaminergic neurons have been performed in mice. Therefore, we provide here a detailed protocol as well as a list of recommendations and guidelines to produce the MPTP mouse model of PD in a reliable and safe manner.

MPTP regimen and safety measures

The fact that MPTP causes parkinsonism in humans is a major incentive for using this toxin to study PD pathogenesis and, at the same time, the reason why safety measures must be so rigorous. The three most important requirements for the safe use of MPTP are personal protective gear, a negative-pressured procedure room and the proper handling, detoxification and disposal of all contaminated samples and materials. A comprehensive discussion of all of these critical aspects can be found elsewhere[8] and should be read carefully and implemented before conducting any actual MPTP experiment.

The administration of MPTP to mice through a number of different routes using different dosing regimens has led to the development of several distinct models, each characterized by some unique biochemical and neuropathological features. However, we believe that the most relevant regimens of MPTP are those that create an overt and stable lesion of the nigrostriatal pathway with the least number of undesirable side effects such as acute death, dehydration and malnutrition. Although MPTP can be given by a number of different routes, including gavage and stereotaxic injection into the brain, the most common, reliable and reproducible lesion is caused by its systemic s.c. or i.p. administration. Note that mice treated with MPTP do not develop parkinsonism, and thus the primary phenotype to be monitored in MPTP mouse studies is the nigrostriatal damage associated with gliosis, whose magnitude depends on the dose and dosing schedule[9]. Furthermore, it seems that unless MPTP is infused by osmotic minipump[10] or is administered with probenecid[11]—a uricosuric agent that decreases the renal excretion of some drugs—it will not produce

proteinaceous inclusions in spared dopaminergic neurons as seen in PD.

Proficient and safe use of an MPTP regimen comes from surrounding oneself with information about the biodisposition of MPTP and about the time course of the nigrostriatal degeneration for a given regimen found either in the literature or generated by one's own pilot work. Information about biodisposition is crucial for determining the period of strict isolation of the animals and of stringent hazardous protective measures. The time course of neuronal degeneration, on the other hand, is critical to determining when the animals must be studied. For instance, if one wishes to study an early molecular or cellular event related to the death of dopaminergic neurons, animals may have to be studied soon after the initiation of intoxication, which is before or during the active phase of neurodegeneration. In contrast, if the main goal of the study is to evaluate the number of surviving dopaminergic neurons, assurance of having reached a stable lesion is a prerequisite for obtaining trustworthy data. How to determine MPTP biodisposition and the time course of degeneration will be explained below.

Selection of the mice

It is crucial to remember that several factors influence the reproducibility of the lesion in mice[12–17]. Different strains of mice, and even mice from a given strain but from different vendors, can exhibit strikingly distinct sensitivity to MPTP, at least with respect to the loss of dopamine in the striatum. As discussed in greater detail elsewhere[8], gender, age and body weight are the factors that modulate MPTP sensitivity and reproducibility of the lesion. The most reproducible results from one experiment to another are obtained when males of at least 8 weeks of age weighing at least 22 g are purchased from the same lot and from the same vendor.

A common problem with the MPTP model is the acute death of the animals that occurs within the first 24 h after the beginning of MPTP administration. It is important to note that the acute death is unrelated to the damage in the dopaminergic systems of the brain; it is most likely due to a peripheral cardiovascular side effect. This side effect is dose dependent and more prevalent in some mouse strains and in female mice. This is often a major problem for researchers because most mice in an experiment can die before any meaningful investigations can be performed, and because those that survive may represent a particular subgroup, which may skew the results and lead to erroneous interpretations. Thus, caution must be taken with the outcome of an experiment in which more than 50% of the animals have died, especially if the proportion of acute deaths is not the same among the various experimental groups.

MPTP metabolism

The metabolism of MPTP is a complex, multistep process[2]. After its systemic administration, MPTP, which is highly lipophilic, rapidly crosses the blood–brain barrier. Once in the brain, the protoxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium by the enzyme monoamine oxidase B (MAO-B) within non-dopaminergic cells, and then (probably by spontaneous oxidation) to 1-methyl-4-phenylpyridinium (MPP^+), which is the active toxic compound. Thereafter, MPP^+ is released into the extracellular space. Since MPP^+ is a polar molecule, unlike its precursor MPTP, it cannot freely enter cells, but depends on the plasma membrane carriers to gain access to neurons such as dopaminergic neurons. MPP^+ has a high affinity for the plasma membrane dopamine transporter[18], as well as for norepinephrine and serotonin transporters. Once inside dopaminergic neurons, MPP^+ can be sequestrated into synaptosomal vesicles[19] or be concentrated within the mitochondria[20]. This complex toxicokinetics implies that a proper interpretation of results from an MPTP study will require assessment of key aspects of MPTP metabolism to ensure that the observed effects are related to a true and meaningful molecular event and not to interference with the pharmacology of the toxin. The assessment may involve, but should not be limited to, assessing striatal MPP^+ levels (discussed below) as well as MAO-B activity, lactate levels and [^{3}H]dopamine and [^{3}H]MPP^+ uptake. Protocols for these analyses can be found in references cited below.

Injection schedules

Several different MPTP dosing regimens are commonly used, according to the literature, ranging from what is commonly called an *acute* intoxication, encompassing one to multiple injections in 1 d, to what is commonly called a *sub-acute* or *chronic* intoxication regimen corresponding to one injection per day for several consecutive or non-consecutive days or even weeks. However, because MPTP has rapid toxicokinetics, the regimens called sub-acute and chronic are, in reality, neither a sub-acute nor a chronic intoxication, but merely a serial acute insult. In our opinion, the only true chronic regimen is the one produced by infusion of MPTP with osmotic pumps[10]. That being said, this regimen has not been well validated yet and its suitability for the investigation of dopaminergic neuronal death mechanisms remains to be established. Nevertheless, it is so technically distinct from any of the other published protocols of MPTP intoxication that detailed information as to how the infusion method works is given below.

One common regimen involves one injection of MPTP every 2 h for a total of four doses over an 8 h period in 1 d. Depending on the doses given, striatal dopamine depletion can range from 40% (14 mg kg^{-1} per dose × 4) to approximately 90% (20 mg kg^{-1} per dose × 4), as assayed by HPLC 7 d after the last dose of MPTP (**Fig. 1**). As we have reported previously[21], with this acute regimen, dopaminergic neurodegeneration occurs morphologically by a non-apoptotic form of death, striatal dopamine is depleted by at least 40–50% in young adult C57/bl mice, and the loss of dopaminergic cell bodies in the substantia nigra is stable (whether the same can be said for the dopaminergic fibers in the striatum is unknown) by 7 d after MPTP administration (**Fig. 2**). Another quite popular regimen is that developed by Tatton and Kish[22], which involves one injection of 30 mg kg^{-1} free base MPTP daily for five consecutive days. This regimen causes apoptosis and depletes striatal dopamine by 40–50% in young adult C57/bl mice, and the dopaminergic lesion stabilizes by 21 d after MPTP administration.

