Supplemental material for:

"*Footprints of a microbial toxin from the gut microbiome to mesencephalic mitochondria*"

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Supplemental Table S1. List of Reagents

REAGENT or RESOURCE	SOURCE
Antibodies – IHC/IF	
Rabbit anti-Occludin	Invitrogen (Cat. No. 40-4700)
Rabbit anti-α-synuclein aggregate antibody [MJFR-14-6-4-2]	Abcam (Cat. No. ab209538)
Mouse Anti-phosphorylated α-synuclein biotin-conjugated (pSyn#64) (S129P)	Wako (Cat. No. 010-26481)
Rabbit anti-ZO-1	Abcam (Cat. No. ab96587)
Rabbit anti-CD4	Cell Signalling (#25229)
Mouse anti-CD11b	BioRad (MCA711GT)
Rabbit anti-tyrosine hydroxylase (TH)	Millipore (Cat. No. AB152)
Mouse anti-ChAT	ThermoFisher Scientific (Cat. No. MA5-31383)
Rabbit anti-Iba1	FUJIFILM Wako Chemicals (Cat. No. 019-19741)
Sheep anti-Trem2	R&D Systems (Cat. No. AF1729)
Donkey anti-Rabbit IgG H&L Alexa Fluor 488	Abcam (Cat. No. ab150073)
Biotinylated anti-rabbit IgG	Vector Labs (Cat. No. BA-1000)
Biotinylated anti-mouse IgG	Vector Labs (Cat. No. BA-9200)
Donkey anti-sheep IgG H&L Alexa Fluor 647	Abcam (Cat. No. ab150179)
Goat anti-mouse Alexa Fluor 488	Molecular Probes, Life Technologies (Cat. No. A11001)
Goat anti-mouse Alexa Fluor 594	Molecular Probes, Life Technologies (Cat. No. A11005)

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Goat anti-rabbit Alexa Fluor 488	Molecular Probes, Life Technologies (Cat. No. A11008)
Goat anti-chicken 594	Molecular Probes, Life Technologies (Cat. No. ab96948)
Mouse anti-Lamp1	clone H4A3 from the Developmental Studies Hybridoma Bank
Antibodies – Flow Cytometry	
Mouse anti-CD45 PerCP (Clone 30F11)	Miltenyi Biotec (Cat. No. 130-102-469)
Mouse anti-CD3 FITC (Clone REA641)	Miltenyi Biotec (Cat. No. 130-119-798)
Mouse anti-CD4 APC (Clone REA604)	Miltenyi Biotec (Cat. No. 130-116-487)
Mouse anti-CD8a PE (Clone REA601)	Miltenyi Biotec (Cat. No. 130-123-781)
Rat anti-IgG2a PerCP	Miltenyi Biotec (Cat. No. 130-103-094)
REA Control-FITC	Miltenyi Biotec (Cat. No. 130-113-449)
REA Control-PE	Miltenyi Biotec (Cat. No. 130-113-450)
REA Control-APC	Miltenyi Biotec (Cat. No. 130-113-446)
Antibodies – Western Blotting	
Rabbit anti-PSD95	Abcam (Cat. No. ab2723)
Mouse anti-β3-Tubulin	Cell Signaling (Cat. No. 4466)
Mouse anti-α-synuclein LB509	Zymed Laboratories Inc. (Cat. No. 180215)
Rabbit anti-α-synuclein, oligomer specific Syn-33	Sigma (Cat No. ABN2265)
Rabbit anti-LC3B	Cell Signaling (Cat. No. 3868)
Rabbit anti-phospho DRP1 (serine 616)	Cell Signaling (Cat. No. 3455s)
Goat anti-rabbit IgG	GE Healthcare (Cat. No. NIF1317)

Goat anti-mouse IgG	Thermo Fisher Scientific (Cat. No. 31320)
Rabbit anti-TLR7	Boster Biological Technology (Cat. No. PA1733)
Mouse anti-synaptophysin	Sigma (Cat. No. S5768)
Rabbit IL-1β	Santa Cruz Biotechnology (Cat. No. sc-7884)
Mouse anti-TLR4	Santa Cruz Biotechnology (Cat. No. sc-293072)
Mouse anti-SDHA	Abcam (Cat. No. ab137746)
Mouse anti-α-tubulin	Sigma (Cat. No. T6199)
Mouse β-actin	Sigma (Cat. No. A5441)
Rabbit anti-TOM20	Santa Cruz Biotechnology (Cat. No. sc-11415)

Kits

NFκB p65 Total SimpleStep ELISA Kit	Abcam (Cat. No. ab176648)
ELISA Kit for Dopamine	MyBioSource (Cat. No. MBS2700357)
Mouse IL-8 ELISA Kit	MyBioSource (Cat. No. MBS776466)
α Synuclein oligomer (SNCO α) ELISA Kit	MyBioSource (Cat. No. MBS724099)
Mouse IFNɣ Quantikine ELISA	R&D Systems (Cat. No. MIF00)
Mouse IL-6 Quantikine ELISA	R&D Systems (Cat. No.M6000D)
Mouse IL-1β Quantikine ELISA	R&D Systems (Cat. No. MLB00C)
Mouse IL-10 Quantikine ELISA	R&D Systems (Cat. No. PM1000B)
Mouse IL-17 Quantikine ELISA	R&D Systems (Cat. No. DY421-05)
NZY Soil gDNA Isolation kit	Nzytech, Lda (Cat. No. MB21802

Mouse TNF-α Quantikine ELISA	R&D Systems (Cat. No. MTA00B)
Chemicals	
10-N-Nonyl acridine orange (NAO)	Enzo (Cat. No. 08091739)
L-BMAA hydrochloride	iChemical (Cat. No. EBD13091)
MitoTracker Green	Invitrogen (Cat. No. M7514)
Ammonium chloride (NH_4Cl)	Merck KGaA (Cat. No. 9434)
Calcium Green-5N	Molecular Probes, Life Technologies (Cat. No. C3739)
Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM)	Molecular Probes, Life Technologies (Cat. No. T668)
Adenosine 5' diphosphate (ADP) potassium salt	Sigma (Cat. No. A5285)
Antimycin A	Sigma (Cat. No. A8674)
Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)	Sigma (Cat. No. C2920)
Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)	Sigma (Cat. No. C2759)
Caspase 1 substrate	Sigma (Cat. No. SCP0066)
Oligomycin	Sigma (Cat. No. J60211)
Polyethyleneimine (PEI)	Sigma (Cat. No. 408700)
Rotenone	Sigma (Cat. No. R8875)
Succinic acid	Sigma (Cat. No. S3674)
2-deoxy-D-glucose (2DG)	Sigma (Cat. No. D8375)
Glucose	Sigma (Cat. No. G8270)
Leupeptin	Sigma (Cat. No. L2023)
5-Fluoro-2′-deoxyuridine (FDU)	Sigma (Cat. No. L2023)

Hoechst	Invitrogen (Cat. No. H1399)
Sodium pyruvate	Sigma (Cat. No. S8636)
Light (0% sugar), fruits of the forest flavored gelatin	Royal®
Banana flavor	LorAnn Oils (Cat. No. 3510-0500)
Almond flavor	LorAnn Oils (Cat. No. 3500-0500)
3,3'-Diaminobenzidine Tetrahydrochloride hydrate 9%	Thermo Fisher (CAS 868272-85-9)
Histopaque 1083	Sigma (Cat. No. 10831-100mL)
DPX Mountant	Sigma (Cat. No. 06522-100mL)
Vectastain Elite ABC Perox standard kit	Vector Labs. (VCPK-6100)
Normal Goat Serum	Abbkine (Cat. No. BMS0050)
Normal Donkey Serum	Abbkine (Cat. No. BMS0140)
M.O.M.® (Mouse on Mouse) Blocking Reagent	Vector Labs (MKB-2213-1)
OCT mounting medium	Carl Roth (Cat. No. KMA-0100-51A)

DETAILED METHODS

Animal model and experimental design

A total of 36 (20 untreated and 16 treated orally with BMAA) 10-month-old (adult) C57BL/6 male mice were used in this study in different cohorts. Mice were obtained from Charles River (Barcelona, Spain) and maintained at our animal colony (Animal Research Center, University of Coimbra), under controlled light (12h day/night cycle), temperature and humidity (45–65%), with free access to standard hard pellets chow and water. Signs of distress were carefully monitored and although it did not occur, a rapid decrease in body weight >15-20% was defined as a potential humane endpoint for the study. The EU and Portuguese legislation (Directive 2010/63/EU; DL113/2013, August 7) for the care and use of animals were followed. All procedures were in accordance with the ethical standards of the Animal Welfare Committee of the Center for Neuroscience and Cell Biology and Faculty of Medicine, University of Coimbra, and the researchers received adequate training (FELASA certified course) and certification from Portuguese authorities (Direção Geral de Veterinária) before the experiments.

To determine the effects of oral administration of the microbial toxin, BMAA, mice were randomly divided in two groups: 16 C57BL/6 mice were daily orally administered with BMAA (0.1 g/Kg bw, in commercially available gelatin) for 12 weeks (between 26 to 38 weeks of age). The concentration of BMAA was selected according to previous studies available in the literature.[1] The remaining mice (20) used as control group received normal gelatin free of BMAA. Body weight was monitored twice/week throughout the study. Immediately before euthanasia, animals were also weighed. Results were expressed as body weight (g). Immediately after euthanasia total blood was collected from selected animals to determine occasional blood glucose levels by the glucose oxidase reaction, using a glucometer (Glucometer-Elite, Bayer SA, Portugal) and compatible stripes. Results were expressed as mg glucose/dL blood. Fecal pellets from animals placed individually in a clean cage were collected at the end of the experiments (38 weeks).

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Microbiome Profiling

Fecal pellets collected at week 12 and samples of animals´ ileum and cecum mucosa-associated material were used for microbial DNA extraction and microbiome profiling. Microbial genomic DNA of frozen samples was extracted using the NZY Soil gDNA Isolation kit (NZYTech Lda, Portugal), which includes a mechanical lysis step (with glass beads). The amount and quality of genomic DNA extracted were evaluated in a Nanodrop 2000 (Thermo Scientific). DNA integrity was accessed by PCR using universal primers for the 16S rRNA gene [27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AGAAAGGAGGTGATCCAGCC-3')] as previously described.[2] Total DNA was sequenced at our sequencing facilities (Genoinseq, https://www.cnc.uc.pt/en/services) using the Illumina MiSeq® platform (Illumina, USA). Universal forward primer 515F-Y (5'-GTGYCAGCMGCCGCGGTAA-3') and reverse primer 926R (5'-CCGYCAATTYMTTTRAGTTT-3')[3] were used to target the hypervariable V4-V5 region using a standard protocol. Treatment of raw data, clustering and taxonomic annotation were performed with mothur package version 1.44.1 (www.mothur.org)[4] and Silva reference files, release 138.[5] Comprehensive meta-analysis of microbiome data, including community profiling, differential abundance and statistical analyses were performed with the online tool MicrobiomeAnalyst[6] and its R package DESeq2.[7] Alpha diversity, which measures within-sample taxonomic diversity and was used to determine if BMAA-treatment induced an increase or decrease in microbiota diversity, was estimated with unfiltered data using the Shannon index as a measure, and statistical significance was assessed with a Mann-Whitney test. Beta diversity, which measures the similarity or dissimilarity between different populations and was used to assess if BMAA-treatment induced changes in the overall composition of the microbial community, was evaluated by principal coordinate analysis using the Bray-Curtis index, after filtering samples for low abundance features based on the mean abundance of OTUs, and for low variance using the inter-quartile range assessment and, in the case of ileum samples, correcting for uneven sequencing depth using the total sum scaling method. Statistical significance was evaluated using permuted analysis of variance (PERMANOVA) complemented with permuted analysis of dispersion (PERMDISP). Heatmaps, stacked bar plots and pie-charts were obtained with MicrobiomeAnalyst after filtering samples for low abundance based on prevalence, low variance based on inter-quartile range, and

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transforming data using the centered-log ratio method. Differential abundance analysis, including statistical analysis and calculation of fold-changes for differentially abundant genera, was also performed on filtered data using the DESeq2 algorithm as implemented by MicrobiomeAnalyst.

Behavioral analyses

Mice were submitted to a battery of behavioral tests starting at the end of treatments (from week 38 to 40). All tests were performed during night cycle and with a minimum of 60 min of acclimatization to the behavioral testing room.

Beam Walking Test: Balance and fine motor coordination were assessed by the ability of the mice to cross a narrow beam to reach an enclosed escape platform.[8] The test was performed in 1 m long wood round beam, with 8 mm of diameter. Mice were allowed up to 90 s to transverse each beam and performed 2 trials for each beam. A maximum value of 90 s was attributed to any rodent that did cross in the time given. Time to cross the beam was evaluated.

Hindlimb Clasping Test: Hindlimb clasping reflex scoring was used as an indicator of mice neurodegeneration.[9] Mice were suspended by the mid-section of the tail and observed over 10 s. Hindlimb clasping was rated from 0 to 3 based on the extent to which the hindlimbs clasped inward: 0=no clasping, hindlimbs freely splayed outward and away from the abdomen, 1=one hindlimb clasped inward for at least 50% of the observation period, 2=both hindlimbs partially clasped inwards for the majority of the observation period, 3=both hindlimbs completely clasped inwards, showing no signs of flexibility.

Inverted Grid Test: Inverted grid test was used as an indicator of mice grip strength.[10,11] Mice were placed in the center of a wire mesh and the grid was inverted at a height of 40 cm above soft padding. Mice were observed and time spent until they released their grip or hold for 60 s was evaluated.

Open Field Test: Open field test was used for the assessment of locomotor horizontal activity and anxiety-like activity in mice.[8] Behaviors were evaluated in an open field squared arena with 50 cm wide × 50 cm deep × 50 cm high. Mice were placed individually in the center of the arena and activity was recorded for 30 min with Acti-

Track System (PanLab, Barcelona, Spain). During the whole experiment the operator was outside the experimental room. Total distance travelled and number of feces expelled in the experimental time, mean velocity of mice, percentage of time resting and time spent in the center of the arena were evaluated.

