Supplemental Figures and Tables

Fig. S1

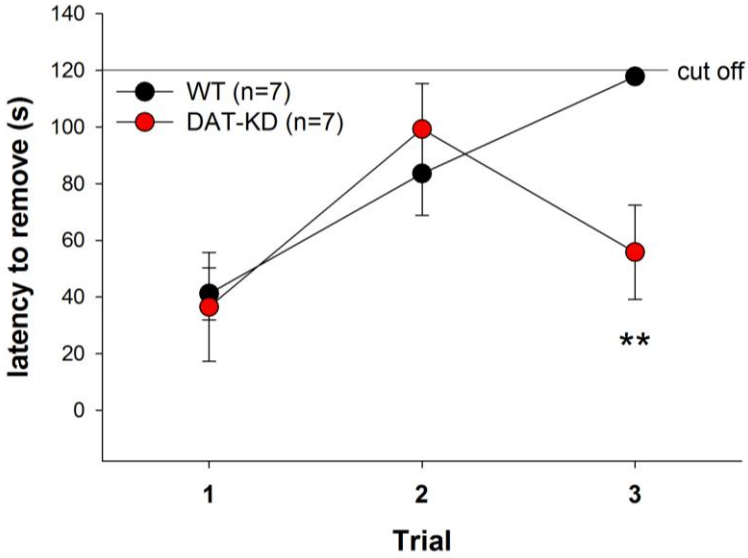

Fig. S1 The adhesive removal test assessed fine motor control and sensitivity in 18 month-old mice (**p<0.01 Mann Whitney U test, n=7/group, mean ± SEM).

