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Original research

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

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ABSTRACT

Objective Idiopathic Parkinson's disease (PD) is characterised by alpha-synuclein (aSyn) aggregation and death of dopaminergic neurons in the midbrain. Recent evidence posits that PD may initiate in the gut by microbes or their toxins that promote chronic gut inflammation that will ultimately impact the brain. In this work, we sought to demonstrate that the effects of the microbial toxin β -N-methylamino-L-alanine (BMAA) in the gut may trigger some PD cases, which is especially worrying as this toxin is present in certain foods but not routinely monitored by public health authorities.

Design To test the hypothesis, we treated wild-type mice, primary neuronal cultures, cell lines and isolated mitochondria with BMAA, and analysed its impact on gut microbiota composition, barrier permeability, inflammation and aSyn aggregation as well as in brain inflammation, dopaminergic neuronal loss and motor behaviour. To further examine the key role of mitochondria, we also determined the specific effects of BMAA on mitochondrial function and on inflammasome activation.

Results BMAA induced extensive depletion of segmented filamentous bacteria (SFB) that regulate gut immunity, thus triggering gut dysbiosis, immune cell migration, increased intestinal inflammation, loss of barrier integrity and caudo-rostral progression of aSyn. Additionally, BMAA induced *in vitro* and *in vivo* mitochondrial dysfunction with cardiolipin exposure and consequent activation of neuronal innate immunity. These events primed neuroinflammation, dopaminergic neuronal loss and motor deficits.

Conclusion Taken together, our results demonstrate that chronic exposure to dietary BMAA can trigger a chain of events that recapitulate the evolution of the PD pathology from the gut to the brain, which is consistent with 'gut-first' PD.

INTRODUCTION

Idiopathic Parkinson's disease (PD) is an age-related neurodegenerative disorder characterised by motor symptoms such as tremor, postural imbalance, bradykinesia and rigidity.^{1,2} Preceding non-motor features also include loss of smell and gastrointestinal (GI) dysfunction.³ Constipation, appearing during the prodromal phase, is perceived as a risk factor for developing PD.³ The histopathological *postmortem* hallmarks of PD are the presence of

SIGNIFICANCE OF THIS STUDY

WHAT IS ALREADY KNOWN ON THIS SUBJECT?

- ⇒ Parkinson's disease (PD) is a multifactorial disease characterised by a long prodromal phase that in at least some cases includes gastrointestinal symptoms.
- ⇒ A direct correlation between gut dysbiosis and disease progression was found in PD patients.
- ⇒ Chronic dietary exposure to BMAA is believed to be the cause of amyotrophic lateral sclerosis/parkinsonism-dementia complex in specific populations.
- ⇒ BMAA targets the cysteine-glutamate antiporter encoded by SLC7A11 (Solute Carrier Family 7 member 11), a gene whose promoter hypermethylation in PD was associated to downregulation of antiporter expression consistent with an environmental exposure linked to PD risk.

WHAT ARE THE NEW FINDINGS?

- ⇒ *In vivo* BMAA administration depleted ileum levels of 'Candidatus Arthromitus', a group of bacteria that regulate gut mucosal immune homeostasis in mice leading to an increase in gut inflammation, disruption of gut barrier integrity and gut aSyn aggregation.
- ⇒ BMAA specifically targeted mesencephalic mitochondria inducing their fragmentation and cardiolipin exposure, which in turn activated innate immune responses such as NOD-like receptor 3 activation and aSyn aggregation.
- ⇒ Gut inflammation potentiated blood-brain barrier permeability and neuroinflammation, which culminated in nigrostriatal neuronal damage and PD-like motor dysfunction.

α -synuclein (aSyn)-containing insoluble fibrous aggregates, termed Lewy bodies and Lewy neurites as well as the loss of dopaminergic neurons in the substantia nigra pars compacta.¹ Aggregated aSyn can also be found in the GI tract and in organs innervated by the vagus nerve^{4,5} years to decades before detectable involvement of the central nervous system (CNS). Those observations allowed the formulation of a hypothesis arguing

SIGNIFICANCE OF THIS STUDY

HOW MIGHT IT IMPACT ON CLINICAL PRACTICE IN THE FORESEEABLE FUTURE?