T-Maze Test: Spontaneous T-maze alternation was used to assess the cognitive ability of rodents.[12] Spatial working memory was evaluated in an enclosed T-maze apparatus with 30 cm length × 10 cm width × 20 cm high. Mice were placed in the base of the T apparatus, with the central partition in place, and allowed to choose one of the goal arms. They were then confined in the chosen arm for 30 s. After that time, the animal was gently removed, as well as the central partition, immediately followed by replacing the rodent in the start area, facing away from the goal arms. Again, mice were allowed to choose between the two open goal arms. 5-6 trials blocks were performed, with each individual trial not taking more than 2 min. The percentage of alternation (defined by, on the second trial choosing the arm not visited before) and time spent to choose one arm were evaluated.

Perfusion

At the end of the experiment, animals were deeply anesthetized with sodium pentobarbital (150 mg/kg) and transcardially perfused with saline (0.9% NaCl) followed by 50 mL of fixative solution (4% paraformaldehyde (PFA) and 0.1% glutaraldehyde in PBS) for 24 h at 4 °C. Brains were then removed and post fixed for 24 h in fixative solution at 4 °C. For TH determination, 6 WT and 5 BMAA-treated mice brains were used, while for aSyn, p-aSyn, CHAT, CD4 and IgG determinations 4 brains were used in both conditions.

Ileum samples were obtained from 4 WT and 4 BMAA-treated mice. The intestines of mice were removed, rinsed with PBS, and sliced in one-centimeter pieces. The ileum was fixed for 24 h in fixative solution at 4 °C. Brain and Ileum were cryoprotected using increasing concentrations of sucrose in PBS (10, 20 and 30%), embedded in Tissue-Tek (Sakura, Finetek, Torrance, CA, USA) and frozen in isopentane with dry ice. Samples were kept at -80 °C until sectioning. Thaw-mounted 20-µm coronal sections were cut on a cryostat (Cryostar NX50, ThermoScientific) at −20 °C and mounted in SuperFrost© microscope slides (Thermofisher).

Immunofluorescence

Sections were thawed for 1 h, washed with PBS and incubated in a solution containing 10% donkey or goat serum (TebuBio) and 0.25% Triton X-100 in PBS for 60 min in a humid chamber at room temperature. In case of using mouse primary antibodies on mouse tissue, M.O.M. Mouse Ig Blocking Reagent was applied for 1h before the blocking step. Slides were drained and further incubated with rabbit-derived anti-ZO-1 (Abcam, 1:300), rabbit-derived anti-Occludin (Life Technologies, 1:300) or mouse-derived anti-CD11b (BioRad, 1:200) in PBS containing 1% donkey serum and 0.25% Triton-X-100 for 24 h at 4 °C for ileum sections. In brain sections, rabbit-derived anti-TH (Millipore, 1:300) and mouse-derived anti-ChAT (ThermoFisher Scientific, 1:100) was diluted in PBS containing 1% goat serum and 0.25% Triton-X-100 for 24 h at 4 °C. Sections were then incubated for 45 min with secondary antibody donkey anti-rabbit or anti-mouse Alexa Fluor 488 (Life Technologies, 1:250) or goat anti-mouse or anti-rabbit Alexa Fluor 594 (Life Technologies, 1:250). Sections were profoundly washed and incubated for 10 min with Hoechst 33342 (Sigma, 1:1000). Finally, sections were mounted with Mowiol© (Sigma).

Immunohistochemistry

Cryosections were thawed for 1 h, hydrated with PBS and treated for antigen retrieval following two cycles of microwave treatment (heating 4 min) with 0.01 M citrate buffer (pH 6.0). Sections were quenched with 1% hydrogen peroxide in methanol for 20 min and incubated in a solution containing PBS, 10% goat serum (TebuBio) and 0.25% Triton X-100 for 60 min in a humid chamber at room temperature. In case of using mouse primary antibodies in mouse tissue, M.O.M. Mouse Ig Blocking Reagent was applied for 1h before the blocking step. Slides were drained and further incubated with rabbit-derived anti-tyrosine hydroxylase (anti-TH, Merck, 1:300), rabbit-derived anti-aSyn (Abcam, 1:500), rabbit-derived anti-CD4 (Cell Signalling, 1:200) or mouse-derived anti-p-aSyn (WAKO, 1:500) in PBS containing 1% goat serum and 0.25% Triton-X-100 for 24 h at 4 °C. Sections were then incubated for 45 min with biotinylated goat anti-rabbit or anti-mouse IgG (Vector, 1:200). The secondary antibody was diluted in PBS containing 0.25% Triton-X-100, followed by incubation with the avidin/biotin complex-HRP (VECTASTAIN Elite ABC Kit Standard, Vector

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Laboratories, CA, USA) for 30 min. The peroxidase was visualized with a standard diaminobenzidine/hydrogen peroxide reaction for 2 min. aSyn, p-aSyn and CD4 sections were counterstained with 1% cresyl violet. Tissue was dehydrated using increasing concentrations of ethanol, cleared in xylene and mounted in DPX mountant (Sigma).

Assessment of intestinal barrier integrity.

Immunofluorescence images of ZO-1 and Occludin staining were acquired in a confocal microscope LSM710 (Zeiss) with a 20× magnification objective at 1024×1024 resolution. Intestinal barrier integrity was assessed by establishing a score system scale where 0 = fluorescence intensity similar to background, 1 = Low fluorescence intensity, 2 = High fluorescence intensity and 3 = High fluorescence intensity + well defined expression in membrane.[13,14] Between 7-10 images with 3-5 villi per image were acquired randomly per animal and blindly scored using this scale.

For the CD11b assessment, images were acquired using confocal microscope LSM710 (Zeiss) with a Plan-Apochromat 40×/1.4 Oil DIC M27 objective at 1024×1024 resolution. A total of 192 villi were analyzed (21-24 villus per animal). To assess the number of CD11b cells, ten images were randomly acquired per animal and CD11b positive cells were counted and divided per the total counting area (mm^2).

For the CD4 assessment, images were acquired in Axio Imager Z2 microscope (Zeiss) at 40× magnification. A total of 169 villi were analyzed with an average of 21 villi per animal. To assess the number of CD4 cells, an average of thirteen images were randomly acquired per animal and CD4 positive cells were counted and divided per the total counting area (mm^2).

All procedures of immunostaining, image acquisition and quantification were blindly performed.

TH immunoreactivity in the Striatum

Slides were scanned at 20× magnification with Slide Scanner AxioScan (Zeiss). A total of eight coronal sections systematically distributed through the anteroposterior axis of

the striatum, with an interval of evaluation of ten per animal, were quantified. The optical density of striatal TH positive fibers was measured using ImageJ software (Version 1.40 National Institute of Health). Images were converted to 8-bit grayscale and the mean intensity of striatal immunoreactivity was quantified. Values were transformed from pixels to optical density (OD) using Kodak No. 3 Calibrated Step Tablet template as pattern curve. To correct the effect of non-specific background staining, the measured values were corrected by subtracting values obtained from adjacent cortical areas.

Stereological analysis of TH^+ cells in the Substantia Nigra

The number of tyrosine hydroxylase-positive (TH) cells in the Substantia nigra (SN) was estimated using the optical fractionator method in combination with the dissector principle and unbiased counting rules.[15] The SN was analyzed with Stereo Investigator software (MBF Bioscience) attached to Axio Imager Z2 microscope (Zeiss). A total of eight sections systematically distributed through the anteroposterior axis of the SN with an interval of evaluation of seven per animal was included in the counting procedure. TH-positive cells were counted using 40× magnification (1.4 numerical aperture, oil immersion) objective. The grid size was 250 × 250 µm and the counting frames were 150 × 150 µm. Coefficient of error was calculated according to Gundersen and coworkers.[15] An error of CE < 0.1 (m=1 class) was accepted for the analysis.

Estimated number of TH^+ and $ChAT^+$ cells in the DMV

Slides were scanned at 20× magnification with Slide Scanner AxioScan (Zeiss). To quantify the number TH cells and ChAT cells in DMV, a total of 3-5 coronal sections systematically distributed through the anteroposterior axis were stained and quantified with an interval of evaluation of five per animal. Images were split in red and green channel after define DMV region. TH-positive cells (red channel) and ChAT-positive cells (green channel) were counted and divided per the total counting area (mm^2).

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aSyn Image Analysis

Images were captured at 20× magnification with Slide Scanner AxioScan (Zeiss). To measure aSyn expression in the DMV, SN, and in the Ileum, images were color deconvoluted using "Colour Deconvolution" plugin available for ImageJ software (https://imagej.net/Colour_Deconvolution). The OD of DAB images in the area of interest was measured as described above in "TH immunoreactivity in the Striatum". In this case, measured values were not corrected from control areas due to the ubiquity of aSyn expression. For the DMV and SN, between five and eight coronal sections systematically distributed through the anteroposterior axis were quantified with an interval of evaluation of five and seven per animal, respectively. For the Ileum, between seven and ten coronal sections per animal.

IgG immunostaining and Quantification

IgG immunostaining was performed as described above except for the use of a directly labelled antibody (biotinylated anti-mouse IgG, Vector, 1:1000). Slides were scanned at 20× magnification with Slide Scanner AxioScan (Zeiss). To quantify the number of brain microvascular vessels with blood-brain barrier breakdown in the Cortex, Striatum and SN. A total of eight coronal sections systematically distributed through the anteroposterior axis were stained and quantified with an interval of evaluation of ten per animal for Cortex and Striatum, and eight per animal for SN. IgG is a protein excluded from the brain parenchyma by the action of the BBB. Its presence in the brain parenchyma is associated with BBB permeability. To assess the BBB integrity, we quantified the number of microvascular leaks (IgG-immunopositive staining in the perivascular area) per total area (mm^2) in Cortex, Striatum and SN.

Trem2-Iba1 quantitative analyses

Frozen sections were thawed for 1 h and hydrated with PBS 1×. Sections were permeabilized with 0.2% Triton X-100 for 20 min, washed thrice with PBS 1× for 10 min each and blocked with 10% donkey serum for 1 h at 37 ºC. Primary antibodies (rabbit-derived anti-Iba1, 1:500, Wako and sheep-derived anti-Trem2 1:200, R&D Systems) were incubated in 1% donkey serum for 48 h at 4 ºC in a humidified chamber.

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Secondary antibodies (donkey anti-rabbit AlexaFluor 488 1:500, Abcam; AlexaFluor 647 1:500, Abcam) were incubated for 2 h at room temperature and washed three times with PBS 1× for 10 min each. Afterwards, sections were incubated with Hoechst 33342 for 15 min at room temperature and rinsed twice with PBS 1×, mounted in Mowiol and sealed with nail polish.

Images from identical stereological regions were acquired using confocal microscope LSM710 (Zeiss) with a Plan-Apochromat 40x/1.4 Oil DIC M27 objective at 1024×1024 resolution. A total of six images per animal across three different sections of SN were randomly acquired for $Iba1^+$ cells quantification. Z-stacks were converted to maximum projection images using Fiji image software. Images were thresholded using the Triangle algorithm and the number of cells were counted and divided by the field of view area.

To quantify the % area of Trem2 contained in $Iba1^+$ cells, images were split in red and green channel and were converted into 8-bit images. To create a binary mask, both images were set a threshold to remove the background. Green channel (Iba1) mask was overlapped with the red mask (Trem2) and the ratio (%) of the red area (Trem2) inside the green area (Iba1) was calculated. This was performed using Fiji image software and the acquisition and analysis was performed blindly.

Flow cytometry

Animals were deeply anesthetized with sodium pentobarbital (150 mg/kg). Blood was collected by cardiac puncture using a syringe with a 23G needle. Blood samples were placed in EDTA (0.5 M) coated tubes and rotary mixed for 1 h. Blood samples were diluted (1:1) in Phosphate-buffered saline (PBS) and transferred to 15 mL tubes containing Histopaque© 1083 solution (Sigma). Tubes were centrifuged at 400×*g* at RT for 30 min. PBMC halo was collected carefully with a Pasteur pipette and transferred to a new tube containing 5 mL PBS. Samples were washed twice with PBS and centrifuged at 250×*g* at 4 ºC for 10 min. The pellet was incubated with Anti-Mouse CD45 PerCP (Clone 30F11), Anti-Mouse CD3 FITC (Clone REA641), Anti-Mouse CD4 APC (Clone REA604) and Anti-Mouse CD8 PE (Clone REA601) (1/50) (Miltenyi biotec) for 10 min at 4 ºC for 10 min. The cell suspension was washed with PBS,

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centrifuged at 250×*g* at 4 ºC for 10 min. the pellet was fixed with 2% PFA solution for 10 min at 4 ºC and washed with PBS. Finally, cells were centrifuged at 250×*g* at 4 ºC for 10 min and the pellet was suspended in PBS ready to be analyzed by flow cytometry.

BD FACSCalibur cytometer (BD Bioscience) was previously set up adjusting voltages, compensated using single-stained cells and the true level of background was defined with the Isotype control antibodies Rat Anti-IgG2a PerCP, REA Control-FITC, REA Control-PE and REA Control-APC (Miltenyi Biotec). The gating strategy was performed by FlowJo© software (BD Bioscience). More than 10000 events were acquired in the region of interest (ROI) identified as the lymphocyte area in the forward versus side scatter dot plot. The percentage of CD4 and CD8 was obtained by gating the $CD45^+CD3^+$ events contained in ROI.

Preparation of Brain Homogenates

After completing the behavioral tests, WT and BMAA-treated mice (a total of 10) were deeply anesthetized under halothane atmosphere before killing by cervical dislocation/displacement for mesencephalon, striatum ileum and cecum isolation. Brain mesencephalon and striatal areas were snap frozen and stored at −80 °C. For western blot analyses of synaptic markers and innate immunity markers the mesencephalon was homogenized in 1% Triton X-100 containing hypotonic lysis buffer (25 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA, pH 7.5) supplemented with 2 mM sodium orthovanadate, 50 mM of sodium fluoride, 2 mM DTT, 0.1 mM PMSF, and a 1:1000 dilution of a protease inhibitor cocktail from Sigma (St. Louis, MO, USA). Tissue suspensions were then frozen three times in liquid nitrogen and centrifuged at 20000×*g* for 10 min. The resulting supernatants were retrieved and stored at −80 °C. For Caspase-1 determination and analysis of innate immunity markers with Elisa kits the mesencephalon was homogenized in lysis buffer (10 mM HEPES; 3 mM $MgCl_2$; 1 mM EGTA; 10 mM NaCl, pH 7.5), supplemented with 2 mM DTT, 0.1 mM PMSF and a 1:1000 dilution of a protease inhibitor cocktail and with 0.1% Triton X-100. Samples were then incubated on ice for 40 min and centrifuged at 2300 ×*g* for 10 min at 4 ºC. The resulting supernatant containing the cytosolic fraction was collected. For the determination of Dopamine levels with an Elisa Kit, striatal tissue was sonicated in ice-

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cold 0.2 M perchloric acid and centrifuged (13000 rpm, 7 min, 4 °C). Supernatants were stored at -80 °C until further analysis whereas the pellet was resuspended in 1 M NaOH and stored at -80 °C. Protein content was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions for plate reader.