Fig. S2

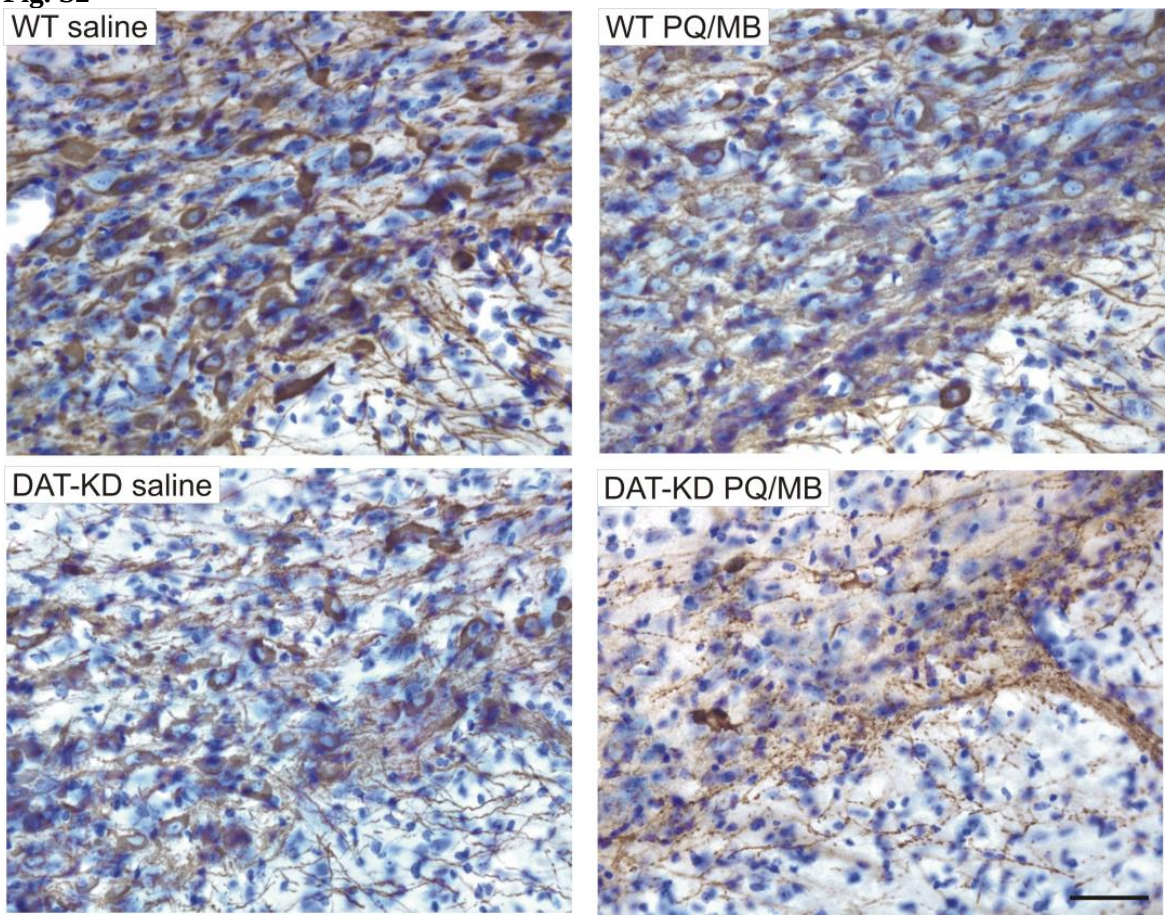

Fig. S2 Representative microscopic images of sections from the medial subregion of the SNc of 5 week-old WT and DAT-KD mice exposed to saline or paraquat/maneb (PQ/MB) (immunostained for TH (brown) and stained for Nissl (blue-purple)). Scale bar = 50 µm.