⇒ This work generated cutting edge knowledge by establishing a direct link between chronic dietary consumption of an environmental microbial toxin and PD features. The specific antibiotic effect of foodborne BMAA identified here and the confirmed role of the dysbiotic gut as a trigger for the cascade of events that culminate in the brain, will have decisive public health implications regarding the revision of food safety guidelines to monitor and control this food toxin. This gives rise to an unprecedented opportunity for new bidirectional synergies between gastroenterology and neurology disciplines, which opens up new innovative possibilities for prevention or early diagnosis of PD.

that microbes or their products could travel from the gut to the brain via vagal retrograde axonal transport.⁶ This hypothesis found indirect support in data from humans who underwent complete truncal vagotomies and who appear to have a lower risk of developing PD.⁷ Additionally, certain microbial products occurring in a dysbiotic gut have been proposed to trigger aSyn overexpression and aggregation.^{8,9} Faecal and mucosa-associated gut microbiota differ between patients with PD and healthy controls,¹⁰ but functional interpretation is still controversial.¹¹ Gut dysbiosis in patients with PD is correlated with intestinal inflammation, inferred from the upregulation of proinflammatory cytokines.^{12,13} In mice, intestinal homeostasis is maintained by tissue-resident T helper 17 (Th17) cells that differentiate in response to symbiont mucosa-associated segmented filamentous bacteria (SFB) '*Candidatus* Arthromitus', which fulfil important roles in barrier integrity protection.^{14,15} SFB adherence to epithelial cells signals the release of serum amyloid A that acts on CD11c⁺ cells in the lamina propria to stimulate the production and release of interleukin (IL)-6 and IL-23 that stimulate differentiation and activation of Th17 cells. IL-17 released from tissue-resident homeostatic intestinal Th17 cells signals epithelial cells to produce antimicrobial peptides and tight-junction proteins.¹⁵ However, gut dysbiosis may also induce non-resident Th17 cells, either by erosion of protective microbes (mice SFB) or by the proliferation of pathobionts, driving the production of pro-inflammatory cytokines with peripheral effects.¹⁴ Recent data showed that inflammatory bowel disease patients may be at significantly higher risk of developing PD.¹⁶ Although PD has often been associated with a leaky intestinal barrier,^{17,18} the mechanisms through which gut dysbiosis disrupts barrier integrity are still incompletely understood. Accumulating evidence suggests that toxins produced by some gut microbes could impact host physiology.¹⁹ β -N-methylamino-L-alanine (BMAA), a natural non-proteinogenic diamino acid produced by cyanobacteria or other microbes and most often detected in aquatic food products, may be involved in neurodegeneration.¹⁹ BMAA was initially discovered in the seeds of cycad plant and its consumption has been proposed to cause amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS-PDC).²⁰ Evidence suggests that BMAA can be misincorporated in proteins in place of serine, or bind electrostatically to protein nascent chains causing misfolding, aggregation, endoplasmic reticulum (ER) stress and apoptosis.¹⁹ Moreover, BMAA can induce mitochondrial dysfunction interfering with oxidative phosphorylation while also deregulating calcium homeostasis and leading

to reactive oxygen species (ROS) overproduction.²¹ Although there is still no evidence that members of the gut microbiota can produce BMAA, hypermethylation of cg06690548 on chromosome 4 in the promoter of *SLC7A11* gene in PD patients is associated with downregulation of this cysteine–glutamate antiporter, a known target of BMAA,²² which is argued to be consistent with a PD-related environmental exposure. In fact, chronic exposure to environmental toxins that may lead to mitochondrial dysfunction has been advanced as a possible cause for PD in genetically susceptible individuals.²³ Building on the mitochondrial cascade hypothesis for PD,²³ we theorised that specific microbial molecules could travel from the gut to the brain and target neuronal mitochondria,²⁴ thus generating a self-amplified harmful cycle primed by neuronal innate immune activation. Accordingly, *in vivo* BMAA administration-induced strong expression of proinflammatory cytokines.²⁵ Our work further revealed that chronic treatment of mice with BMAA significantly eroded the levels of SFB, microbiota abundant in the ileum mucosa, with consequent alteration of local immune cell responses that led to increasing gut inflammation and disruption of gut barrier integrity. Surprisingly, we also observed that mesencephalic mitochondria were dysfunctional and accumulated aSyn aggregates which in turn led to further activation of neuronal innate immunity and neuroinflammation. Finally, we demonstrated a caudo-rostral progression of the disease from the gut to the brain most likely through the vagus nerve, as evidenced by the aSyn trail that culminated in substantia nigra (SN) degeneration and motor impairment, all archetypal features of PD.