Regimen validation

As indicated above, some regimens of MPTP have been used extensively and have been well validated. However, although these facts are important in selecting an MPTP regimen, they should not be taken as evidence that these regimens are either the best or the only ones usable to generate valid data. Indeed, for a specific experiment or situation, one may have a good rationale for using a less common regimen or for developing a new regimen. Before using any less well validated or novel approach to MPTP

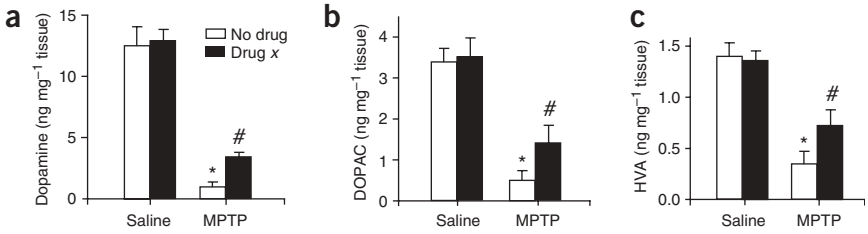

Figure 1 | Mouse brain levels of dopamine (DA) and its main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Mice ($n = 4$ per group and condition) were treated with drug *x* 0.5–1.0 h after the first and the last MPTP i.p. injection (18 mg kg^{-1} as 4 injections 2 hours apart). Drug *x* was continued for 4 additional days. Mice were killed at 7 d after the last dose of MPTP. Brains were collected as described in the protocol for HPLC determination of dopamine and metabolite levels. With this MPTP regimen, there is a profound reduction in striatal DA (**a**), DOPAC (**b**) and HVA (**c**) levels. Drug *x* significantly, though not dramatically, attenuates the loss of DA, DOPAC and HVA caused by MPTP. Bars represent mean ± s.e.m. Statistical analyses were carried out using two-way ANOVA followed by the Newman–Keuls post hoc test. *Lower than saline controls ($P < 0.01$); #lower than saline controls, but higher than MPTP/no drug group ($P < 0.05$).

dosing, it is advisable to define the toxicokinetics of MPTP and the time course of dopaminergic damage either by HPLC or immunostaining with quantitative morphology or both. Investigations of the striatal contents of MPP^+ must indicate when its highest tissue concentration is reached and when it becomes undetectable. These two parameters will help in determining the best set of time points that cover the tissue kinetics of MPP^+, allowing a reliable calculation of the peak and the half-life of the toxin for a new regimen. With this information in hand, for each selected time point, 3–5 control (e.g., MPTP wild-type mice) and experimental mice (e.g., MPTP knockout mice) should be killed during the actual study and compared to ensure that none of the differential toxicity observed is related to a simple interference with MPTP toxicokinetics. If the regimen involves one or multiple injections, the peak MPP^+ is often found approximately 90 min after MPTP administration and MPP^+ is no longer detectable by 8 h after the last injection of the drug. Thus, in addition to 90 min and 8 h, two time points on both sides of these values, such as 50, 70, 110, 130 min and 6, 7, 9, 10 h can be tried. Always assess striatal MPP^+ levels after the exact number of injections and dose to be used in the study to produce dopaminergic neurodegeneration.

The next step is to determine the time course of neurodegeneration; for this, 5–6 mice per time point should be killed at 2, 4, 7, 14, 21 and 30 d after the last injection and tissue should then be harvested and processed for the assays used to evaluate the status of the dopaminergic system. In the case of infusion of MPTP, it may be suggested that the time course of dopaminergic neurodegeneration be evaluated by killing animals every 3–5 d during the infusion period and then at 4, 7, 14, 21 and 30 d after its cessation. On the basis of these results, it is possible to determine when the lesion starts and when it no longer progresses. If a greater temporal resolution is needed to refine the time course of degeneration, then additional time points should be tested.

Use of other drugs with MPTP

For the investigation of neuroprotection by using other drugs with MPTP (**Fig. 1**), testing that these drugs do not interfere with MPTP metabolism is crucial. This is achieved by giving the drug under investigation either before or after MPTP administration and then collecting the striatum to determine the half-life of MPP^+, as described above. Often, however, to assess completely the potential effect (or the lack thereof) of a drug on MPTP metabolism, other assays, including MAO-B activity and MPP^+ uptake, should be tested. Always assess striatal MPP^+ levels after the exact number of injections and doses of MPTP and of the drug to be used in the study. If the pharmacologic intervention under investigation alters MPTP toxicokinetics (e.g., striatal MPP^+ levels) when administrated before or with MPTP, try delaying administration of the drug in question for at least 8 h after the last injection of MPTP to allow the unperturbed metabolism of MPTP/MPP^+ to take place.

MATERIALS

REAGENTS

- Mice (see REAGENT SETUP)
- Sterile saline
- MPTP · HCl powder **! CAUTION** MPTP is extremely toxic. Therefore (i) always wear the personal protection gear described in reference 8 when handling it, and (ii) order multiple small-quantity vials of MPTP · HCl rather than one large vial. Small-quantity vials are available in 10 and 100 mg and need only the calculation of the volume of solvent (e.g., saline) that has to be added to the vial to get to the required concentration, thus avoiding any weighing of the MPTP · HCl powder.

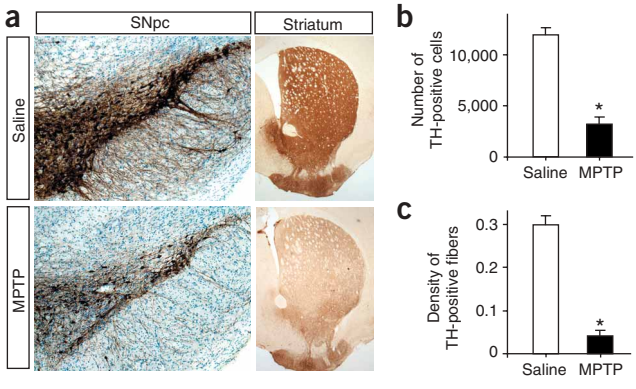

Figure 2 | Immunostaining of tyrosine hydroxylase (TH)–positive neurons in the substantia nigra pars compacta (SNpc) and terminals in the striatum. Mice were intoxicated with MPTP as in **Figure 1** and tissues were immunostained for TH following our standard protocol[26]. (**a**) Illustration of the effect of MPTP on TH immunostaining in both the mouse SNpc and striatum at 7 d after injection. In a saline-injected control, there is a dense TH-positive network of cell bodies and fibers in the SNpc and terminals in the striatum. After the MPTP injections, there is a dramatic reduction in TH immunoreactivity at the levels of the SNpc and the striatum. Ventral midbrain sections are counterstained with Nissl (blue) for anatomical reference. Panel (**b**) shows the loss of SNpc neurons in MPTP-dosed mice counted by stereology, and panel (**c**) shows the loss of striatal fibers in MPTP-dosed mice assessed by optical density. Bars represent means ± s.e.m. of five mice per group. Statistical analyses were carried out using the unpaired Student's *t*-test. *Lower than saline controls ($P < 0.01$).