Fig. S3

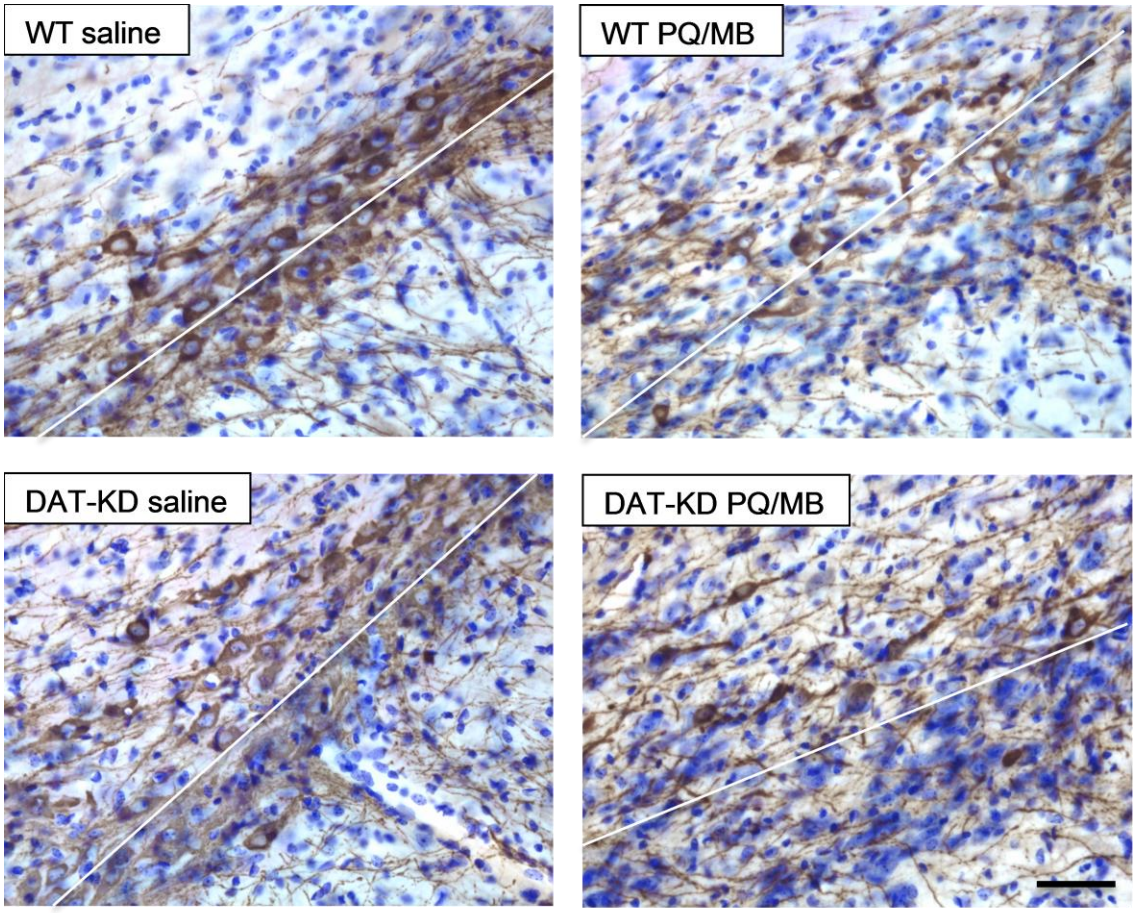

Fig. S3 Representative microscopic images of sections from the dorsal (above the white line) and ventral (below the white line) subregions of the SNc of 5 week-old WT and DAT-KD mice exposed to saline or paraquat/maneb (PQ/MB) (immunostained for TH (brown) and stained for Nissl (blue-purple)). Scale bar = 50 µm.

Fig. S4

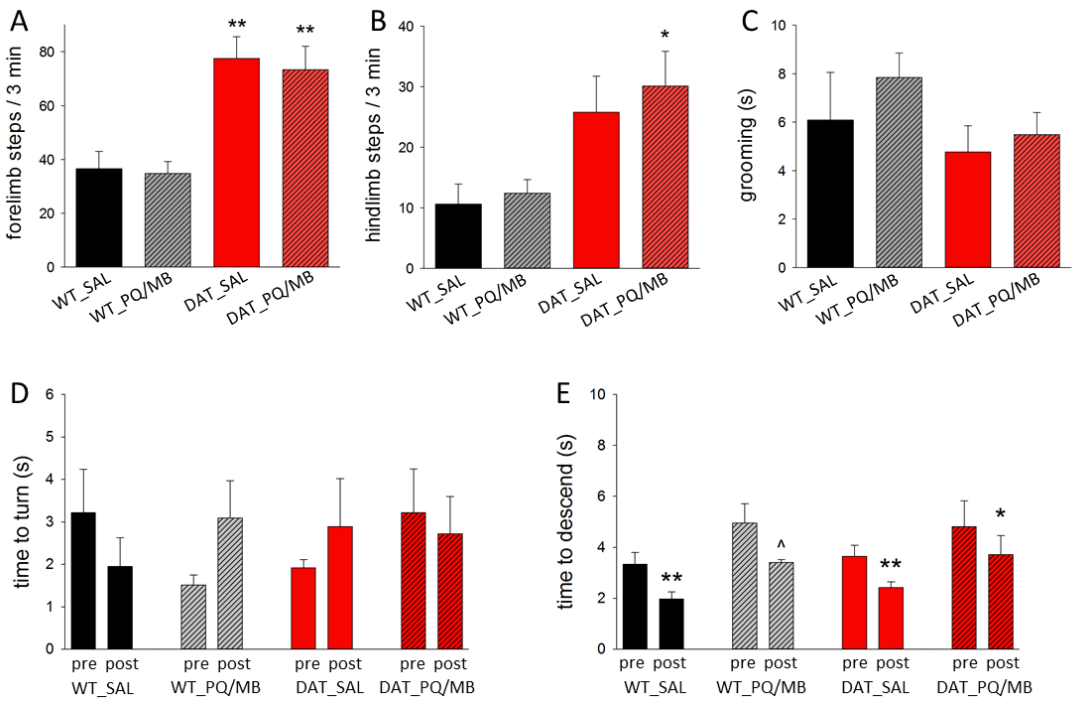

Fig. S4 Young adult DAT-KD mice make more forelimb (A) and hindlimb (B) steps in the cylinder, while grooming (C) was unchanged (2x2 ANOVA genotype x toxin, *p<0.05, **p<0.01 Holm-Sidak DAT-KD versus respective WT) . On the vertical pole time to turn face down was not changed by genotype (WT vs DAT-KD), time (pre vs post toxin exposure) of test or toxin exposure (saline vs PQ/MB). During the second trial (post toxin exposure) the time to descend after the turn decreased in all groups but only non-significantly in WT injected with PQ/MB (*p<0.05, **p<0.01 Wilcoxon signed rank test). These mice were significantly slower compared to corresponding saline injected mice (^p<0.05 Mann Whitney U). N=5 (WT_SAL), 5 (WT_PQ/MB), 10 (DAT_SAL), 8 (DAT_PQ/MB). Means + SEM.