Preparation of Intestine homogenates

The intestines of mice were removed, rinsed with PBS, snap frozen and stored at −80 °C. One-centimeter pieces of cecum and ileum were sliced. For homogenization, tissue was first rinsed again in PBS and then homogenized in lysis buffer (25 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA, pH 7.5) supplemented with 0.1% Triton X-100, 2 mM DTT, 0.1 mM PMSF and a 1:1000 dilution of a protease inhibitor cocktail as above. Tissue suspensions were frozen three times in liquid nitrogen, sonicated on ice and centrifuged at 17968×*g* for 10 min, at 4 ºC. Protein content of the resulting supernatants was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Maintenance and treatment of cell lines

NT2 (teratocarcinoma) cells containing mitochondrial DNA (Rho+) and depleted of mtDNA (Rho0) were used.[16] NT2 Rho+ cells were cultured in 75 cm^2 tissue culture flasks containing Optimem medium and 10% heat-inactivated fetal calf serum, penicillin (50 U/mL), and streptomycin (50 µg/mL). The media for the NT2-ρ0 cells consisted in Optimem medium containing 10% heat-inactivated fetal calf serum, penicillin (50 U/mL), and streptomycin (50 µg/mL), further supplemented with uridine (50 µg/mL) and pyruvate (200 µg/mL). Cells were maintained at 37 °C in a humidified incubator containing 95% air and 5% CO_2. 24 h after plating, cells were treated with 3 mM BMAA for 48 h the higher concentration that did not reduce NT2 Rho+ cell viability determined by the MTT-reduction test (data not shown). Afterwards, 2 h before harvesting, 5 µM CCCP was added in the culture medium where indicated. For all experimental procedures, controls were performed in the absence of those agents.

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Preparation and Treatment of Primary Mesencephalic neurons

Primary neurons were prepared from mesencephalon of C57Bl/6 mice embryos brains at gestation day 14/15 and cultured as described previously with some modifications.[17] Embryos were carefully removed under aseptic conditions and collected in Hanks' balanced salt solution (HBSS) [5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $NaH_2PO_4.H_2O$, 5 mM glucose, 5.36 mM sodium pyruvate, 5.36 mM Hepes, 0.001% Fenol Red, (pH 7.2)] at room temperature. Brains were then dissected and the ventral mesencephalon excised. Briefly, after careful removal of the meninges, tissues were mechanically sliced into small pieces in HBSS. The removed ventral mesencephalon tissue was incubated in HBSS solution containing trypsin (0.5 g/L) and DNase I (0.04 g/L) for 15 min at 37 °C. Tissue digestion was stopped by the addition of trypsin inhibitor (type II-S; 0.75 g/L) in HBSS containing DNase I (0.04 g/L), followed by a centrifugation at 140×*g* for 5 min. After washing the pellet once with HBSS, the cells were dissociated mechanically and suspended in fresh Neurobasal medium supplemented with 2 mM L-glutamine, 2% B-27 supplement, penicillin (100000 U/L), and streptomycin (100 mg/L) and 1% heat-inactivated FBS. Cells were then seeded on poly-L-lysine (0.1 g/L)-coated dishes. For western blotting analyses, caspase-1 determination and ELISA kits, mesencephalic neurons were seeded on poly-L lysine (0.1 mg/mL) coated six-well plates at a density of 1.3×10^6 cells/mL. For immunocytochemistry, mesencephalic neurons were seeded on poly-L-lysine (0.1 mg/mL)-coated coverslips at a density of 0.6×10^6 cells/mL. For the Seahorse experiments mesencephalic neurons were seeded on poly-L-lysine (0.1 mg/mL)-coated microplates at a density of 0.6×10^6 cells/mL. For cardiolipin and live imaging analyses, mesencephalic neurons were seeded on poly-L-lysine (0.1 mg/mL)-coated ibidi µ-Slide 8-well plates at a density of 0.6×10^6 cells/mL. For determination of mitochondrial membrane potential mesencephalic neurons were seeded on poly-L lysine (0.1 mg/mL) coated 24-well plates at a density of 1.3×10^6 cells/mL. Cultures were grown at 37 °C in a fully humidified air atmosphere containing 5% CO_2. On the 4th day *in vitro* half of the medium was replaced with serum-free medium and incubated with 1:2000 5-Fluoro-2′-deoxyuridine (FDU) to inhibit proliferating glial cells. We observed a low level of glial cell contamination in primary mesencephalic neuronal cultures (less than 1% of Iba1-, Trem2-, CD11b-positive cells and less than 20% of GFAP-positive cells). Half of the medium was changed on the 6th and 8th day to serum-free medium.

Immunocytochemistry was performed to observe the ratio between neurons and microglia in cultures and no contamination with microglial cells was observed (data not shown). After 14 days *in vitro*, cultured neurons were treated with 3 mM BMAA for 48 h, the higher concentration that did not reduce cell viability determined by the MTT-reduction test (data not shown). 2 h before cell harvesting, 1 μM CCCP was added to the culture medium where indicated. For all experimental procedures, controls were performed in the absence of those agents. Wherever indicated, 20 mM NH_4Cl and/or 20 μM Leupeptin (Sigma, St. Louis, MO, USA) were added for 4 h to the culture medium. The combination of NH_4Cl with Leupeptin blocks all types of autophagy, as it reduces the activity of all lysosomal proteases by increasing the lysosomal lumen pH without affecting the activity of other intracellular proteolysis systems.[18]

Preparation of Cellular Extracts

For the analysis of aSyn protein levels, of LC3 autophagic marker and of innate immunity markers by western blot, mesencephalic neurons and NT2 cells were washed in ice-cold PBS (1×) and lysed in 1% Triton X-100 containing hypotonic lysis buffer (25 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA, pH 7.5 supplemented with 2 mM sodium orthovanadate, 50 mM of sodium fluoride, 2 mM DTT, 0.1 mM PMSF, and a 1:1000 dilution of a protease inhibitor cocktail from Sigma (St. Louis, MO, USA). Cell suspensions were then frozen three times in liquid nitrogen and centrifuged at 20000×*g* for 10 min. The resulting supernatants were removed and stored at −80 °C. To prepare mitochondrial fractions for the analysis of phospho-Drp1 levels by western blot, neurons were washed in ice-cold PBS (1×) and scraped in a buffer containing 250 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, supplemented with 2 mM sodium orthovanadate, 50 mM of sodium fluoride, 0.1 mM PMSF, 2 mM DTT and 1:1000 dilution of a protease inhibitor cocktail followed by homogenization. Cells were centrifuged at 492×*g* for 12 min at 4 °C and the resulting supernatant was further centrifuged at 11431×*g* for 20 min at 4 °C. Pellets resulting from this step constitute a crude mitochondrial fraction. The mitochondrial fractions were then frozen three times with liquid nitrogen. To prepare cytosolic fractions for the analysis of innate immunity markers with Elisa kits, neurons were washed in ice-cold PBS and disrupted in lysis buffer (10 mM HEPES; 3 mM $MgCl_2$; 1 mM EGTA; 10 mM

NaCl, pH 7.5) supplemented with 2 mM DTT, 0.1 mM PMSF and a 1:1000 dilution of a protease inhibitor cocktail and supplemented with 0.1% Triton X-100. Neurons were scraped, transferred to the respective tubes and incubated on ice for 40 min. Samples were then centrifuged at 2300×*g* for 10 min at 4 ºC and the resulting supernatant contained the cytosolic fraction. For Caspase-1 determination neurons were washed once in ice-cold PBS (1×) and harvested on ice with a lysis buffer containing 25 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA, and 2 mM $MgCl_2$, supplemented with 2 mM DTT, 0.1 mM PMSF, and a 1:1000 dilution of a protease inhibitor cocktail. The cellular suspension was frozen/thawed three times on liquid nitrogen and centrifuged at 20000×*g*, for 10 min at 4 °C. The resulting supernatant was collected. Protein content was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions for plate reader.

Mitochondria isolation by Percoll gradient

Mesencephalon and cortex from mice were washed with ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.2/KOH. Mesencephalon and cortical mitochondria were then isolated using a discontinuous Percoll density gradient centrifugation. For this purpose, tissue was homogenized with 25 up and down strokes in Dounce All-Glass Tissue Grinder (Kontes Glass Co., Vineland, NJ, USA) using pestle A (clearance: 0.07-0.12 mm) followed 25 up and down strokes with pestle B (clearance: 0.02-0.056 mm). After a brief centrifugation at 1100×*g* for 2 min at 4 °C, the supernatant was mixed with freshly made 80% Percoll prepared in 1 M sucrose, 50 mM HEPES, 10 mM EGTA, pH 7.0, then carefully layered on the top of freshly made 10% Percoll (prepared from 80% Percoll) and further centrifuged at 18500×*g* for 10 min at 4 °C. The supernatant was discarded including the cloudy myelin containing fraction but leaving the mitochondria-enriched pellet in the bottom of the tube. The pellet was suspended in 1 mL washing buffer containing 250 mM sucrose, 5 mM HEPES-KOH, 0.1 mM EGTA, pH 7.2 and centrifuged again at 10000×*g* for 5 min at 4 °C. Finally, the mitochondrial pellet was suspended in ice-cold washing buffer and the amount of protein quantified by the Bio-Rad protein assay. Isolated mitochondria were kept on ice until further use for functional analysis. Alternatively, samples were frozen at -80°C.

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Analysis of Oxygen Consumption (OCR)

Mesencephalic neurons

Neurons were seeded in 24-well XF culture plates and placed in a 37 °C, 5% CO_2 incubator to determine OCR with a Seahorse XF24 Extracellular Flux analyzer (Seahorse Bioscience, Billerica, MA, USA). On the assay day, neurons were washed and placed in unbuffered medium (DMEM without glucose and without pyruvate, 2 mM glutamine, 31.6 mM NaCl and penstrep). The microculture plates were then degassed in a non-CO_2 incubator at 37 °C for 1 h before placement into the Seahorse Analyzer. The wells were analyzed according to the procedure described in the Seahorse OCR Test kit. Briefly, the respiratory analyses were performed by sequentially adding 1 μM oligomycin (inhibitor of ATP synthase), 2 μM CCCP (protonophore that uncouples oxidative phosphorylation) and 2 μM Antimycin A (Complex III inhibitor).

Isolated Mitochondria

OCR was measured in fresh mesencephalic or cortical mitochondria with a Seahorse XF24 Extracellular Flux analyzer (Seahorse Bioscience, Billerica, MA, USA). Where indicated, mitochondria were isolated from the mesencephalon or cortex of mice treated with or without BMAA, or isolated from the mesencephalon of wild-type mice (5 μg of protein per well) and incubated with 0.5, 1 or 3 mM BMAA for 30 min at 30 ºC. Mitochondria were then attached to 24-well XF culture plates pre-coated with polyethyleneimine (PEI, 1:15000 dilution from a 50% solution) in mitochondrial assay solution (MAS) containing 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA, pH 7.2, at 37 °C.[19,20] For this purpose, the multiwell plate was centrifuged at 2200×*g* for 20 min at 4 °C. In order to evaluate the mitochondria attachment efficiency, the plates were analyzed under light microscopy using 20× magnification to ensure consistent adherence to the wells (data not shown). After an incubation period of 8 min at 37°C, the multiwell plate was transferred to the Seahorse XF24 flux analyzer. The levels of respiratory coupling in isolated mitochondria and basal mitochondrial OCR were analyzed in MAS containing succinate (10 mM; Complex II substrate) plus rotenone (2 μM; Complex I inhibitor). Mitochondria were then energized by adding ADP (4 mM); respiration derived by ATP synthesis was then prevented by the addition of oligomycin (2.5 μg/mL; inhibitor of

ATP synthase). The addition of the uncoupler FCCP (4 μM) caused an increase in OCR reflecting the maximal respiratory chain activity as well as the maximal substrate oxidation rate. Finally, antimycin A (4 μM; Complex III inhibitor) was added to fully block the respiratory chain and the residual OCR.

Analysis of Glycolytic Fluxes (ECAR)

Mesencephalic neurons were seeded in 24-well XF culture plates and were placed in a 37 °C, 5% CO_2 incubator to determine glycolytic fluxes with a Seahorse XF (Seahorse Bioscience, Billerica, MA, USA). On the assay day, neurons were washed and placed in non-buffered Medium (DMEM without glucose, 2 mM glutamine, 5 mM pyruvate, 31.6 mM NaCl and penstrep). The microculture plates were then degassed in a non-CO_2 incubator at 37 °C for 1 h before placement into the Seahorse Analyzer. The wells were analyzed according to the procedure described in the Seahorse Glycolysis Stress Test kit. Briefly, the measure of protons produced indirectly via lactate released from cultured cells was used as an indicator of glycolysis and is provided by measuring the acidification of the medium (extracellular acidification rate—ECAR). The glycolytic stress test performed by sequentially adding 10 mM glucose, 1 μM oligomycin (to block mitochondrial respiration and force cells to rely on glycolysis for ATP production), and 100 mM 2-deoxyglucose (2-DG; glucose analog and inhibitor of glycolytic ATP production) provided information on glycolysis, the glycolytic capacity, and the glycolytic reserve.[21]

Seahorse data analysis

For the respiratory coupling experiments, the following determinations were calculated according to the ensuing “rate measurement equation”.[19]

Mesencephalic neurons

Nonmitochondrial respiration: minimum rate measurement after antimycin A injection; Basal Respiration: last rate measurement before first injection minus nonmitochondrial respiration; Maximal respiration: maximum rate measurement after FCCP injection

minus nonmitochondrial respiration; ATP synthesis: last rate measurement before oligomycin injection minus minimum rate measurement after oligomycin injection; Mitochondrial coupling efficiency: ratio between ATP production and Basal Respiration × 100).