▲ CRITICAL In the United States, MPTP can be purchased only as MPTP · HCl (Sigma Chemical Company, St. Louis, MO).
- Commercial bleach
- Paraformaldehyde powder ! CAUTION As this is a light powder that can cause respiratory tract irritation, wear a dust mask such as the 3M 8511 particulate respirator N95 dust mask (3M Corp., St. Paul, MN).
- Sodium phosphate buffer
- Sucrose, minimum 99.5%
- Isopentane (2-methylbutane)
- Animal care–approved anesthetic that does not interfere with monoamines, such as pentobarbital
- Antiseptic, such as betadine, to clean and disinfect incision area; required for MPTP infusion only.
- Topical local anesthetic medication such as bupivacaine · HCl applied 8–12 h after surgery and every 12 h for up to 48 h thereafter; required for MPTP infusion only ! CAUTION Most pain medications exert anti-inflammatory effects that can attenuate MPTP-induced toxicity. Thus, injection of pain medications should not be used.
- Trichloracetic acid ! CAUTION Very corrosive—causes severe burns. Its use requires safety glasses, gloves and good ventilation. ▲ CRITICAL Must be of highest purity.
- 4-Phenylpyridine ▲ CRITICAL Must be of highest purity.
- Potassium phosphate, monobasic, anhydrous
- Phosphoric acid ! CAUTION Corrosive—causes burns. Its use requires safety glasses, adequate ventilation and rubber or plastic gloves. ▲ CRITICAL Must be of highest purity.
- Acetonitrile ! CAUTION Irritant. Toxic by inhalation, ingestion or skin absorption. May cause serious damage to the eyes. Its use requires safety glasses and good ventilation. ▲ CRITICAL Must be HPLC grade.

EQUIPMENT
- A negative-pressure procedure room (temperature 22–27 °C) with a sink and a fume hood approved by the institution's animal care and use committee (see ref. 8 for detail)
- A fixed safe-box to store the stock of MPTP · HCl powder
- Covering material that is absorbent on one side and non-absorbent on the other (e.g., VersiDry super-absorbent underpads, NPS Corp., Green Bay, WI) (see EQUIPMENT SETUP)
- Plastic-backed absorbent sheets (e.g., SuperSorb underpads, Kendall Medical Products, Mansfield, MA) to cover the entire floor of the procedure room
- Protective gear (see ref. 8 for a complete description)
- Scale for weighing mice
- Microbalance for weighing MPTP (if necessary)
- Disposable plastic tuberculin syringes with 27-gauge needles for the injections
- Disposable sterile glass tubes (14 ml, 30 ml)
- Osmotic minipumps (Alzet 2004, DURECT Corporation, Cupertino, CA); required for MPTP infusion only
- Flow moderators (Alzet 0002486); required for MPTP infusion only
- Filling tubes (Alzet 0007987); required for MPTP infusion only
- Dissolvable sutures or autoclips plus autoclip applicator and remover; required for MPTP infusion only ! CAUTION Non-dissolvable sutures should be removed 10–14 d after surgery.
- Warming pad (Vetko thermal barrier, Harvard Apparatus, South Natick, MA); required for MPTP infusion only
- Indelible markers
- Clean mouse cages
- Magnifying glass
- Wet ice for dissection and dry ice for freezing dissected tissues
- Aluminum foil for wrapping and storage of tissues
- Refrigerated centrifuge
- A –80 °C freezer to store samples
- A peristaltic perfusion apparatus (e.g., Masterflex adjustable pump) calibrated to deliver 10 ml min^{-1}
- Light microscope attached, if possible, to a stereology system (to count neurons; see ref. 23 for details)
- HPLC system with UV-detector and a C18-reverse phase column (to quantify MPTP/MPP^+; see below for details)
- HPLC system with electrochemical detector and a C18-reverse phase column (to quantify monoamines; see ref. 24 for details)
- Scintillation counter (to asses MAO-B activity and [^{3}H]dopamine or [^{3}H]MPP^+ uptake; see refs. 24,25 for details)
- A metabolic water bath/shaker (for lactate levels; see ref. 26 for details)

REAGENT SETUP

Mice Adult male C57bl/6 mice of approximately 25–30 g are the preferred strain, gender and weight for MPTP studies. Selected mice should be at least 8 weeks of age to obtain a reproducible lesion. Although many investigators use approximately 10-week-old mice, others argue that older mice (e.g., 12–15 months old) should be used to model the age-related situation found in PD better. ! CAUTION For some unknown reason, there is a higher death rate among female mice following acute MPTP administration. Also, mice weighing less than 22 g have a higher acute death rate than those weighing more than 22 g. ! CAUTION At the age of 6 weeks, mice exhibit a greater variability in MPTP-induced dopaminergic damage, perhaps because of the still maturing MAO function. In addition, mice aged 12–15 months are more sensitive to MPTP than those aged 10–12 weeks, and thus dosages may have to be reduced to obtain the desired magnitude of neuronal loss. ! CAUTION All experiments involving animals must be performed according to national and institutional regulations. ▲ CRITICAL MPTP-related fatality is not only strain dependent but also breeding house dependent. For instance, the Charles River C57bl/6 mice from Kingston, North Carolina, are highly sensitive to MPTP in that they exhibit an 80–90% reduction in striatal dopamine levels 7 d after the 20 mg kg^{-1} × 4 doses injection schedule and a 5–10% death rate. On the other hand, C57bl/6 mice from the other Charles River breeding houses have the same dopaminergic sensitivity but a higher death rate after the same MPTP regimen. C57bl/6J mice, which are the Jackson C57 line, have a peripheral sensitivity to MPTP and exhibit a significant death rate with the 20 mg kg^{-1} × 4 doses injection schedule, but they are fine if the dose per injection is reduced to 18 mg kg^{-1} free base. In our hands, the DBA/2 mice cannot be used with an acute dosing schedule greater than 16 mg kg^{-1} × 4 doses over 8 h. Thus, before engaging in any important experiment, test dopaminergic sensitivity and death rate in the strain of mice to be used for the selected regimen if this information is not already known.

EQUIPMENT SETUP

Covering material This material should be used to cover all work surfaces, including the fume hood and the animal rack that holds mouse cages.

PROCEDURE

Housing and acclimation

1| Receive mice, place them in a restricted procedure room[8] (temperature 22–27 °C) and allow them to acclimate for 1 week before injection.