Table S1. Body weight in DAT-KD and WT mice

	WT	**DAT-KD**
5 weeks	17.1±0.44 (n=11)	17.7±0.39 (n=20)
18 months	27.9±0.74^^ (n=7)	34.1±2.76**^^ (n=7)

Body weights as mean±SEM, N given in brackets (**p<0.01, compared to WT; ^^p<0.01 compared to 5 weeks, 2x2 genotype x age ANOVA, Holm-Sidak).

Table S2. Effect of paraquat/maneb (PQ/MB) exposure and DAT knock-down on microglial activation and alpha-synuclein aggregation

	WT saline		WT PQ/MB		DAT-KD saline		DAT-KD PQ/MB	
	resting	activated	resting	activated	resting	activated	resting	activated
IBA-1 cells in SN (n/mm^2)	2.75 ±0.22	0.26 ±0.01	2.8 ±0.16	0.27 ±0.03	2.73 ±0.17	0.25 ±0.02	2.55 ±0.23	0.20 ±0.02
IBA-1 IF in Striatum (mean pixel intensity)	25.9±1.93		27.5±2.20		24.2±1.71		22.7±0.87	
alpha-synuclein IF in SNc (mean pixel intensity)	23.4±1.81		25.7±2.65		28.9±4.00		21.2±2.57	
alpha-synuclein IF in SNr (mean pixel intensity)	41.0±6.01		43.6±4.29		46.0±6.47		37.3±3.71	

Quantification of IBA-1 cells (n/mm^2) with activated or resting morphology in the SN, IBA-1 mean immunofluorescence (IF) intensity per pixel (arbitrary units) in the striatum and alpha-synuclein mean IF intensity per pixel (arbitrary units) in the SNc and SNr of WT and DAT-KD mice (5 weeks old) injected with saline or PQ/MB. Data are shown as the mean±SEM; 2X2 genotype x toxin between-subject ANOVA, Holm-Sidak, not significant.

Supplemental methods

Behavior

Spontaneous activity and motor coordination

Fore- and hindlimb use was measured in 5 week-old DAT-KD and WT mice in a small transparent cylinder (height, 15.5 cm, diameter, 12.7 cm) placed on glass with a mirror positioned at an angle to allow for a view of movements along the ground and walls of the cylinder (Fleming et al. 2004). Videotapes were viewed and rated in slow motion by an experimenter blind to the mouse genotype and drug condition to assess forelimb and hindlimb steps, and time spent grooming. For the pole test mice were put head up on a vertical wooden pole with a diameter of 10 mm in the home cage (Fleming et al. 2004). Time to turn and time to descend after the turn

were recorded in 5 consecutive trials. If the mouse failed to turn or slid downwards, a cutoff time of 30 seconds for time to turn and for time to descend was noted.

Adhesive removal test

Animals were tested in their home cage; nesting material, food pellets and littermates were removed from the cage to minimize distraction. A round 1/4" adhesive-backed sticker was placed on top of the snout of the mouse. The mouse was placed back into the home cage and the latency for the mouse to first touch the sticker with the forepaws (contact latency) and to fully remove the sticker (removal latency) was recorded. A cut-off of 2 minutes was used if the mouse did not remove the sticker. The procedure was repeated for a total of 3 trials, and the mouse was given a 1 minute inter-trial interval to reduce the impact of stress. This test has been shown to be sensitive to dopamine loss in another mouse model where deficits can be improved by administration of the dopamine precursor L-DOPA (L-3,4-dihydroxyphenylalanine) (Lam et al. 2011).

Histology

Mice were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and intracardially perfused with 0.1 M phosphate buffered saline (PBS) at room temperature followed by 4% paraformaldehyde. Brains were quickly removed, post-fixed for 24 hours in 4% paraformaldehyde at 4°C, cryoprotected in 30% sucrose in 0.1 M PBS, frozen on powdered dry ice and stored at -80°C. Free-floating coronal sections (40 μm) were cut on a Leica CM1850 cryostat. Investigators blind to genotypes and treatments performed histological processing and analyses.