For the glycolysis experiments, the following determinations were calculated: Glycolysis, minimum rate measurement after glucose injection minus maximum measurement after 2DG injection; Glycolytic capacity rate, minimum rate measurement after oligomycin injection minus maximum measurement after 2DG injection; Spare Glycolytic Capacity, minimum rate measurement after oligomycin injection minus minimum measurement after Glucose injection.

Isolated mitochondria

Basal respiration: last rate measurement before first injection; Maximal respiration: last rate measurement after FCCP injection; ATP synthesis: last rate measurement before oligomycin injection minus minimum rate measurement after oligomycin injection; H+ leak: minimum rate measurement after oligomycin injection minus measurement after antimycin A.

Mitochondrial Ca^{2+} handling capacity

Mitochondrial Ca^{2+} uptake was measured fluorometrically in fresh mesencephalic or cortical mitochondria, in the presence of the Ca^{2+}-sensitive fluorescent dye Calcium Green 5N (150 nM), using excitation and emission wavelengths of 506 nm and 532 nm, respectively, according to Pellman and coworkers[22] with minor modifications. Where indicated, mitochondria were isolated from the mesencephalon or cortex of mice treated with or without BMAA, or isolated from the mesencephalon of wild-type mice (5 μg of protein per well) which were incubated with 0.5, 1 or 3 mM BMAA for 30 min at 30 ºC. Calcium Green is a cell-impermeant visible light-excitable Ca^{2+} indicator that exhibits an increase in fluorescence emission intensity upon binding to Ca^{2+}; thus, a decrease in the Calcium Green fluorescence is function of external Ca^{2+} concentration which indicates the capacity of mitochondria to handle Ca^{2+}. Briefly, 5 μg of isolated mitochondria were added to the standard incubation medium containing 125 mM KCl,

0.5 mM $MgCl_2$, 3 mM KH_2PO_4, 10 mM HEPES, pH 7.4, 10 μM EGTA, supplemented either with 3 mM pyruvate, 1 mM malate, 3 mM succinate, 3 mM glutamate, 0.1 mM ADP and 1 μM oligomycin. After a basal fluorescence record four pulses of 10 μM $CaCl_2$, were added every 4 min and Ca^{2+} handling capacity plotted as a decrease in fluorescence units (RFU), which reflects the rate of decrease of Calcium Green-5N fluorescence. Calcium mitochondrial uptake levels were quantified determining the area under the curve using GraphPad software.

Western Blotting

Samples were suspended in 6× sample buffer (4× Tris-Cl/SDS, pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) under reducing conditions. For the analysis of aSyn oligomers, samples were suspended in 2× sample buffer (40% glycerol, 2% SDS, 0.2 M Tris-HCl pH 6.8, 0.005% Coomassie Blue) and loaded under non-denaturing conditions. Depending on the protein molecular weight, samples were loaded onto adequate % PAGE gels for the analysis of aSyn oligomers or SDS-PAGE gels for the remaining proteins. After transfer, the PVDF membranes (Millipore, Billerica, MA, USA) were incubated for 1 h in Tris-buffered solution (TBS) containing 0.1% Tween 20 and 5% BSA, followed by an overnight incubation with the respective primary antibodies at 4 °C with gentle agitation: 1:100 monoclonal anti-aSyn LB509 from Zymed Laboratories Inc. (South San Francisco, CA, USA); 1:1000 polyclonal anti-aSyn, oligomer specific Syn-33 from Sigma (St. Louis, MO, USA); 1:1000 polyclonal anti-LC3B from Cell Signaling (Danvers, MA, USA); 1:1000 anti-TH from Millipore (Billerica, MA, USA); 1:1000 monoclonal anti-synaptophysin from Sigma (St. Louis, MO, USA); 1:1000 anti-PSD95 antibody from Abcam (Cambridge; UK); 1:1000 anti-phospho-Drp1 from Cell Signaling (Danvers, MA, USA); 1:1000 polyclonal anti-Tom20 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 1:1000 anti-SDHA from Abcam (Cambridge; UK); 1:500 anti-pro-IL1β from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 1:100 anti-TLR7 from Abcam (Cambridge; UK); and 1:100 anti-TLR4 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 1:10000 monoclonal anti-α-tubulin from Sigma (St. Louis, MO, USA), 1:1000 β-III tubulin from Cell Signaling (Danvers, MA, USA) or 1:5000 β-actin from Sigma (St. Louis, MO, USA) were used for loading control. Membranes were washed with TBS

containing 3% BSA and 0.1% Tween three times (each time for 5 min), and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at RT with gentle agitation. After three washes, specific bands of interest were detected by developing with an alkaline phosphatase enhanced chemical fluorescence reagent (ECF from GE Healthcare, Piscataway, NJ, USA). Fluorescence signals were detected using a Bio-Rad Versa-Doc Imager. Analysis of Western blot band densities were determined using Quantity One Software (Bio-Rad). Regions of interest were drawn around bands of interest and the background was automatically subtracted. Relative densities were calculated in relation to WT or untreated conditions for each membrane and normalized to housekeeping genes (β-actin, β-III tubulin for cytosolic samples and SDHA and TOM20 for mitochondrial samples).

Immunocytochemistry and Confocal Microscopy Analysis

Mesencephalic neurons and NT2 cells were grown on glass coverslips (16 mm diameter) in 12-well plates. Following treatment, neurons and cells were washed twice with serum-free medium and fixed with 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed again with PBS, permeabilized with methanol for 20 min at −20 °C (for LC3B) or with 0.2% Triton X-100 for 2 min at room temperature, and incubated with 3% BSA, to prevent non-specific binding, for 30 min. Cells were incubated with primary antibodies: 1:400 rabbit monoclonal anti-LC3 XP® from Cell Signaling (Danvers, MA, USA); 1:200 anti-SDHA from Abcam (Cambridge; UK); 1:100 polyclonal anti-Tom20 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 1:100 anti-Lamp1 clone H4A3 from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). Cells were then incubated with the appropriate secondary antibody (1:250 Alexa Fluor 594 or 1:250 Alexa Fluor 488 from Molecular Probes (Eugene, OR, USA)). Subsequently, cells were incubated with Hoechst 15 μg/μL for 5 min at room temperature and protected from light. After a final wash, the coverslips were immobilized on a glass slide with mounting medium Dako Cytomation (Dako, Glostrup, Denmark). Negative controls omitting each primary antibody were performed in each case, and no staining was seen. Images were acquired using a Plan-Apochromat/1.4NA 63× lens on an Axio Observer.Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software. Co-localization of

Tom20/Lamp1 and LC3/SDHA was quantified in threshold images with the JACoP plug-in of the ImageJ software, as previously described.[23, 24] A freely available ImageJ macro tool was used to analyze mitochondrial network as described in Valente and co-workers.[23] Briefly, images were pre-processed to improve quality prior to binarizing and skeletonizing. Mitochondrial footprint, the area occupied by mitochondrial structures, was calculated from the binarized image prior to skeletonizing. The remaining descriptive parameters were calculated from the skeletonized image. At least 20 cells were examined for each condition.

Mitochondrial movement analysis

Mesencephalic neurons were seeded on ibidi µ-Slide 8-well plates and washed twice with HBSS [5.36 mM KCl, 0.44 mM KH_2PO4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $NaH_2PO_4.H_2O$, 5 mM glucose, 5.36 mM HEPES, 0.001% Phenol Red, (pH 7.2)], and mitochondria were labeled with 100 nM MitoTracker Green (Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C in the dark, as previously described.[23] After a gentle wash, cells were kept in HBSS and imaged for mitochondrial movements. Timelapse images were captured under a Plan-Apochromat/1.4NA 63× lens on an Axio Observer.Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software with a stage-based chamber (5% CO2, 37 °C). The inverted microscope was driven by LSM software and images were taken every 2 s for a total of 10 min under 63× magnification (Zeiss Plan-ApoChromat 63×, 1.4NA). For transport analysis, mitochondria were considered immobile if they remained stationary for the entire recording period. Movement was registered only if the displacement was above the length of the mitochondrion (about 2 µm). For each time-lapse movie, mitochondria were manually tracked and transport parameters were generated using the ImageJ software plug-in Multiple Kymograph, submitted by J. Rietdorf and A. Seitz (European Molecular Biology Laboratory, Heidelberg, Germany). Movement velocity data were determined from the kymographic images and calculated based on the slope ($v = dx/dt$) obtained for each profile along the recording time. Each series of images was recorded for at least three randomly selected MitoTracker Green neurons per culture and three independent cultures per condition.

Analysis of Mitochondrial Membrane Potential (Δψm) with TMRM Probe

The tetramethylrhodamine methyl ester dye (TMRM) (Molecular Probes, Eugene, OR, USA) was used to monitor changes in mitochondrial membrane potential.[25] TMRM is a cell permeable fluorescent indicator that accumulates in the highly negatively charged matrix of the mitochondria. The accumulation in functional mitochondria takes place as a consequence of TMRM positive charge and a decrease in TMRM cellular retention is associated with a decrease in Δψm. After treatments, mesencephalic neurons and NT2 cells were washed with PBS (1×) and loaded in the dark with 300 nM TMRM in Krebs buffer (pH 7.4) composed of 132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 6 mM glucose, 10 mM HEPES, 10 mM $NaHCO_3$, and 1 mM $CaCl_2$. Basal fluorescence was recorded for 5 min at 37 °C (λex = 540 nm and λem = 590 nm). Subsequently, 1 μM FCCP (protonophore) and 2 μg/mL oligomycin (inhibitor of H^+ transporting ATP synthase and an inhibitor of Na^+/K^+ transporting ATPase) were added to each well in order to achieve maximal mitochondrial depolarization and to prevent ATP synthase reversal, respectively. Measurements were recorded for another 3 min at 37 °C. TMRM retention ability was calculated by the difference between the total fluorescence (after depolarization) and the initial value of fluorescence (basal fluorescence). Results were expressed as a percentage of the dye retained within the untreated WT neurons or untreated NT2 cells. Measurements were performed using a Spectramax Plus 384 spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA).

Determination of Cardiolipin fluorescence intensity

Cardiolipin is an important component of the inner mitochondrial membrane but also found in the membrane of most bacteria. Cardiolipin distribution to the outer membrane leads to apoptosis and inflammasome activation.[26] Cardiolipin distribution and fluorescence was measured by using the 10-N-Nonyl acridine orange (NAO) (Enzo, Lausen, Switzerland) that is generally used as a fluorescent marker of the inner mitochondrial membrane in whole cells and believed to bind negatively charged phospholipids. NAO accumulation in the cell seems to be related to specific interactions with mitochondrial membrane proteins and/or lipids, such as cardiolipin, and is largely independent of mitochondrial membrane potential. After treatments, mesencephalic neurons were washed with HBSS and loaded in the dark with 100 nM Cardiolipin in

HBSS for 1 h. After a gentle wash, cells were kept in HBSS and imaged. Images were obtained using a Plan-Apochromat/1.4NA 63× lens on an Axio Observer.Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software. Cardiolipin fluorescence was quantified in threshold images using the ImageJ software.

Caspase-1 Activation Assay

To evaluate caspase-1 activation, extracts containing 40 μg of protein were incubated in a reaction buffer (25 mM HEPES pH 7.5, 0.1% (w/v) 3[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS), 10% (w/v) sucrose, 2 mM DTT) with 100 μM of the colorimetric substrate for caspase-1 from Sigma Chemical Co. (St. Louis, MO, USA), for 2 h at 37°C. Detection was at 405 nm using a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Evaluation of inflammation markers by ELISA

Inflammation markers were evaluated in 25 μg from intestinal (ileum and cecum), brain mesencephalon, mesencephalic neurons homogenates and 50 μL of plasma by using the NFκB p65, IL-1β, IL-8, IL-10, IL-17, TNF-α, IFNγ and IL-6 ELISA kits. Absorbance was registered at 450 nm in a SpectraMax Plus 384 multiplate reader. Results were expressed as μg/ml protein for NFκB p65 and as pg/mL for the remaining markers.

Assessment of dopamine levels

Measurement of dopamine in the Striatum was performed with the MyBioSource' ELISA kit for Dopamine, in 50 μL homogenates according to manufacturer's instructions. Absorbance was registered at 450 nm in a SpectraMax Plus 384 multiplate reader. Results were expressed as pg/mL.

Assessment of aSyn oligomers levels

The concentration of aSyn oligomers in each sample was determined in 25 μg of brain mesencephalon and intestinal homogenates with the mouse aSyn oligomer ELISA kit.

Absorbance was read at 450 nm in a SpectraMax Plus 384 multiplate reader. Results were expressed as pg/mL.

Statistical analysis

Microbiome population statistics are described in detail above. Statistical analysis of datasets was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) software and is summarized in Supplementary Table 2. All data are represented as the mean ± SEM. Normality distribution analysis (Shapiro-Wilk test) was applied to determine the subsequent parametric or non-parametric tests. Pair-wise comparisons were performed by unpaired Student t test or Mann-Whitney test. Comparisons of multiple groups were performed with one-way ANOVA followed by Dunnett post-hoc test or Kruskal-Wallis test followed by Dunn post-hoc test. Correlation analysis between two variables was performed by Pearson correlation test. All statistical tests were two-tailed and the annotation for significance values was: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. P- and N-values are indicated at each figure legend.

Detailed Methods References

1 Karamyan VT, Speth RC. Animal models of BMAA neurotoxicity: A critical review. *Life Sciences* 2008;82:233–246.

2 Tiago I, Veríssimo A. Microbial and functional diversity of a subterrestrial high pH groundwater associated to serpentinization. *Environ Microbiol* 2013;15:1687–706.

3 Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 2016;18:1403–14.

4 Schloss PD, Westcott SL, Ryabin T, *et al.* Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl Environ Microbiol* 2009;75:7537 LP – 7541.

5 Glöckner FO, Yilmaz P, Quast C, *et al.* 25 years of serving the community with ribosomal RNA gene reference databases and tools. *J Biotechnol* 2017;261:169–76.

6 Chong J, Liu P, Zhou G, *et al.* Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat Protoc* 2020;15:799–821.

7 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550.

8 Cunha-Santos J, Duarte-Neves J, Carmona V, *et al.* Caloric restriction blocks neuropathology and motor deficits in Machado–Joseph disease mouse models through SIRT1 pathway. *Nat Commun* 2016;7:11445.