METHODS

Animal model and experimental design

C57BL/6 male mice obtained from Charles River (Barcelona, Spain) were housed under a 12 hours light/dark cycle with free access to water and food. At 26 weeks mice were randomly divided in two groups (untreated (Unt) and BMAA-treated) and daily orally administered with BMAA (0.1g/kg bw) for 12 weeks in gelatin pellets, until they were sacrificed. This BMAA dose was selected according to previous studies.²⁶ Untreated animals received vehicle. Behavioural analyses were performed (30–40 weeks), mice were sacrificed, and samples were collected for analysis, as described in detail in online supplemental material. All reagents used are listed in online supplemental table S1.

Microbiome profiling

Faecal, ileum and cecum mucosa-associated material were collected to assess microbiome profiles using 16S rRNA gene sequencing as previously described^{27,28} and using the mothur package V.1.44.1²⁹ and Silva reference files (release 138)³⁰ for treatment of raw data, clustering and taxonomic annotation. Data analyses were performed with the online tool MicrobiomeAnalyst³¹ and its R package DESeq2³² software as described in online supplemental material.

Behavioural analyses

Mice behavioural analyses were assessed as described before for beam walking,³³ hindlimb clasping,^{34,35} inverted grid,^{9,36} open field³³ and T-maze³⁷ as described in online supplemental material.

Immunohistochemistry, immunofluorescence and microscopy analyses

At the end of the experiment, animals were transcardially perfused. Brain and ileum samples were collected, cryoprotected and stored at -80°C . Immunohistochemistry and immunofluorescence staining

for brain and gut samples were performed as published³⁸ and as described in detail in online supplemental material. In gut samples we assessed (a) the integrity of intestinal barrier by immunofluorescence, using a score system scale^{18,39}; (b) The number of CD11b divided per the total counting area (mm²) and by immunohistochemistry (c) the number of CD4 cells divided per the total counting area (mm²); and (d) the optical density (OD) of aSyn aggregates and the presence of phosphorylated-aSyn (p-aSyn) inclusions. In brain samples, we used immunofluorescence to determine (a) the number of in Iba1⁺ cells in and Trem2 expression in SN and (b) the number of TH + and ChAT + cells in dorsal motor nucleus of the vagus (DMV) divided per the total counting area (mm²). We used immunohistochemistry to assess (c) the OD of aSyn aggregates in striatum (STR), SN and DMV, the presence of p-aSyn in DMV and SN and the OD of tyrosine hydroxylase (TH) in STR, (d) the stereological quantification of TH + cells in SN and (e) the number of IgG-immunopositive staining in the perivascular area per total area (mm²) in the cortex, STR and SN to assess the blood-brain barrier (BBB) integrity. All image acquisitions were performed under well-established blinded protocols and randomised processes, and with different technical and biological replicates, as comprehensively described in online supplemental material.

Flow cytometry

Blood was collected by cardiac puncture, transferred to Histo-paque 1083 solution (Sigma) and centrifuged. The peripheral blood mononuclear cell (PBMC) fraction was isolated, washed and 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). Cell suspension acquisition was performed in BD FACSCalibur cytometer (BD Bioscience) and analysed in FlowJo software (BD Bioscience) (see online supplemental material for comprehensive description).

Western blotting, spectrophotometry and ELISA determinations in brain, intestine homogenates and blood

After completing the behavioural tests, Unt and BMAA-treated mice were euthanised and blood, mesencephalon, striatum, ileum and cecum samples were isolated. Tissues were snap frozen, stored at -80°C and homogenised as described in online supplemental material. Plasma interferon (IFN) γ and IL-6 levels were determined. In mesencephalic homogenates we determined synaptic markers and innate immunity markers by western blot, caspase-1 activity by spectrophotometry and innate immunity markers and aSyn oligomers with ELISA kits and western blot. In striatal homogenates we determined dopamine levels with ELISA Kit. In ileum and cecum homogenates we determined caspase-1 activity by spectrophotometry, and innate immunity markers and aSyn oligomers with ELISA kits and by western blot (see online supplemental material).