Immunohistochemisty for tyrosine hydroxylase

Sections were washed in 50 mM tris buffer solution (TBS), incubated in 0.5% hydrogen peroxide for 15 minutes, washed in 50 mM TBS and then transferred into a blocking solution containing

50 mM TBS, 10% Normal Goat Serum (NGS) and 0.5% Triton X. Sections were incubated at 4°C overnight with an anti-tyrosine hydroxylase (TH) antibody (primary rabbit polyclonal antibody 1:600, Millipore AB152) in a 5% NGS and 0.5% Triton X solution. Sections were equilibrated at room temperature for 1 hour, washed in TBS, incubated with a goat anti-rabbit biotinylated secondary antibody (1:600, Vector BA-1000) in TBS with 2% NGS and 0.5% Triton X, washed in TBS and then incubated for 45 minutes in a 50 mM TBS solution containing an avidin-biotin complex (Vectastain ABC Peroxidase Kit Elite Series, Vector). Tissue sections were subsequently washed in TBS and then incubated in a 50 mM TBS solution containing 3,3'-diaminobenzidine (DAB; Sigma) and 0.3% H_2O_2 (Sigma) to reveal staining. Sections were rinsed with TBS and mounted on charged glass slides. Nissl staining was performed 24 hours later by rehydrating the sections in a series of graded ethanol solutions and water. Next, tissue sections were incubated in a filtered cresyl violet solution (FD Cresyl Violet Solution, FD Neurotechnologies PS102-2) for 2 minutes. Slides were then quickly washed and placed in a solution of 95% ethanol with 0.1% glacial acetic acid. Sections were dehydrated in increasing concentrations of ethanol, placed in xylenes and then mounted with Eukitt mounting medium (Calibrated Instruments, Hawthorne, NY).

Quantification of iron-containing cells in the substantia nigra

Briefly, sections were washed for 30 s in double-distilled water prior to staining, then incubated for 30 min in a freshly prepared solution of equal parts 2% HCl and 2% potassium ferrocyanide. After washing with 50 mM TBS, sections were immersed in 50mM TBS with 0.5% hydrogen peroxide for 15 min, to quench endogenous peroxidase activity, and then incubated in DAB/ hydrogen peroxide solution. Stereo Investigator software (MicroBrightField, Williston, VT) coupled to a Leica DM-LB microscope with a Ludl XYZ motorized stage and z-axis microcator (MT12, Hendenheim, Traunreut, Germany) was used to count iron-positive cells in the substantia nigra (SN). The SN was delineated at 5X magnification and iron-positive cells were counted at

40X magnification within counting frames randomly chosen by the software. Both hemispheres of one nigral section per mouse were counted and the total number of iron-positive cells was compared between groups (total counts and not a stereological estimate).

Quantification of striatal TH, DAT, IBA-1 and striatal and nigral alpha-synuclein protein

Striatal sections (40 µm; rostral, medial and caudal striatum) and sections of the SNc (for alpha-synuclein staining) were rinsed in 50 mM TBS, placed in a blocking solution of 10% NGS and 0.5% Triton X for one hour and incubated overnight at 4°C in the primary antibody (TH, Millipore; DAT, Millipore; anti-IBA-1, Wako, 019-19741; alpha-synuclein, BD Biosciences) in TBS with 2% NGS and 0.5% Triton X. Sections were equilibrated at room temperature for one hour, washed in TBS, incubated for 90 minutes in secondary antibody (1:600, Goat anti-rabbit Cy3, Millipore AP132C; goat anti-mouse Cy5) in 50 mM TBS and 2% NGS, washed in TBS, rinsed in tap water and mounted on charged glass slides. The slides were stored at 4°C protected from light. For image acquisition, slides were loaded into a Agilent microarray scanner equipped with 2 photomultiplier tubes which simultaneously detected cyanine-3 (Cy-3, 550-610 nm) and cyanine-5 (Cy-5, 650-650 nm). Images for Cy3 and Cy5 immunofluorescent excitation were transferred to ImageJ (NIH) and the mean signal intensity per striatum or SNc was quantified. The left and right hemispheres of each sample were averaged for quantification.