9 Zhu J-W, Li Y-F, Wang Z-T, *et al.* Toll-Like Receptor 4 Deficiency Impairs Motor Coordination. Front. Neurosci. 2016;10:33.

10 Sampson TR, Debelius JW, Thron T, *et al.* Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell* 2016;167:1469-1480.e12.

11 Deacon RMJ. Measuring motor coordination in mice. *J Vis Exp* 2013;:e2609–e2609.

12 Deacon RMJ, Rawlins JNP. T-maze alternation in the rodent. *Nat Protoc* 2006;1:7–12.

13 Perez-Pardo P, Dodiya HB, Engen PA, *et al.* Role of TLR4 in the gut-brain axis in Parkinson's disease: a translational study from men to mice. *Gut* 2019;68:829 LP – 843.

14 Engen PA, Dodiya HB, Naqib A, *et al.* The Potential Role of Gut-Derived Inflammation in Multiple System Atrophy. *J Parkinsons Dis* 2017;7:331–46.

15 Gundersen HJ, Jensen EB V, Kieu K, *et al.* The efficiency of systematic sampling in stereology — reconsidered. *J Microsc* 1999;193:199–211.

16 Binder DR, Dunn WH, Swerdlow RH. Molecular characterization of mtDNA depleted and repleted NT2 cell lines. *Mitochondrion* 2005;5:255–65.

17 Gao H-M, Liu B, Zhang W, *et al.* Synergistic dopaminergic neurotoxicity of MPTP and inflammogen lipopolysaccharide: relevance to the etiology of Parkinson's disease. *FASEB J* 2003;17:1–25.

18 Arduíno DM, Esteves AR, Cortes L, *et al.* Mitochondrial metabolism in Parkinson's disease impairs quality control autophagy by hampering microtubule-dependent traffic. *Hum Mol Genet* 2012;21:4680–702.

19 Ferreira IL, Carmo C, Naia L, *et al.* Assessing mitochondrial function in in vitro and ex vivo models of Huntington's disease. *Methods Mol Biol* 2018;1780:415-442.

20 Rogers GW, Brand MD, Petrosyan S, *et al.* High Throughput Microplate Respiratory Measurements Using Minimal Quantities Of Isolated Mitochondria. *PLoS One* 2011;6:e21746.

21 Silva DF, Selfridge JE, Lu J, *et al.* Bioenergetic flux, mitochondrial mass and mitochondrial morphology dynamics in AD and MCI cybrid cell lines. *Hum Mol Genet* 2013;22:3931–46.

22 Pellman JJ, Hamilton J, Brustovetsky T, *et al.* Ca2+ handling in isolated brain mitochondria and cultured neurons derived from the YAC128 mouse model of Huntington's disease. *J Neurochem* 2015;134:652–67.

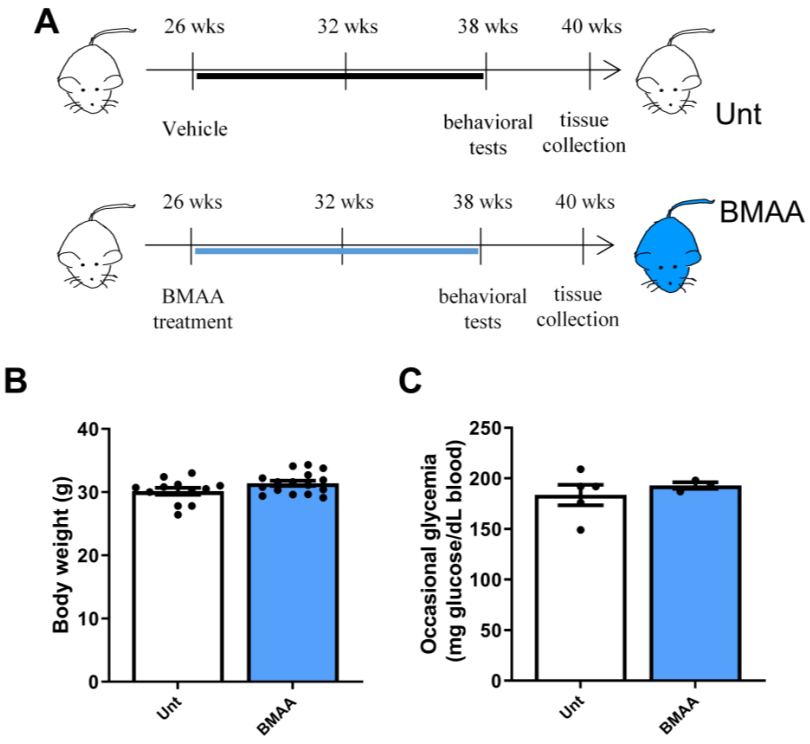
23 Esteves AR, Gozes I, Cardoso SM. The rescue of microtubule-dependent traffic recovers mitochondrial function in Parkinson's disease. *Biochim Biophys Acta - Mol Basis Dis* 2014;1842:7–21.

24 Valente AJ, Maddalena LA, Robb EL, *et al.* A simple ImageJ macro tool for analyzing mitochondrial network morphology in mammalian cell culture. *Acta Histochem* 2017;119:315–26.

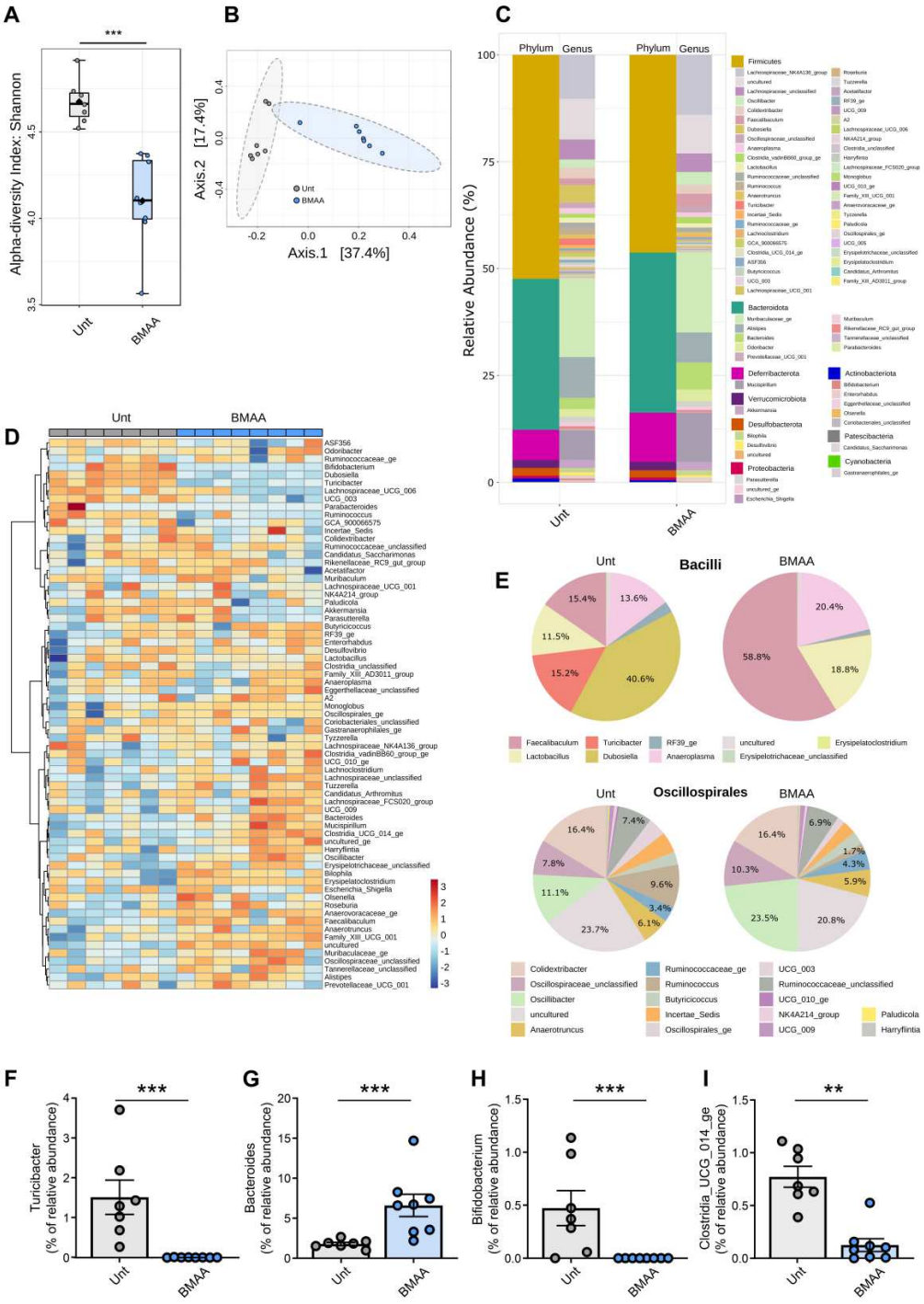
25 Scaduto RC, Grotyohann LW. Measurement of Mitochondrial Membrane Potential Using Fluorescent Rhodamine Derivatives. *Biophys J* 1999;76:469–77.

26 Hsu Y-H, Dumlao DS, Cao J, *et al.* Assessing Phospholipase A2 Activity toward Cardiolipin by Mass Spectrometry. *PLoS One* 2013;8:e59267.

Supplemental figures and respective legends



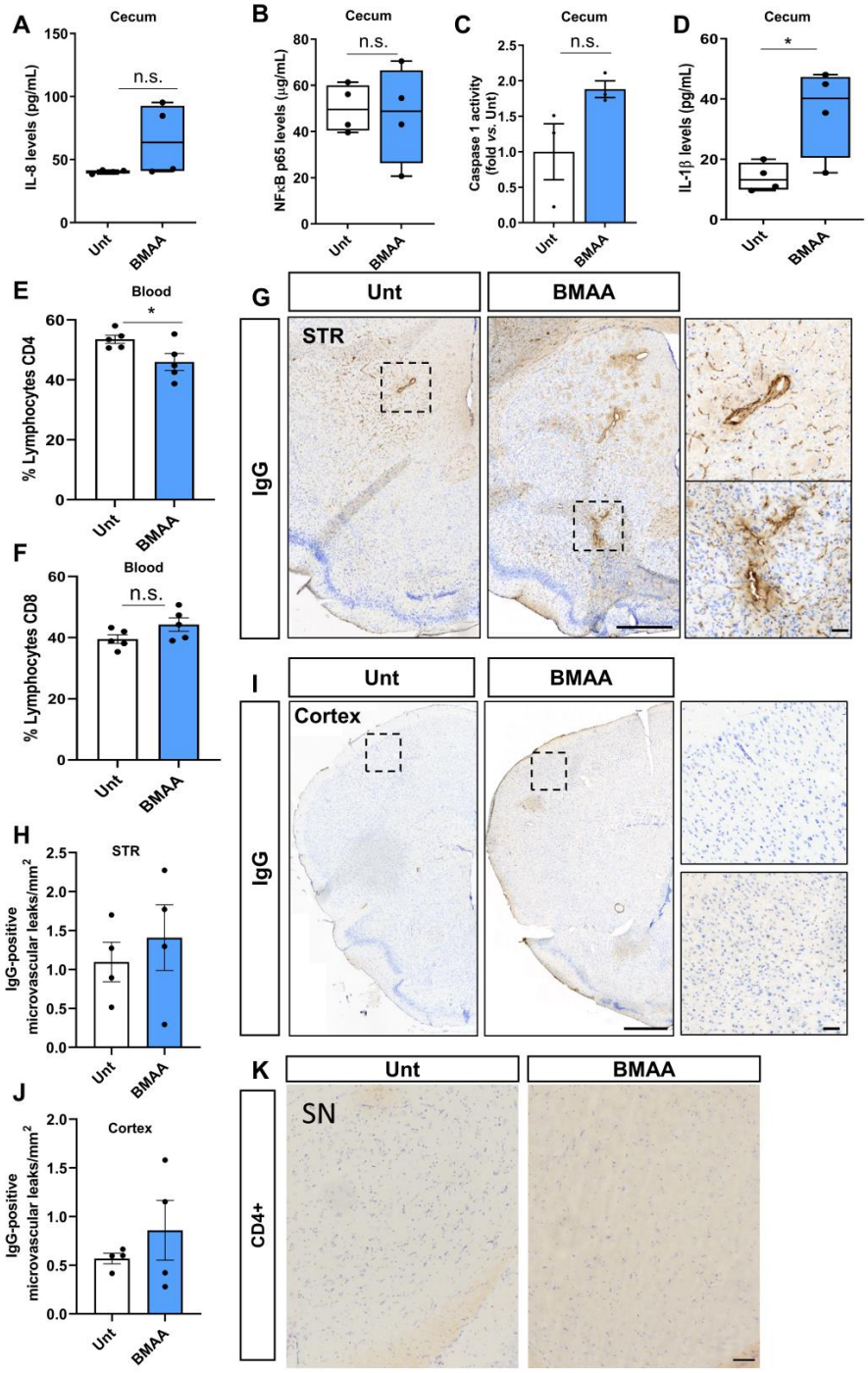
Supplemental Fig. S1. Mice body weight and glycemia were unaltered during the treatment. (**A**) Schematic representation of experimental design. **(B)** The body weights of mice were measured twice a week during the treatment. (**C**) Blood glucose was measured at the end of treatment and occasional glycemia were calculated.



Supplemental Fig. S2. Cecum mucosa-associated microbiota diversity in BMAA-treated mice.