! CAUTION Travel alters brain chemistry, including catecholamine levels; hence, stabilization of brain chemistry for 5–7 d is recommended for reproducible results. Also, a new environment is not conducive to good results either; thus clean cages, which represent a new environment, should be changed 2–3 d before MPTP injection.

2| The day before starting MPTP administration, weigh, group and code mice (See **Supplementary Information** online). House no more than five mice per cage. Because of the dominance situation, it is unwise to house two mice per cage.

! CAUTION C57bl/6 mice, which are the strain of choice for most MPTP experiments, tend to fight in order to establish dominance in the cage. The fighting can sometimes be vicious and end in severe wounding, infections and even death, making the mice unsuitable for experimentation.

? TROUBLESHOOTING

3| Use a simple marking system on the tails using an indelible marker to help distinguish the mice.

MPTP administration

4| Choose route of administration: s.c. or i.p. In addition, MPTP can be either injected (option A) or infused (option B).
▲ CRITICAL STEP As discussed above, the preferred route for systemic administration of MPTP in mice is s.c. or i.p. Although some investigators are more comfortable administering drugs and toxins such as MPTP via one of these routes rather than the other, it seems that both are equally effective as far as the nigrostriatal damage is concerned. A notable difference does exist, however, between these two routes. Any substance administered i.p., whether by injection or by infusion, is absorbed primarily via the hepatic portal circulation rather than by the capillaries. Substances such as MPTP that are extensively metabolized by the liver will readily be detoxified by xenobiotic-metabolizing cytochrome P450 enzymes[27], thereby possibly producing more variable concentrations in the blood and in the brain and consequently more variable effects. Investigators must be aware of this fact as it can be of significance in particular experiments, especially when drugs known to modify cytochrome P450 activity are used.

(A) MPTP injection

(i) Estimate the total volume of MPTP solution needed for the experiment. This can be derived from adding the weights (in grams) of all mice to be injected with MPTP (**Supplementary Information**). MPTP can be purchased in the United States only as MPTP·HCl, which has a molecular weight of 209.7. In the literature, the doses of MPTP are often reported for the free base not for the conjugated salt, and we recommend following this practice. Thus, in the case of MPTP·HCl, the presence of HCl has to be taken into account in making the injection solution. HCl has a molecular weight of 35.4 and represents 17% of the MPTP·HCl molecular mass. Thus, to make a 20 mg kg^{-1} dose of free base MPTP, the dose of MPTP·HCl is 20 mg kg^{-1} × 1.17 = 23.4 mg kg^{-1}. Dosing is calculated as described below. The **Supplementary Data** online include an example of a dosing schedule worksheet that is essentially a checklist to prevent double-dosing or other accidents. Unless you have strong justifications, avoid using uncommon regimens found in the literature that may preclude making informed decisions about critical aspects of an experimental design and comparison with other published studies.
▲ CRITICAL STEP To avoid overloading animals with fluid (which can lead to fatal heart failure), inject approximately 10 µl per 1 g body weight (i.e., approximately 0.25 ml for a 25 g mouse). The total volume is then calculated by multiplying the total weight of MPTP-treated mice by 10 µl and by the number of injections in the regimen. For example, if a MPTP experiment includes five mice weighing 25 g each and involves four injections per mouse, then the total volume of the solution needed is 125 g × 10 µl × 4 = 5 ml.
▲ CRITICAL STEP It is advisable to prepare at least 10% more solution to avoid any shortage because of the dead space in the syringe.

(ii) Weigh the necessary amount of MPTP·HCl. First convert the dose of MPTP·HCl into a concentration. Given the recommended injection volume of 10 µl per 1 g body weight, which corresponds to 10 ml kg^{-1}, the dose of MPTP·HCl in milligrams per kilogram can be simply converted into a concentration of MPTP·HCl expressed in milligrams per 10 ml. Then, multiply the total volume needed by the desired concentration of MPTP·HCl. For example, if the total volume is 5 ml and the concentration is 23.4 mg per 10 ml, then the total amount of MPTP·HCl is (5 ml × 23.4 mg)/10 ml = 11.7 mg.
! CAUTION All weighing of MPTP powder should be performed in a fume hood following the procedure outlined in ref. 8.
▲ CRITICAL STEP If several injections are to be made on the same day, enough MPTP solution can be made up for the entire injection schedule and can be kept on ice.

(iii) Wearing protective gear[8], dissolve the calculated amount of MPTP·HCl in the calculated volume of sterile saline. Cover the vial with several layers of parafilm and mix the solution.
! CAUTION Animals should be injected with a sterile solution of MPTP. The weighed MPTP is diluted with sterile saline. Do not autoclave MPTP solutions to avoid vaporizing the compound, which may lead to exposure from inhalation.

(iv) Inject mice as per the dosing schedule worksheet, administering injections precisely 2 h apart or at the same time daily. For consistent results, injections should be made in the peritoneal area up and slightly to the left to avoid puncturing the intestine or up and slightly to the right to avoid hitting the bladder. Subcutaneous injections are best given on the back between the blades of the scapula. If multiple injections are given in 1 d, it is good practice to alternate sides of the peritoneum or the back to decrease irritation in these areas.
? TROUBLESHOOTING

(B) MPTP infusion

(i) Determine the amount of MPTP·HCl needed for the study. First, calculate the mass of MPTP·HCl to be flowed per hour.
▲ CRITICAL STEP At this point, only infusions of 5.85 and 35.10 mg kg^{-1} d^{-1} MPTP·HCl (i.e., 5 and 30 mg kg^{-1} d^{-1} free base) for up to 28 d have been documented as producing dopaminergic damage in mice[10]. As the volume flow rate for the osmotic pump model 2004 is 6 µl d^{-1}, the required concentrations of MPTP·HCl are 5.85/6 = 0.975 mg kg^{-1} $µl^{-1}$ and 35.1/6 = 5.85 mg kg^{-1} $µl^{-1}$. Assuming that we have mice weighing 26 g, the total mass of powder needed for the study is therefore 0.975 × 0.026 = 0.025 mg $µl^{-1}$ or 5.85 × 0.026 = 0.152 mg $µl^{-1}$.