Quantification of activated versus resting IBA-1-positive microglia in the SN

Briefly, sections were washed in 0.1M PBS, incubated in 0.5% H2O2 in methanol for 30 min, washed in PBS, incubated for 1 hour in a blocking solution (0.1M PBS, 10% normal goat serum, 0.5% Triton-X), incubated overnight with a primary antibody against IBA-1 (polyclonal rabbit anti-IBA-1; 1:500 dilution; Wako Pure Chemical Industries Ltd., Japan), washed in 0.1M PBS, incubated in biotinylated secondary antibody solution (goat anti-rabbit IgG, 1:200 dilution; Vector Laboratories, Inc., Burlingame, CA), washed in 0.1M PBS, incubated in avidin-biotin

complex (ABC; Vector Laboratories, Burlingame, CA), washed in 0.1M PBS, incubated in DAB/H2O2 (Sigma) solution to reveal staining, mounted and coverslipped with Eukit mounting medium (Calibrated Instruments, Hawthorne, NY). Stereo Investigator software (MicroBrightField, Colchester, VT) and a Leica DM-LB microscope were used to outline the SN and count IBA-1 positive cells within counting frames randomly selected by the software. As previously described, activated microglia were identified by their large (>6 μm), round cell bodies and few projections, whereas resting microglia were identified by their small (<4 μm) cell bodies and multiple processes (Watson et al. 2012). The numbers of resting and activated microglia counted were normalized to the area of the SN for each hemisphere, and the normalized values were averaged for each mouse.

Analysis of aggregated alpha-synuclein

Briefly, sections were incubated at room temperature for 10 minutes in 0.1 M PBS containing 10 μg/mL of Proteinase K (Invitrogen, Carlsbad, CA) prior to incubation in a "mouse-on-mouse" blocking solution (Vector Laboratories, Burlingame, CA). Sections were then incubated overnight with a primary antibody for alpha-synuclein (1:250, mouse anti-alpha-synuclein, BD Biosciences, San Jose, CA) that recognizes both mouse and human alpha-synuclein (van der Putten et al. 2000). Following incubation with a secondary antibody (biotinylated goat anti-mouse IgG) and 50 mM TBS solution containing an avidin-biotin complex (Vectastain ABC Peroxidase Kit Elite Series, Vector), staining was revealed with DAB (Sigma) and 0.3% H_2O_2 (Sigma). Sections were mounted (Eukitt mounting medium, Calibrated Instruments, Hawthorne, NY) and qualitatively examined for the presence of alpha-synuclein-positive aggregates using light microscopy with a magnification between 5-60X.

HPLC analysis of monoamine content in the striatum

Electrochemical recordings were obtained and analyzed using EzChrom Software (Agilent, Santa Clara, CA) and compared against standards of known concentration every 24 hours. Two preparation methods were used. Striatal tissue from the left hemisphere was sonicated in an extraction buffer containing 0.2 M PCA, 100 µM EDTA and 2 µM isoproterenol (internal standard). Dopamine (DA), serotonin (5-HT) and norepinephrine (NE) were resolved using a mobile phase consisting of 50 mM sodium acetate, 0.50 mM sodium dodecane sulfonate, 10 µM EDTA, 12% acetonitrile, 9% methanol, pH 5.5, pumped at a rate of 200 µL/min (model LC-10AD; Shimadzu, Columbia, MD) through a 100 x 2 mm column (2 µm, Super-ODS C18, Tosoh Bioscience, Montgomeryville, PA). The metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) were resolved using a mobile phase consisting of 50 mM sodium phosphate buffer, pH 2.85, 6% methanol, 1.4 mM heptane sulphonic acid, 10 µM EDTA through a 100 X 2 mm (3 µm, Hypersil C18, Keystone Scientific) column. A different method was used for tissue from the right striata in order to improve isolation and detection of an additional dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL). In this case, tissue was sonicated in an extraction buffer containing 0.2 M PCA, 100 µM EDTA and 100 nM DHBA (internal standard) followed by a subsequent alumina extraction. Dopamine, DOPAC, and DOPAL were resolved using a mobile phase consisting of 0.071 M citric acid, 0.014 M sodium acetate, 15% methanol, 423 mg/mL sodium-1-octane sulfonate, 5 mg/L disodium EDTA, pumped at 0.5 mL/min through an Eicom SC-5ODS column maintained at 25°C fitted with an AC-ODS precolumn (Eicom, San Diego, CA). Due to the lack of a commercial standard, we synthesized DOPAL by incubating 200 uM dopamine with 0.1 mg/mL monoamine oxidase in 100 mM PBS (pH 7.2) at 37°C.