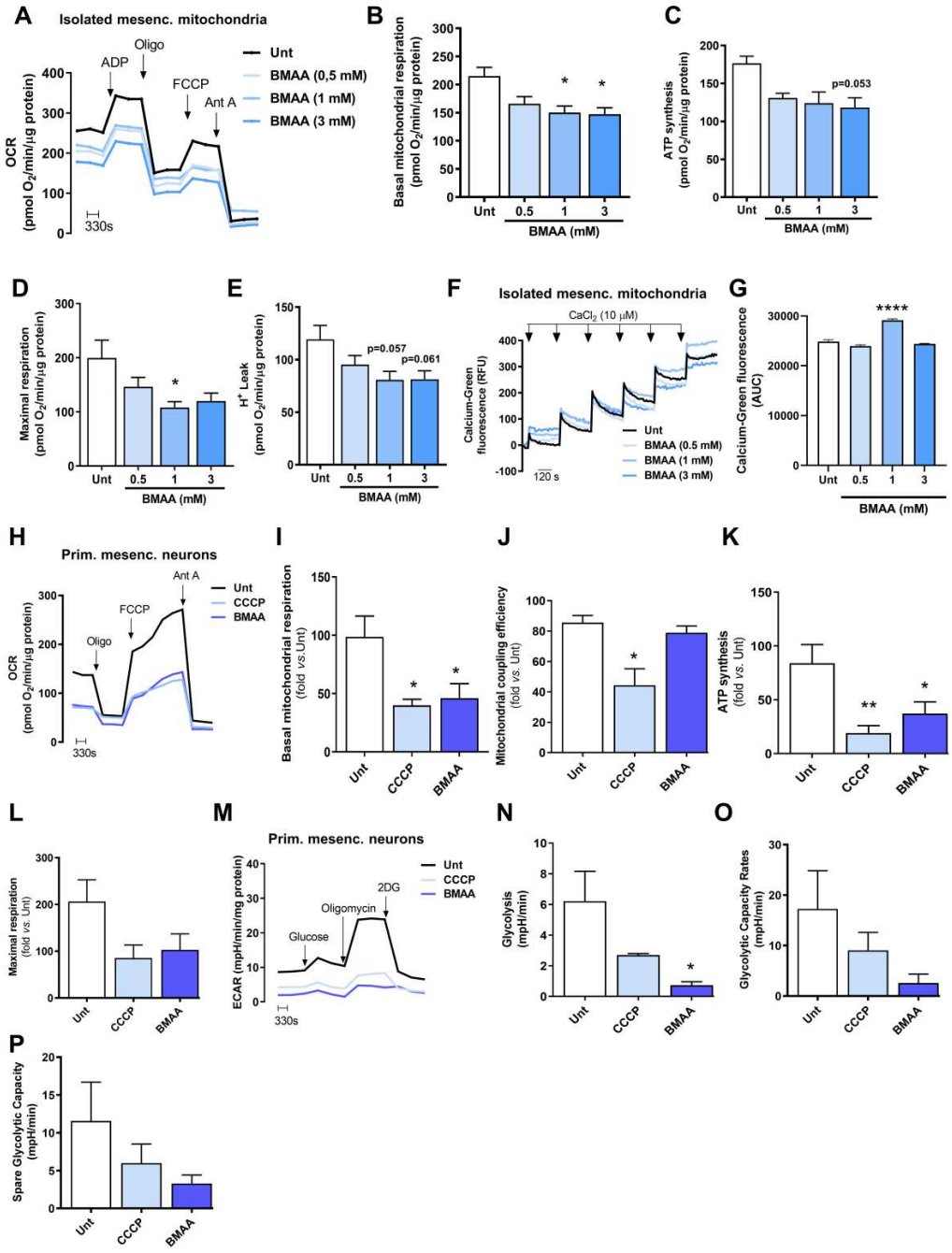
(A) Alpha-diversity measured using the Shannon index at OTU level derived from 16S rDNA sequencing of cecum intestinal samples from untreated (Unt) or BMAA-treated mice (n values for Unt = 7 and BMAA = 8, Unt vs BMAA, Mann-Whitney test, ***p = 0.00031). **(B)** Beta diversity evaluated by principal coordinate analysis (PCoA) based on Bray-Curtis index of OTUs derived from 16S rDNA sequencing of cecum intestinal samples from untreated (Unt) or BMAA-treated mice (n values for Unt = 7 and BMAA = 7; PERMANOVA: $r^2 = 0.352$, **$p < 0.002$; PERMDISP: $F = 0.269$, $p = 0.614$). **(C)** Taxonomic diversity of cecum intestinal samples from untreated (Unt) or BMAA-treated mice at phylum and genus level. **(D)** Heatmap of genera relative abundances obtained for cecum intestinal samples from untreated (Unt) or BMAA-treated mice using Pearson's correlation coefficient as a distance metric, with clustering based on Ward's algorithm. **(E)** Pie-charts showing proportional taxonomic composition at genus level of cecum intestinal microbiota samples from untreated (Unt) or BMAA-treated mice for two selected taxa affected by BMAA treatment, the class Bacilli and the order Oscillospiralles. **(F-I)** Differential abundance of selected bacterial genera in cecum intestinal samples from untreated (Unt) or BMAA-treated mice (n values for Unt = 7 and BMAA = 8, Unt vs BMAA, DESeq2 statistical analysis). **(F)** *Turicibacter* (***padj = 3.44×10^{-22}). **(G)** *Bacteroides* (***padj = 2.49×10^{-7}). **(H)** *Bifidobacterium* (***padj = 6.82×10^{-10}). **(I)** Clostridia_UCG_014_ge (**padj = 0.00826).

Esteves AR, *et al. Gut* 2022; 72:73–89. doi: 10.1136/gutjnl-2021-326023



Supplemental Fig. S3. The effect of BMAA in cecum inflammation, CD4 and CD8 blood percentages, striatum and cortical microvascular leaks and SN CD4+ cells infiltration.

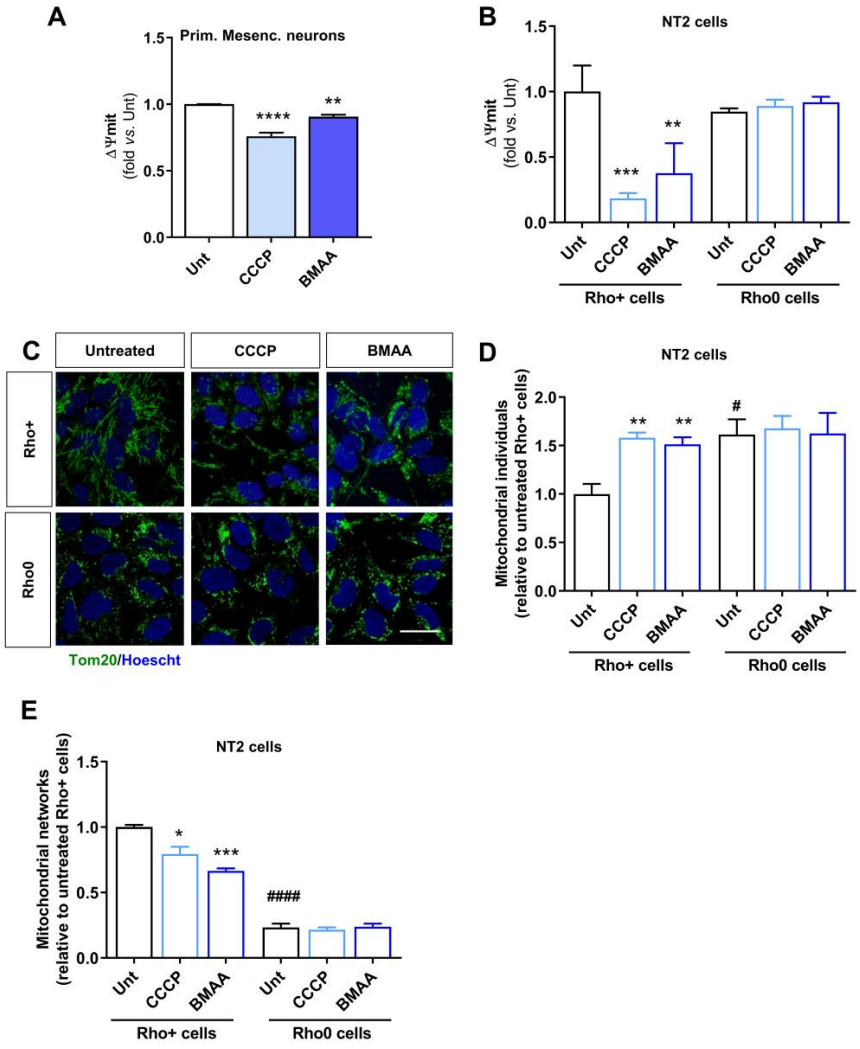
(A-D) Measurement of specific mediators involved in the inflammatory response in cecum intestinal samples from untreated (Unt) or BMAA-treated mice measured by ELISA. **(A)** IL-8 (n values for all conditions = 4, Unt vs BMAA, p = 0.123), **(B)** NFkβ (n values for all conditions = 4, Unt vs BMAA, p = 0.8162), **(C)** Caspase-1 activation was measured using a colorimetric substrate as described in Material and Methods (n values for all conditions = 3, Unt vs BMAA, p= 0.099), **(D)** IL-1β (n values for all conditions = 4, Unt vs BMAA, *p= 0.0291). **(E)** Percentage of CD4 lymphocytes in $CD45^{+}/CD3^{+}$ cell population in blood samples by flow cytometry (n values for all conditions = 5, Unt vs BMAA, *p = 0.0427). **(F)** Percentage of CD8 lymphocytes in $CD45^{+/}CD3^{+}$ cell population in blood samples by flow cytometry n values for all conditions = 5, Unt vs BMAA, p = 0.1077. **(G-J)** Assessment of IgG-positive microvascular leaks in Striatum (STR) (**G**-**H**) and Cortex (**I**-**J**) in untreated and BMAA-treated mice. Representative images of coronal sections stained with IgG in STR (**G**) and Cortex (**I**). Quantification of IgG-positive microvascular leaks per mm^2 in STR (**H**) (n values for all conditions = 4, Unt vs BMAA, p = 0.5489) and Cortex (**J**) (n values for all conditions = 4, Unt vs BMAA, p = 0.389). **(K)** Representative images of SN coronal sections stained with anti-CD4 in untreated (Unt) and BMAA-treated mice. Scale bar are 50 µm (enlarged inner boxes) and 500 µm. Data represent mean+SEM. Statistical analysis: Unpaired Student's t test was performed in all analyses.



Supplemental Fig. S4. BMAA targets the mitochondria

(A) Mesencephalic isolated mice mitochondria treated with different concentrations of BMAA (0.5; 1 and 3 mM) for 30 min. Representative graph showing OCR **(B)** Basal Respiration (Unt vs 1 mM BMAA, *p = 0.0188, Unt vs 3 mM BMAA, *p = 0.0147);

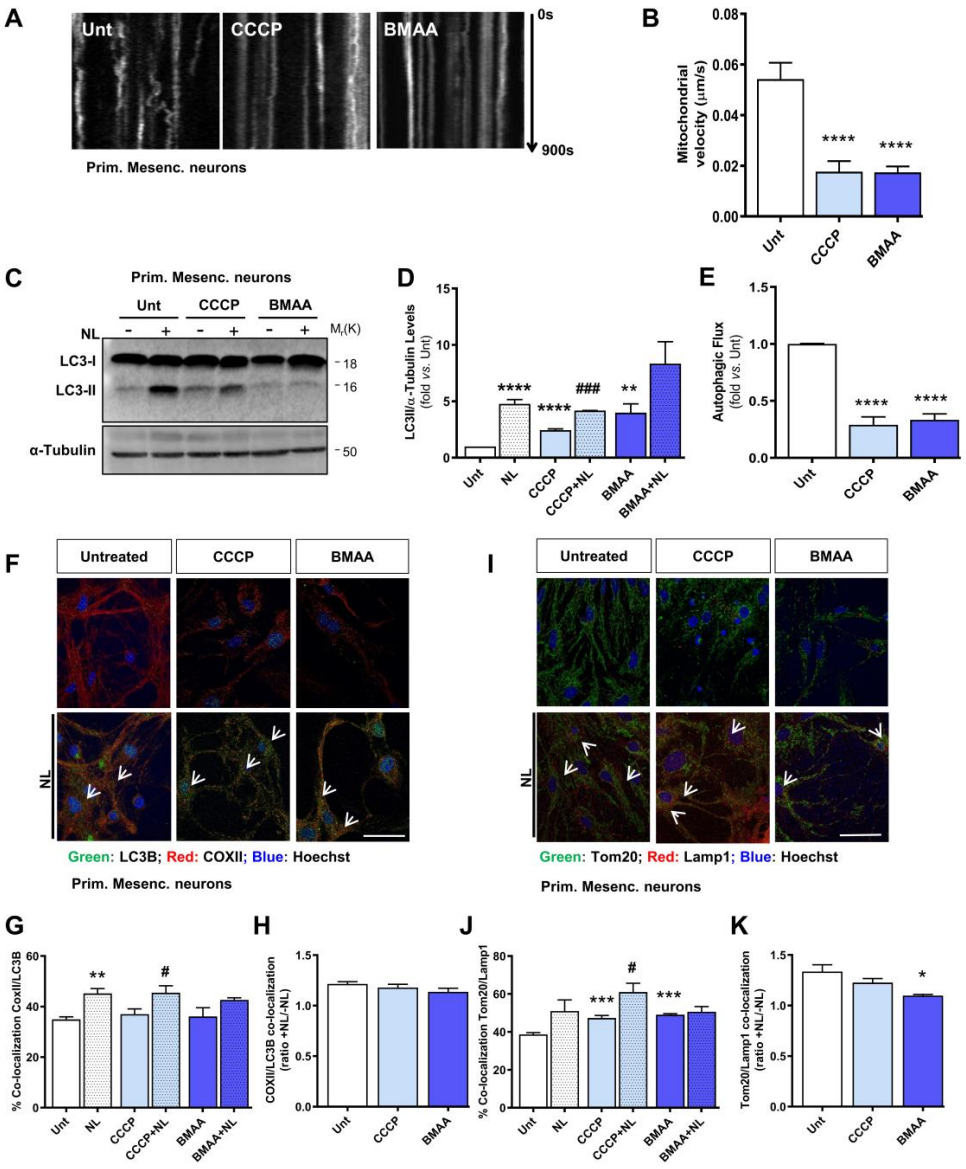
(C) ATP synthesis; **(D)** maximal respiration (Unt vs 1 mM BMAA, *p = 0.0343); **(E)** proton Leak. Values are pmol O_2/min/µg protein. n values for all conditions = 3, **(F-G)** Isolated mesencephalic mitochondria's ability to uptake calcium was evaluated with the fluorescent probe Calcium-green (n values for Unt = 6, 0.5 mM BMAA = 5, 1 mM BMAA = 5, 3 mM BMAA = 4; Unt vs 1 mM BMAA, ****p < 0.0001). Primary mice mesencephalic neurons treated with 1 µM CCCP for 2 h and 3 mM BMAA for 48 h. **(H)** Representative graph showing OCR; **(I)** Basal Respiration (Unt vs CCCP**,** *p = 0.0191, Unt vs BMAA**,** *p = 0.0325); **(J)** Mitochondrial coupling efficiency (Unt vs CCCP**,** *p = 0.0372); **(K)** ATP synthesis (Unt vs CCCP**,** **p = 0.0093, Unt vs BMAA**,** *p = 0.0468); **(L)** maximal respiration. Values are mean ± S.E.M and n values for all conditions = 4. **(M)** Representative graph showing extracellular acidification rate (ECAR). **(N)** Basal glycolysis rate (Unt vs BMAA**,** *p = 0.0251); **(O)** Glycolytic capacity rate (Unt vs BMAA**,** p = 0.189); **(P),** Spare glycolytic capacity (Unt vs BMAA**,** p = 0.203). Values are mpH/min/mg protein and n values for all conditions = 3. Statistical analysis: One-way ANOVA followed by Dunnett's test was performed in **B**, **D-E, G, I, K** and **N-P**. Kruskal-Wallis test followed by Dunn's test was performed in **C, J** and **L**.