(ii) Estimate the total volume of MPTP solution needed for the experiment by multiplying the number of mice with the same body weight (e.g., 10 mice weighing 26 g) by 200 µl, which is the nominal volume of the reservoir of the osmotic pump model 2004. Based on this example, the total volume is 200 µl × 10 = 2 ml.
▲ CRITICAL STEP It is advisable to prepare a larger than required volume of solution (e.g., 2.2 ml). Thus, in this case, 55 and 334.4 mg MPTP·HCl will be mixed with 2.2 ml sterile saline.
(iii) Fill the osmotic pump as follows.
! CAUTION The following steps must be performed in the fume hood using a sterile technique.
(iv) Weigh the empty pump together with its flow moderator.
(v) Attach the filling tube (supplied with each package of pumps) to a tuberculin syringe and draw up the room temperature solution.
! CAUTION Make sure that the syringe and the attached tube are free of air bubbles.
(vi) Insert the filling tube through the opening at the top of the pump and push it gently to the bottom of the pump reservoir. Then, slowly fill the pump while holding it in an upright position.
(vii) When the solution appears at the outlet, stop filling and carefully remove the filling tube. Wipe off overflow with a tissue.
! CAUTION Do not wipe pump with tissue dampened with the bleach solution.
(viii) Insert the flow moderator until the cap or flange is flush with the top of the pump.
(ix) Weigh the filled pump with the flow moderator in place. The difference in the weights between Steps 4B(iv) and 4B(ix) gives the net weight of the solution loaded.
! CAUTION Under the conditions outlined above, 1 µl MPTP solution is equivalent to 1 mg. The fill volume should be more than 90% of the reservoir volume specified on the instruction sheet. If this is not the case, there may be some air trapped inside the pump.
! CAUTION Because of the hazardous nature of the MPTP solution, do not evacuate the incompletely filled pump and refill. Instead, discard this pump by sealing it in a biohazard bag, incinerate it and use another one.
(x) Prepare a surgery area in the fume hood of the MPTP procedure room.
(xi) Anesthetize each mouse. Once the mouse is properly anesthetized (i.e., no response to tail pinch or toe pinch), shave and wash the abdominal area with betadine. Then, with a scalpel, make 1–1.5-cm long midline incision in the skin in the mid abdomen below the rib cage.
(xii) With forceps, carefully tent the musculoperitoneal layer to avoid any damage to the bowel and incise the peritoneal wall directly beneath the cutaneous incision.
(xiii) Gently insert a filled pump, delivery portal first, into the peritoneal cavity, making adjustments where necessary.
(xiv) Close the musculoperitoneal layer with absorbable sutures taking care to avoid perforating the underlying bowel.
(xv) Close the skin incision with 2–3 wound clips or sutures.
▲ CRITICAL STEP Although the method has not been published yet, infusion of MPTP by implanting osmotic pumps s.c. should be easily adaptable from this i.p. protocol.
(xvi) Place mouse on a 37 °C warming pad until it awakens, then place the animal in a clean cage. It is not necessary to house mice separately.
! CAUTION Do not use a heating lamp as this can cause the mouse to overheat and possibly die.
? TROUBLESHOOTING

Mouse monitoring after MPTP injections and during osmotic pump infusion

5| Leave mice to rest in the restricted procedure room after the last injection of MPTP or during the MPTP infusion for the required period of high-risk isolation.

! CAUTION Isolate the mice while MPTP and its metabolites are being excreted. After MPTP administration to mice, the interior surfaces of the cage and the surfaces that the animals and their excreta physically touch, including food and drinking bottle, are contaminated with MPTP and its metabolites[28]. After one injection, 70% of the total injected dose of MPTP and its metabolites is recovered 2 d later from the inside cage-wash, urine and feces; of this, approximately 15% is unmetabolized MPTP, and the rest consists of MPTP metabolites, such as MPP^+. The excretion of unmetabolized MPTP occurs mainly during the first day after injection; mainly MPTP metabolites are excreted up to 3 d after injection[28]. One day after an injection of radiolabeled MPTP into mice, most of the radioactivity is localized in the brain and the adrenal gland, and the other organs show 50–75% lower amounts of radioactivity[29]. Analyses of the radioactive species recovered from different organs and body fluids such as bile, urine, blood and CSF demonstrate variable amounts of unmetabolized MPTP soon after injection, but by 12–24 h after injection, virtually all of the radioactivity corresponds to MPP^+ (refs. 29,30). Thus, following a 1-d schedule, there is no evidence in mice that MPTP and its metabolites are still being excreted after 3 d. From this, it appears that the potential human risks of exposure to MPTP and its metabolite MPP^+ are through direct contact with the animal, the animal cage's inner surfaces and the bedding material.

There is minimal risk from exposure due to airborne or vapor-borne forms of MPTP. Although the safety procedures outlined in ref. 8 must always be followed, the period of maximal risk of MPTP contamination is from the moment of injection to the time when MPTP or its metabolites are no longer found in the excreta of treated animals; as a precautionary measure, we define the period of high risk to last up to the fourth day after MPTP injection if the 14–20 mg kg^{-1} × 4 doses regimen is used. If another regimen is used, such as MPTP infusion, the duration of this high-risk period must be determined by collecting urine and by extracting $MPTP/MPP^+$ from the interior of the cages, as done in reference 28, over time; the levels of MPTP and MPP^+ in the exacts must be quantified by HPLC so that the period of high-risk can be determined before embarking on any actual MPTP study.

6| As indicated above, it is prudent to prepare a small excess of MPTP · HCl solution. Mix any left over with an excess of 1% bleach solution in water, which will oxidize the remaining MPTP within a few seconds. This can then be discarded as liquid hazardous waste.

7| Clean tools, decontaminate work area with bleach and discard all disposables in a biohazard bag. Non-disposable items (i.e., surgical tools) must be decontaminated with bleach and then with copious amounts of water.

8| After the isolation period, transfer mice to a clean cage and house in a regular animal room.
▲ **CRITICAL STEP** Mice subjected to the infusion protocol should be treated in the same way as those receiving the injections of MPTP in that cages should be changed weekly and decontaminated before normal cage-washing operations.

9| At the end of the isolation period, tissue can be harvested and processed in regular laboratory space. For specific studies, mice may have to be killed before the end of the high-risk period. In this case, tissue must be harvested and processed in the restricted procedure room using the full set of personal protection measures[8].

10| Clean and detoxify or dispose of the contents of the procedure room at the end of the high-risk period as described in ref. 8.

Processing of tissue samples

11| Regardless of the MPTP regimen used, tissue samples are either fixed or fresh-frozen. Although fixation can be achieved by various means and using various fixatives, we find that transcardial perfusion of 70–90 ml freshly prepared 4% paraformadehyde in 0.1 M sodium phosphate buffer (pH 7.1–7.4) followed by an overnight immersion in the same fixative solution at 4 °C provides excellent and reproducible results, especially for tyrosine hydroxylase immunostaining in mice (option A). Information about the preparation of the fixative solutions and transcardial perfusion can be found elsewhere[31]. Alternatively, brain regions can be dissected (option B). A tutorial film of the procedure used to dissect the brain regions in mice for the MPTP studies can be found online as **Supplementary Information**, in addition to the step-by-step description of this procedure below. Brain levels of MPTP and MPP^+ can be quantified by HPLC, as described in option C. Tissue collection during the high-risk period (i.e., the period during which mice still excrete $MPTP/MPP^+$) relies on the same methods, but collection of samples must be done in the restricted procedure room with covered surfaces and while wearing personal protection equipment. Perfusion for fixed tissues should be performed under a fume hood and the perfusion solution should be collected, treated with an excess of 1% bleach solution and discarded as hazardous waste. When tissue is dissected, tissue remains and carcasses must be collected and discarded as hazardous waste. Covered surfaces where killing has taken place must be sprayed with 1% bleach before discarding as medical waste. All non-disposable instruments must be soaked in 1% bleach for 5 min then rinsed thoroughly with water.

(A) Perfusion and fixation

(i) Deeply anesthetize mice (e.g., pentobarbital at 35–45 mg kg^{-1}).

(ii) Perfuse mice transcardially at a flow rate of 10 ml min^{-1} first with saline for 3 min followed by 4% paraformaldehyde solution for 8 min using a peristaltic pump.

(iii) Remove brains quickly and immerse overnight in at least ten times the brain's own volume of the same fixative.
▲ **CRITICAL STEP** Some antigens (e.g., microglial markers) are quite sensitive to fixation, and so fixation conditions may have to be optimized before the initiation of studies. This may mean determining the optimal volume of fixative solutions for perfusion and length of time for fixation by immersion.

(iv) Cryoprotect the fixed brains by discarding the fixative solution and replacing it with 30% sucrose in 0.1 M sodium phosphate buffer (pH 7.1–7.4). It is important to cryoprotect until the brains sink quickly to the bottom of the container after inversion of the container (usually more than 48 h). Several alternative protocols and cocktails for cryoprotection exist, including the use of graded sucrose solutions in 0.1 M phosphate buffer.

(v) Freeze brains by immersion in isopentane (2-methylbutane) cooled on powdered dry ice. For best results, insert a thermometer into the isopentane to gauge its temperature. At –40 °C, it takes approximately 35 s to freeze a mouse brain.

! CAUTION If the isopentane is too cold (colder than –60 °C) and the brain is left in the cold isopentane too long, the brain will crack.

(vi) Wrap frozen brains in aluminum foil with the sample identification on the outside of the foil (use indelible marker) and store at –80 °C until they are processed.

(vii) To prepare the sections for immunostaining, solidly bond whole fixed, frozen brains to the metal object holder with a tissue-freezing medium (e.g., OCT Tissue-Tek) and cut on a cryostat or a freezing-sliding microtome. Collected sections 30–40 µm sections work best for us) can be used for immunohistochemistry and the numbers of stained cellular elements (e.g., neurons) can be counted using stereology, as described in references 26,32.

(B) Dissection of brain regions

(i) Decapitate mouse with a pair of sharp scissors. If you remove the head properly, you should be able to see the cerebellum at the back of the cranium. If not, trim away the excess spinal tissue until you reach this point.

! CAUTION When harvesting tissues for catecholamine measurement and for mitochondrial assays, do not use anesthesia without prior evidence that anesthetic does not affect catecholamine levels and turnover as well as mitochondrial respiration.

(ii) Cut the skin over the skull in the middle with a scalpel, starting from between the eyes and going backward to where the skin ends. Pull skin sideways to reveal the skull. Insert the tip of one blade of a pair of pointed angular scissors into the dorsal aspect of the foramen magnum and cut, in a forward direction, the occipital and interparietal bones midline above the cerebellum. Continue cutting along the sagittal suture of the skull until you reach between the eyes.

! CAUTION Keep scissor tips pointing upward to avoid injuring the brain.

(iii) With the curved back of a pair of medium-tipped forceps, pull the skull apart and up at the sagittal suture without nicking the brain.

(iv) With the same pair of medium-tipped forceps, peel off any remaining meninges, especially the tentorium, which is interposed between the cerebellum and the cerebrum.

(v) Remove the brain by gently tilting the skull forward, allowing the brain to be released from the skull by gravity. Sequentially, snip the olfactory bulbs, the optic nerves and the fifth nerves with the same medium-tipped forceps. Grab the brain by the caudal end of the brainstem, dip it in chilled saline to wash out excess blood and then place it on a Petri dish sitting in wet ice and covered with dH_2O-dampened filter paper. Blot brain with a paper towel to remove excess saline.

(vi) Using fine-tipped forceps, gently grab the cerebellum at its base and pull it away from the brainstem. Immediately place it on dry ice–chilled foil (to be used as a negative control). Then sever the brainstem from the rest of the brain by cutting behind the caudal aspect of the cerebral cortex but in front of the inferior colliculus with either the back of the fine forceps or a scalpel.

(vii) Turn the brain so that its dorsal surface sits on the filter paper. Locate the cerebral peduncles (small white strips on each side behind the hypothalamus). Starting at the anterior aspect of the cerebral peduncles, gently tease the entire midbrain structure away from the cerebrum using the fine-tipped forceps.

(viii) Place the caudal aspect of this block of tissue containing the midbrain on the filter paper with the ventral aspect facing you. Place the blade of the scalpel a third of the way from the top of the dorsal aspect of the midbrain block and start to cut, slanting the blade at a 45° angle toward you while cutting. Once you have made this cut, you will have isolated all of the ventral midbrain containing the substantia nigra plus the ventral tegmental area. Place this structure on dry ice–chilled foil.

▲ CRITICAL STEP To optimize the ventral midbrain dissection, it is necessary to stain an entire midbrain for tyrosine hydroxylase and practice cutting before embarking on any experiments using this structure. To do this, fix the dissected block by immersion in the same fixative solution described above and perform tyrosine hydroxylase immunostaining on the intact block. Then use tyrosine hydroxylase–defined landmarks to refine your technique.

(ix) For the striatum, turn the brain so that it sits on its ventral surface and its posterior aspect is pointing toward you. Cut the brain down the middle with a scalpel to separate the two hemispheres. Using the corpus callosum as a landmark, with the butt end of the fine forceps pull back the corpus callosum and open the third ventricle to reveal the striatum, which appears as a bulging circular structure with a tail. Gently score around the striatum with the tips of the fine forceps and then scoop it out (do not scoop too deeply) and place it on the dry ice–chilled, numbered foil. Repeat this procedure for the other half of the brain.

! CAUTION Placing fresh tissue on a room-temperature piece of foil can oxidize monoamines. Thus, place all foils for a particular mouse on dry ice before dissection. Wrap all tissues with the sample number exposed, and cover the foil containing each sample with powdered dry ice.

■ PAUSE POINT Store tissues at –80 °C until analysis.