Supplemental Fig. S5. Functional mitochondria are required for BMAA-induced mitochondrial fragmentation.

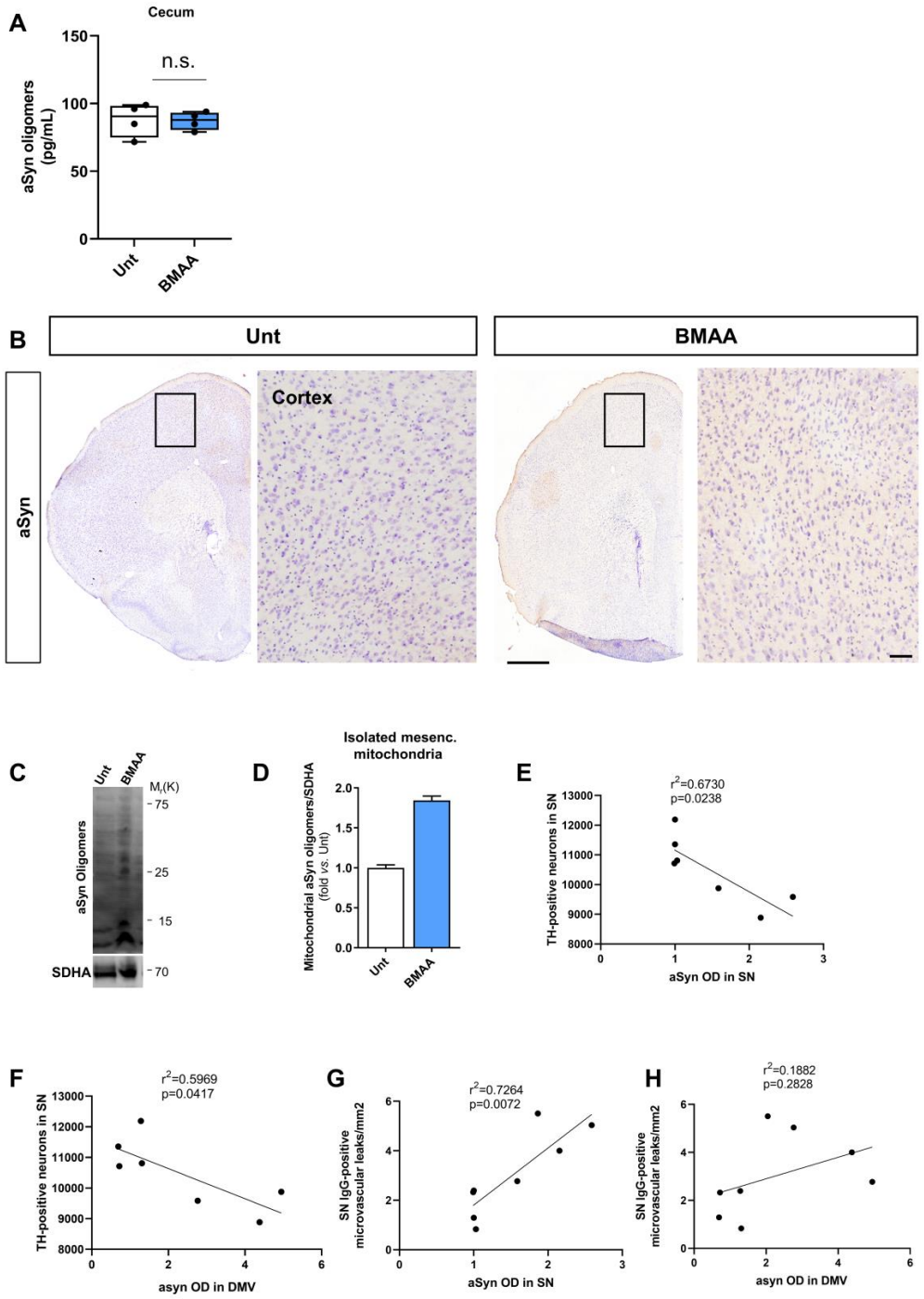
(A) Primary mice mesencephalic neurons treated with 1 μM CCCP for 2 h and 3 mM BMAA for 48 h. Changes in mitochondrial membrane potential (ΔΨm) were assessed using the fluorescent cationic dye TMRM. Values are mean ± S.E.M. (n values for Unt = 5, CCCP = 4 and BMAA = 5, Unt vs CCCP, ****$p < 0.0001$, Unt vs BMAA, **$p = 0.0025$). **(B)** NT2-Rho+ and -Rho0 cells were treated with 5 μM CCCP for 2 h and with 3 mM BMAA for 48 h. Changes in mitochondrial membrane potential (ΔΨm) were

assessed using the fluorescent cationic dye TMRM. Values are mean ± S.E.M (n values for Unt, CCCP and BMAA = 3 in Rho+, and Unt, CCCP and BMAA = 5 in Rho0; Unt vs CCCP, ***p = 0.0005, Unt vs BMAA, **p = 0.0067 in Rho+). **(C)** Cells were immunostained with Tom20. Alterations in mitochondrial network were calculated with an ImageJ Macro tool as described in Materials and Methods section. **(D-E),** mitochondrial network was quantified. **(D)** Number of mitochondria individuals in Rho+ cells (n values for all conditions = 3, Unt vs CCCP, **p= 0.0037, Unt vs BMAA, **p = 0.0068, Unt Rho+ vs Rho0 cells, $^{\#}$p=0.0309); **(E)** Number of mitochondrial networks in Rho+ cells (n values for all conditions = 3, Unt vs CCCP, *p= 0.01, Unt vs BMAA, ***p = 0.0009, Unt Rho+ vs Rho0 cells, $^{\#\#\#\#}$p<0.0001). Statistical analysis: One-way ANOVA followed by Dunnet's test was performed to compare different treatments against Unt group, and unpaired Student's t test was performed to compare Rho+ vs Rho0 cells.



Supplemental Fig. S6. Mitochondrial trafficking and degradation are affected by BMAA. Primary mice mesencephalic neurons were treated with 3 mM BMAA for 48 h and 1 µM CCCP for 2 h. **(A)** Representative kymograph images of mitochondria movement **(B)** Average transport velocity of mitochondria was calculated using an Image J Macro tool as described in Material and Methods. Data is reported as absolute values (µm/s) (n values for all conditions = 6, Unt vs CCCP**,** ****p < 0.0001, Unt vs BMAA**,** ****p < 0.0001). **(C)** Lysates from primary mesencephalic neurons in the

presence or absence of lysosomal inhibitors (NL, last 4 h) were examined by immunoblotting. Representative immunoblot for LC3B-I and II levels. **(D)** Autophagic vacuoles basal levels (LC3-II basal densitometric values) were determined. Values are mean ± S.E.M. (n values for all conditions = 3, apart from Unt and NL = 4, Unt vs NL, ****p<0.0001; Unt vs CCCP**,** ****p<0.0001; Unt vs BMAA, **p = 0.0021; CCCP vs CCCP+NL**,** $^{###}$p = 0.001) **(E)** Autophagic flux was determined (ratio of LC3-II densitometric value of NL-treated samples over the corresponding untreated samples). Values are mean ± S.E.M. (n values for all conditions = 4, Unt vs CCCP**,** ****p < 0.0001, Unt vs BMAA**,** ****p < 0.0001). The blots were re-probed for α-tubulin to confirm equal protein loading. **(F)** Co-localization between autophagic vacuoles (labeled in green with LC3B antibody) and mitochondria (labeled in red with COXII antibody) was visualized by immunofluorescence. Hoechst 33342-stained nuclei are in blue. **(G-H)** Assessment of LC3B and COXII co-localization was calculated using Image J as described in Material and Methods (n values for all conditions = 4, **(G)** Unt vs NL**,** **p = 0.002; CCCP vs CCCP+NL**,** #p = 0.05; BMAA vs BMAA+NL, p = 0.212). **I,** Co-localization between mitochondria (labeled in green with Tom20 antibody) and lysosomes (labeled in red with Lamp1 antibody) was visualized by immunofluorescence. Hoechst 33342- stained nuclei are in blue. **(J-K)** Assessment of Tom20 and Lamp1 co-localization was calculated using Image J as described in Material and Methods. (n values for all conditions = 4, **(J)** Unt vs NL, p = 0.0823; CCCP vs CCCP+NL**,** $^{#}$p = 0.032, Unt vs CCCP, **p = 0.006, Unt vs BMAA, **p = 0.002, **(K)** Unt vs BMAA, *p = 0.02). Statistical analysis: One-way ANOVA followed by Dunnett's test was performed to compare different treatments against Unt group, and unpaired Student's t test was performed to compare NL treatments vs respective control group.



Supplemental Fig. S7. a**Syn aggregation in the cecum, cortex and in mesencephalic mitochondria and Pearson Correlations.**

(A) aSyn oligomers in cecum intestinal samples from untreated (Unt) or BMAA-treated mice measured by ELISA (n values for all conditions = 4, Unt vs BMAA, p= 0.9153). **(B)** Photomicrographs represent histology for aSyn immunoreactivity in Cortex from untreated and BMAA-treated mice. Histology samples were counter-stained with cresyl violet. Scale bars are 50 µm (enlarged inner square) and 1 mm. **(C)** Representative immunoblot showing aSyn monomer and oligomers in mitochondrial homogenates isolated from the mesencephalon of untreated and BMAA-treated mice. The blots were re-probed for SDHA to confirm equal protein loading and mitochondrial fraction purity. **(D)** Densitometric analyses of mitochondrial levels of aSyn normalized against SDHA. Data are expressed relatively to untreated mice (n values for all conditions = 2). Statistical analysis: Unpaired Student's t test was performed in all analysis. **(E-H)** Pearson correlation between SN TH-positive neurons and aSyn OD in the SN and DMV and between IgG-positive microvascular leaks in SN and aSyn OD in the SN and DMV. (**E**) Correlation between the loss of TH-positive neurons in SN with the increase of aSyn in the SN (n = 7, p= 0.0238, r^2= 0.6730); (**F**) Correlation between the loss of TH-positive neurons in SN with the increase of aSyn in the DMV (n = 7, p= 0.0417, r^2= 0.5969); (**G**) Correlation between IgG-positive microvascular leaks in SN with the increase of aSyn in SN (n = 8, p= 0.0072, r^2= 0.7264); (**H**) Correlation between IgG-positive microvascular leaks in SN with the increase of aSyn in SN (n = 8, p= 0.2828, r^2= 0.1882).

Whole representative WB membranes

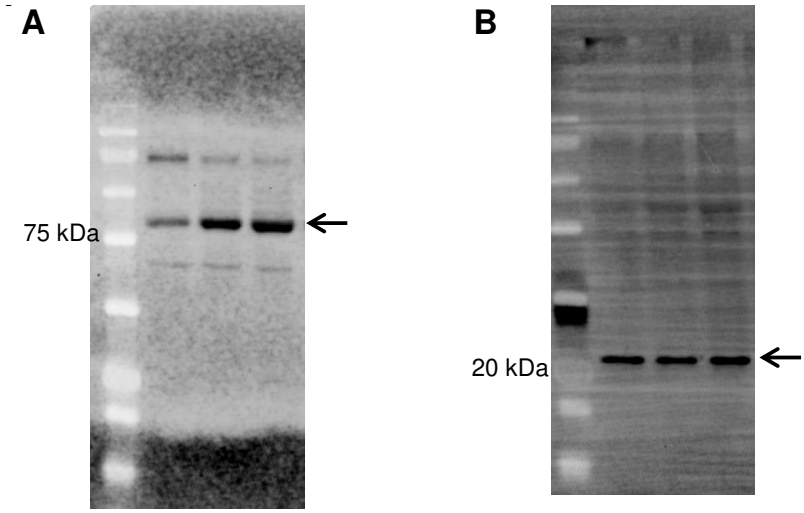


Figure 1. Whole representative western blots of Figure 4M showing phospho-Drp1 at 78 kDa (**A**) and Tom20 at 20 kDa (**B**).

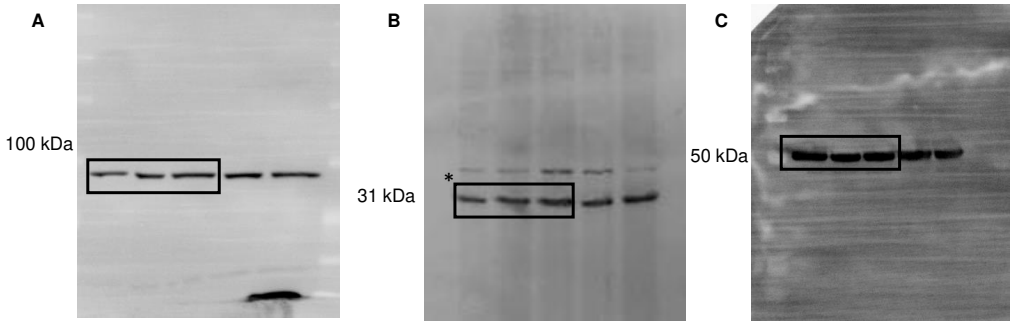


Figure 2. Whole representative western blots of Figure 5A showing TLR4 at 95 kDa (**A**), ProIL1β at 31 kDa (**B**) and βIII-Tubulin at 50 kDa (**C**). * This representative blot of ProIL1β those not match the inset in figure 5B, which was lost.

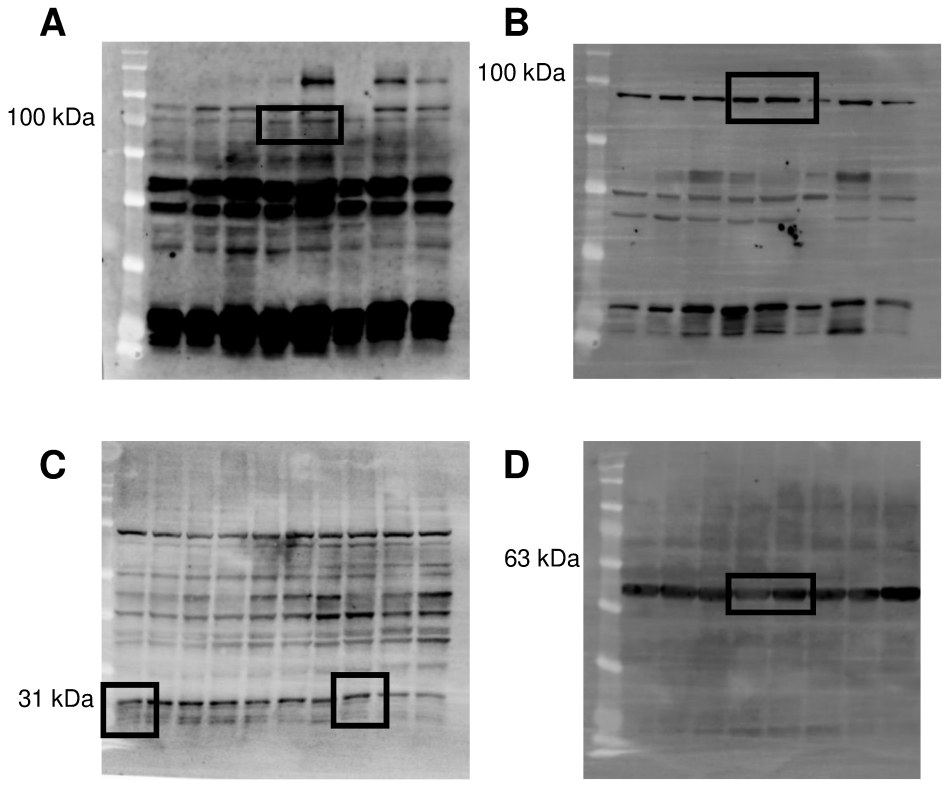


Figure 3. Whole representative western blots of Figure 5G showing TLR7 at 116 kDa (**A**), TLR4 at 95 kDa (**B**), ProIL1β at 31 kDa (**C**) and respective βIII-Tubulin at 50 kDa (**D**).

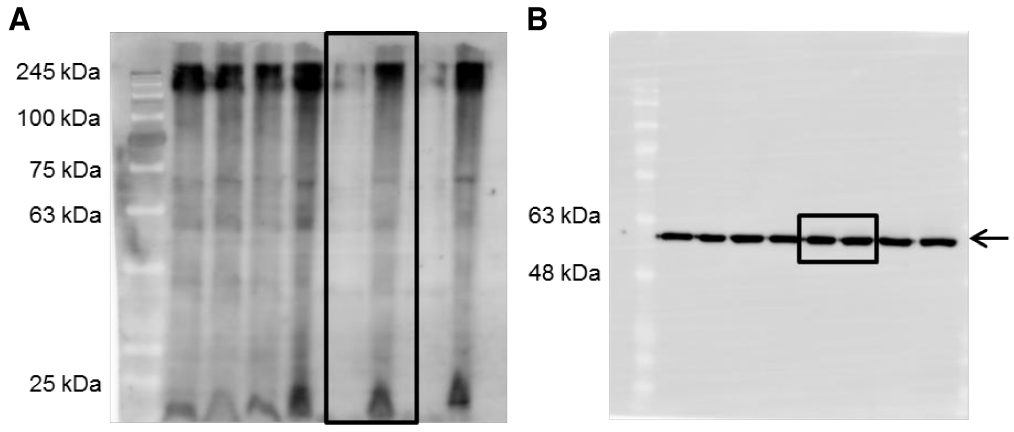


Figure 4. Whole representative western blots of Figure 6D showing aSyn Oligomers (**A**) and βIII-Tubulin at 50 kDa (**B**).

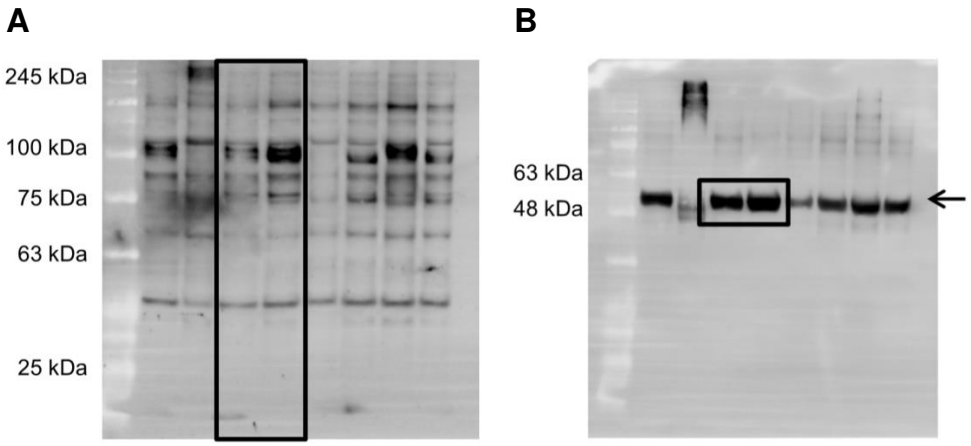


Figure 5. Whole representative western blots of Figure 6K showing aSyn Oligomers (**A**) and βIII-Tubulin at 50 kDa (**B**).

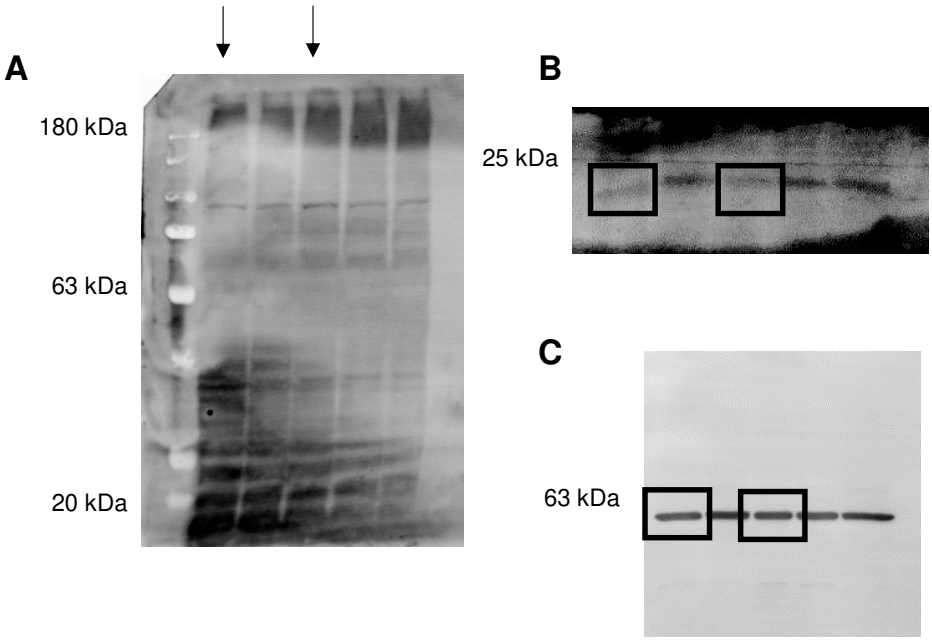


Figure 6. Whole representative western blots of Figure 6N showing aSyn Oligomers (**A**), aSyn monomer (**B**) and β-actin at 40 kDa (**C**).

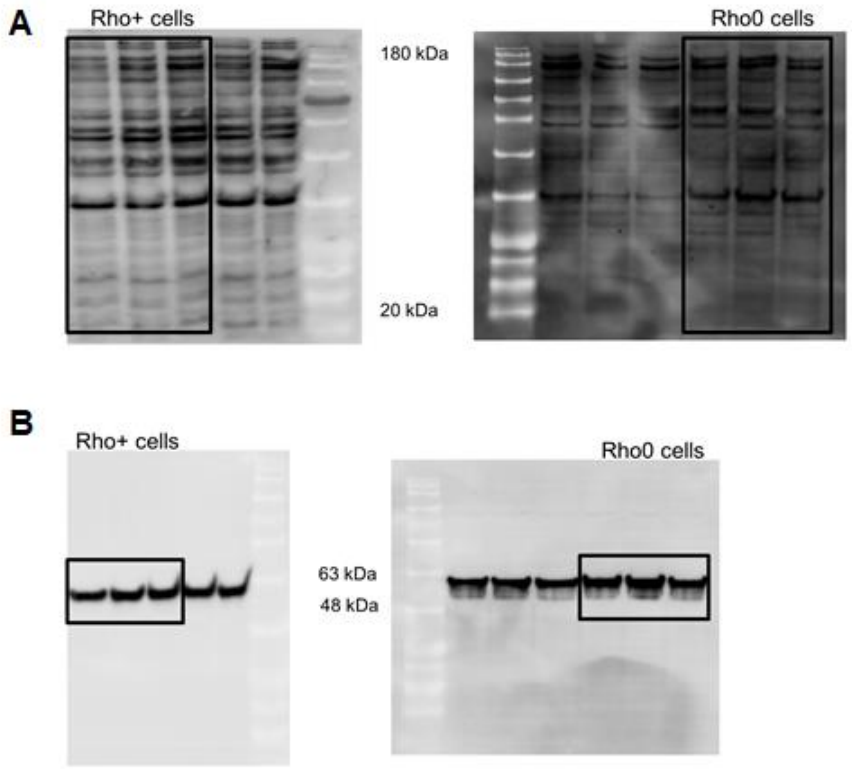


Figure 7. Whole representative western blots of Figure 6P showing aSyn Oligomers (**A**) and α-Tubulin at 50 kDa (**B**).

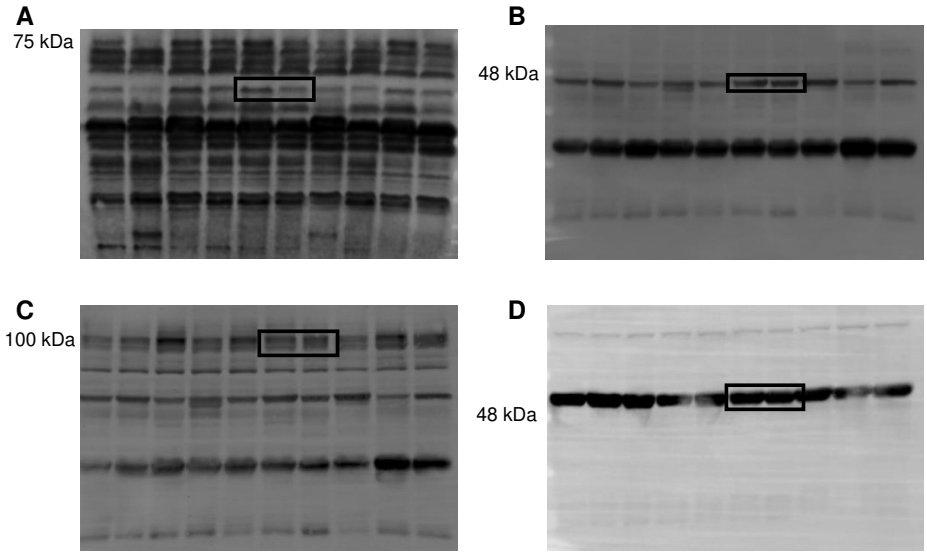


Figure 8. Whole representative western blots of Figure 7D showing TH at 62kDa (**A**), Synaptophysin at 38 kDa (**B**), PSD95 at 95 kDa (**C**) and βIII-Tubulin at 50 kDa (**D**).

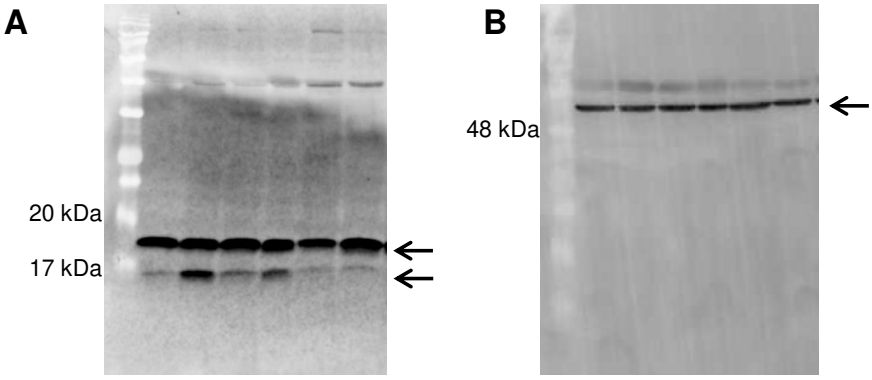


Figure 9. Whole representative western blots of Supplementary Figure S6C showing LC3B at 16 and 18 kDa (**A**) and α-Tubulin at 50 kDa (**B**).

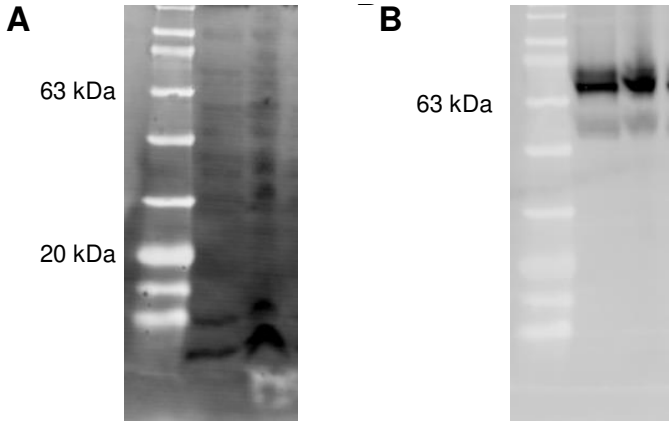


Figure 10. Whole representative western blots of Supplementary Figure S7C showing mitochondrial aSyn Oligomers (**A**) and SDHA at 70 kDa (**B**).