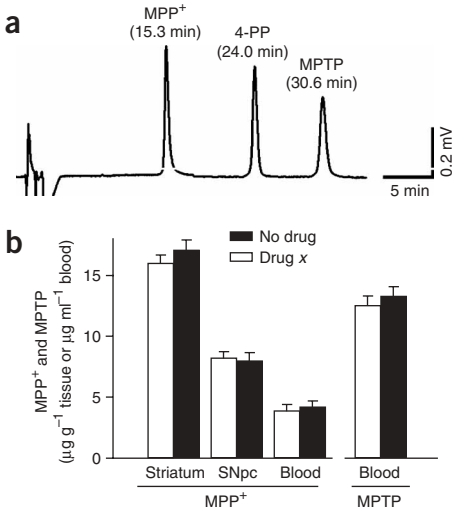

Figure 3 | MPTP and MPP+ quantification in blood and brain tissues. Mice ($n = 5$ per group and condition) were treated with drug *x* and MPTP as in **Figure 1**. Striata and ventral midbrain (Vmbr) as well as whole blood were collected 90 min after the last injection of MPTP. Samples were processed for MPTP and MPP+ levels as described in the protocol. Panel (**a**) shows a typical chromatograph generated by UV detection of MPP+ and MPTP. 4-Phenylpyridine (4-PP) is used as the internal standard. Panel (**b**) shows calculated levels of MPP+ in the striatum, Vmbr and blood as well as levels of MPTP in the blood. It shows that drug *x* does not interfere with MPTP metabolism as there is no significant difference ($P > 0.05$) between the MPTP/no drug *x* and the MPTP/drug *x* groups. Bars represent means ± s.e.m. Statistical analyses were carried out using two-ANOVA followed by the Newman–Keuls post hoc test.

(C) Quantification of brain levels of MPTP and MPP+

(i) As MPTP and MPP+ fluoresce under UV illumination at 245 and 295 nm, respectively[33], both can be quantified at the same time by UV detection. First mix brain samples or urine or cage extracts in 9 volumes of 5% trichloracetic acid in HPLC-grade water containing 20 μg ml^{-1} 4-phenylpyridine as internal standard.

(ii) Centrifuge mixtures at 15,000 *g* for 20 min at 4 °C and transfer supernatants to clean 1.5-ml centrifuge tubes.

(iii) Inject an aliquot (20–100 μl) of each supernatant onto a C18 reverse phase column attached to an HPLC system consisting of a pump, an injector and a UV detector. The mobile phase should consist of 50 mM potassium phosphate adjusted to pH 3.2 with ultrapure 18 M phosphoric acid.

(iv) After filtering the aqueous phase through a 0.22 μm hydrophilic filter system (e.g., disposable Millipore Express PLUS Membrane), mix 9 volumes with 1 volume of HPLC-grade acetonitrile and degas (20–30 min) before use. Set flow rate to 1.6 ml min^{-1}, which gives with a 5 μm, 250 × 4.6 mm C18 reverse phase column, typical retention times of 15.3 min for MPP+, 24.0 min for 4-phenylpyridine and 30.6 min for MPTP. With this method, our detection limit is 0.32 ng ml^{-1}. Recoveries range from 84 to 90%, and this method provides good reproducibility.

(v) Manual or computerized quantification for each compound can be carried out by generating standard curves and by correcting for recovery based on the internal standard. **Figure 3** shows a typical chromatogram for MPTP and MPP+ analyses.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Reason	Solutions
Housing (Steps 1 and 2)	Fighting and injuries	New environment	Put a handful of dirty shavings from the old cage into the clean cage before transferring the mice
		Establishing dominance	Separate out the aggressor (only mouse in the cage with unruffled fur)
		Housing only two mice in a cage	
Injection (Step 4A)	High rate of acute death (>20%)	Error in the dosage of MPTP (too high)	Confirm the dose of MPTP or the strain of mice
		New lot of MPTP with higher potency is used	Reduce the dose per injection
		Different lots of mice or animal vendors	Confirm the proper and safe practice of injection
		Pharmacologic or genetic interventions under investigation render mice more sensitive to the cardiovascular side effects of MPTP	Modify the time of drug administration with respect to MPTP regimen
		MPTP injections may have lesioned a vital organ	Practice injecting on a sacrificed mouse with an open peritoneum
		Too cold in the procedure room	Ensure that the temperature in the procedure room is maintained between 22 and 27 °C

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Reason	Solutions
Infusion (Step 4B)	Difficulty filling the pumps	Air trapped in the reservoir	Try filling pump with the filling tube at a slight angle
		Bevel of the filling tube pressing against the reservoir wall	Insert and remove the flow moderator several times before reinserting the filling tube Rotate the filling tube
	Shift of the pump in the i.p. space	Too large of an i.p. pocket	Reduce the size of the i.p. pocket
Analyses (Step 11C)	High variability in the extent of the dopaminergic lesion among animals	Inconsistent injection method	Improve and test proper injection technique
		I.p. injection	Change from i.p. to s.c. injection
		MPTP injections hitting the bladder, the intestines or the muscles	Practice injection on a sacrificed mouse with an open peritoneum
		Inconsistent MPTP solution preparation	Standardize solution preparation Perform important experiments within run rather than between runs
		Leakage of the MPTP solution from the injection site	Keep the syringe in place for a few seconds after completing each injection
		Mismatching of age, gender and weight of mice among the various experimental groups	Pay attention to proper matching for MPTP and use outliers (age, gender, weight) if necessary for vehicle controls

ANTICIPATED RESULTS

The first sign of a successful injection is piloerection. On acute dosing, after the third injection, mice appear immobile. This behavior continues after the fourth injection and lasts for 24–48 h, after which mice appear normal. The lack of activity can translate into decreased food and water intake and a possible slight weight loss. Mice may exhibit a small (approximately 1 g) weight loss by day 3 of the sub-acute injection schedule; thus mice should be weighed on day 3 for dosage adjustment. In any event, mice should be checked at least once per day. Pump infusion does not seem to cause any weight loss problems; however, mice should still be checked once per day. Seven days after the last injection, if the 20 mg kg^{-1} free base $\times$ 4 doses schedule of MPTP is used, there is an approximately 80–90% loss of dopamine levels in the striatum on HPLC analysis and an approximately 70% loss of dopaminergic neurons. If the sub-acute dosing schedule is used, with the exception of piloerection, which is seen after the first dose, mice appear normal. As the sub-acute dosing schedule is mild compared with the acute dosing schedule, there is a population of dopaminergic neurons that appear sick and do not die immediately. Thus, stabilization of the loss of striatal dopamine levels (approximately 50%) and of dopaminergic neuron number (approximately 40%) occurs 21 d after the last administration of MPTP instead of after 7 d. **Figures 1** and **2** are examples of dopamine levels before and after MPTP administration and of tyrosine hydroxylase–positive neuron number loss in the substantia nigra pars compacta.

Note: Supplementary information is available via the HTML version of this article.

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1. Fahn, S., Przedborski, S. Parkinsonism, In *Merritt's Neurology* (ed. Rowland, L.P.) 828–846 (Lippincott Williams & Wilkins, New York, 2005).
2. Dauer, W. & Przedborski, S. Parkinson's disease: mechanisms and models. *Neuron* **39**, 889–909 (2003).
3. Przedborski, S. & Tieu, K. Toxic animal models. In *Neurodegenerative diseases: Neurobiology, pathogenesis and therapeutics* (eds. Beal, M.F., Lang, A.E. & Ludolph, A.) 196–221 (Cambridge, New York, 2005).
4. Langston, J.W., Ballard, P. & Irwin, I. Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**, 979–980 (1983).
5. Sotelo, C., Javoy, F., Agid, Y. & Glowinski, J. Injection of 6-hydroxydopamine in the substantia nigra of the rat. I. Morphological study. *Brain Res.* **58**, 269–290 (1973).
6. Betarbet, R. *et al.* Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.* **3**, 1301–1306 (2000).
7. Bove, J. *et al.* Proteasome inhibition and Parkinson's disease modeling. *Ann. Neurol.* **60**, 260–264 (2006).

8. Przedborski, S. *et al.* The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. *J. Neurochem.* **76**, 1265–1274 (2001).
9. Sonsalla, P.K. & Heikkila, R.E. The influence of dose and dosing interval on MPTP-induced dopaminergic neurotoxicity in mice. *Eur. J. Pharmacol.* **129**, 339–345 (1986).
10. Fornai, F. *et al.* Parkinson-like syndrome induced by continuous MPTP infusion: Convergent roles of the ubiquitin-proteasome system and \{alpha\}-synuclein. *Proc. Natl. Acad. Sci. USA* **102**, 3413–3418 (2005).
11. Meredith, G.E. *et al.* Lysosomal malfunction accompanies alpha-synuclein aggregation in a progressive mouse model of Parkinson's disease. *Brain Res.* **956**, 156–165 (2002).
12. Heikkila, R.E., Sieber, B.A., Manzino, L. & Sonsalla, P.K. Some features of the nigrostriatal dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the mouse. *Mol. Chemic. Neuropathol.* **10**, 171–183 (1989).
13. Giovanni, A., Sieber, B.A., Heikkila, R.E. & Sonsalla, P.K. Correlation between the neostriatal content of the 1-methyl-4-phenylpyridinium species and dopaminergic neurotoxicity following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration to several strains of mice. *J. Pharmacol. Exp. Ther.* **257**, 691–697 (1991).
14. Giovanni, A., Sieber, B.-A., Heikkila, R.E. & Sonsalla, P.K. Studies on species sensitivity to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Part 1: Systemic administration. *J. Pharmacol. Exp. Ther.* **270**, 1000–1007 (1994).
15. Giovanni, A., Sonsalla, P.K. & Heikkila, R.E. Studies on species sensitivity to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Part 2: Central administration of 1-methyl-4-phenylpyridinium. *J. Pharmacol. Exp. Ther.* **270**, 1008–1014 (1994).
16. Miller, D.B., Ali, S.F., O'Callaghan, J.P. & Laws, S.C. The impact of gender and estrogen on striatal dopaminergic neurotoxicity. *Ann. N. Y. Acad. Sci.* **844**, 153–165 (1998).
17. Staal, R.G. & Sonsalla, P.K. Inhibition of brain vesicular monoamine transporter (VMAT2) enhances 1-methyl-4-phenylpyridinium neurotoxicity *in vivo* in rat striata. *J. Pharmacol. Exp. Ther.* **293**, 336–342 (2000).
18. Javitch, J.A., D'Amato, R.J., Strittmatter, S.M. & Snyder, S.H. Parkinsonism-inducing neurotoxin, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite *N*-methyl-4-phenylpyridinium by dopamine neurons explain selective toxicity. *Proc. Natl. Acad. Sci. USA* **82**, 2173–2177 (1985).
19. Liu, Y., Roghani, A. & Edwards, R.H. Gene transfer of a reserpine-sensitive mechanism of resistance to *N*-methyl-4-phenylpyridinium. *Proc. Natl. Acad. Sci. USA* **89**, 9074–9078 (1992).
20. Ramsay, R.R. & Singer, T.P. Energy-dependent uptake of *N*-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria. *J. Biol. Chem.* **261**, 7585–7587 (1986).
21. Jackson-Lewis, V., Jakowec, M., Burke, R.E. & Przedborski, S. Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Neurodegeneration* **4**, 257–269 (1995).
22. Tatton, N.A. & Kish, S.J. *In situ* detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience.* **77**, 1037–1048 (1997).
23. Liberatore, G. *et al.* Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. *Nat. Med.* **5**, 1403–1409 (1999).
24. Przedborski, S. *et al.* Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J. Neurosci.* **12**, 1658–1667 (1992).
25. Youdim, M.B.H. Assay and purification of brain monoamine oxidase, in Eds. Marks, N. & Rodnight, R. *Research Methods in Neurochemistry*, Vol. 3, 167–208 (Plenum Press, New York, 1975).
26. Tieu, K. *et al.* D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. *J. Clin. Invest.* **112**, 892–901 (2003).
27. Chiba, K., Kubota, E., Miyakawa, T., Kato, Y. & Ishizaki, T. Characterization of hepatic microsomal metabolism as an *in vivo* detoxication pathway of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice. *J. Pharmacol. Exp. Ther.* **246**, 1108–1115 (1988).
28. Yang, S.C., Markey, S.P., Bankiewicz, K.S., London, W.T. & Lunn, G. Recommended safe practices for using the neurotoxin MPTP in animal experiments. *Lab. Anim. Sci.* **38**, 563–567 (1988).
29. Johannessen, J.N., Chiueh, C.C., Herkenham, M. & Markey, C.J. Relationship of the *in vivo* metabolism of MPTP to toxicity. In *MPTP: A Neurotoxin Producing a Parkinsonian Syndrome* (eds. Markey, S.P., Castagnoli, N. Jr., Trevor, A.J. & Kopin, I.J.) 173–189 (Academic Press, Orlando, 1986).
30. Markey, S.P., Johannessen, J.N., Chiueh, C.C., Burns, R.S. & Herkenham, M.A. Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. *Nature* **311**, 464–467 (1984).
31. Kiernan, J.A. *Histological and Histochemical Methods Theory and Practice* (Butterworth Heinemann, Oxford, 1999).
32. Vila, M. *et al.* Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Proc. Natl. Acad. Sci. USA* **98**, 2837–2842 (2001).
33. Shinka, T., Castagnoli, N., Jr., Wu, E.Y., Hoag, M.K. & Trevor, A.J. Cation-exchange high-performance liquid chromatography assay for the nigrostriatal toxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and its monoamine oxidase B generated metabolites in brain tissues. *J. Chromatogr.* **398**, 279–287 (1987).