Western blotting, spectrophotometry and ELISA determinations in cellular extracts

NT2 Rho+, Rho0 cells⁴⁰ and primary mesencephalic neurons obtained from mesencephala of C57Bl/6 mice embryos brains at gestation day 14/15 were cultured as described previously with some modifications^{41,42} and treated with 3 mM BMAA for 48 hours with 1 μ M CCCP (cells) or 5 μ M (neurons) for 2 hours before cell harvesting or with 20 mM NH₄Cl and/or 20 μ M Leupeptin for 4 hours to the culture medium wherever indicated. Cells were washed in ice-cold phosphate-buffered saline (PBS 1 \times), scraped and homogenised as described in

online supplemental material. In cellular extracts we determined aSyn oligomers, LC3II and innate immunity markers by western blot. In neuronal mitochondrial fractions we determined phospho-Drp1 levels and aSyn oligomers by western blot. In neuronal cytosolic fractions we analysed innate immunity markers with ELISA kits and Caspase-1 activation by spectrophotometry (see online supplemental material).

Immunocytochemistry and confocal microscopy analysis

Primary mesencephalic neurons, NT2 Rho+ and Rho0 cells were grown on glass coverslips (16 mm diameter) in 12-well plates or in ibidi μ -Slide 8-well plates. Following treatments, we determined mitochondrial movements with MitoTracker Green in neurons, mitochondrial membrane potential with TMRM Probe in both cells and neurons and cardiolipin distribution and fluorescence using the 10-N-Nonyl acridine orange in neurons. Immunocytochemistry and confocal microscopy procedures are described in online supplemental material.

Mitochondria isolation, oxygen consumption rate, Ca²⁺ handling capacity and glycolytic fluxes (ECAR) analyses

Mouse mesencephalic and cortical mitochondria were isolated by Percoll gradient (see online supplemental material). Oxygen consumption rate (OCR) was measured in fresh mesencephalic or cortical mitochondria with a Seahorse XF24 Extracellular Flux analyser (Seahorse Bioscience, Billerica, Massachusetts, USA). For the respiratory coupling experiments, the following determinations were calculated according to the ensuing 'rate measurement equation' (see online supplemental material).⁴³ Mitochondrial Ca²⁺ uptake was measured fluorometrically in the presence of the Ca²⁺-sensitive fluorescent dye Calcium Green 5N (150 nM), using excitation and emission wavelengths of 506 nm and 532 nm, respectively.⁴⁴ In primary mesencephalic neurons the OCR and ECAR were determined using a Seahorse XF24 Extracellular Flux analyser (Seahorse Bioscience). The experimental set-up is described in online supplemental material.

Statistical analyses

Microbiome population statistics are described in the detailed online supplemental materials and methods. Statistical analyses of data sets were performed using GraphPad Prism V.8 (GraphPad Software) software as summarised in online supplemental table 2. All data are represented as the mean \pm 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's t test or Mann-Whitney test. Comparisons of multiple groups were performed with one-way analysis of variance followed by Dunnett's post-hoc test or Kruskal-Wallis test followed by Dunn's post-hoc test. Correlation analysis between two variables was performed by Pearson's 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.

RESULTS

Gut microbiota alterations in mice treated orally with BMAA

BMAA is a microbial neurotoxin that can be ingested through the diet due to its accumulation in the food chain, especially from aquatic ecosystems.¹⁹ We have orally administered BMAA for 12 weeks to wild-type (WT) C57Bl/6 male mice and observed no alterations in their weight or glycaemia (online supplemental figure S1A-C) although a significant increase in stool water

content was observed (data not shown). After treatment, faecal material was collected, and mice were sacrificed to isolate the ileum and the cecum. A comprehensive analysis of the ileum-associated (figure 1), faecal (figure 2) and cecum-associated bacteria (online supplemental figure S2) was performed using Illumina MiSeq technology at our Next Generation Sequencing Unit Genoinseq (<https://www.cnc.uc.pt/en/services>). We obtained on average 1876 ± 1650 reads per ileum sample, and detected 102 genera, 59 families, 40 orders, 14 classes and 10 bacterial phyla. From the stools we obtained $52\,952 \pm 12\,996$ reads per sample and identified 165 genera, 82 families, 56 orders, 18 classes and 11 phyla. From the cecum samples we obtained on average $31\,001 \pm 7140$ reads per sample and detected 121 genera, 62 families, 39 orders, 15 classes and 11 phyla. We observed an increase in the diversity of ileum-associated bacteria in BMAA-treated mice (alpha-diversity, which measures within-sample taxonomic diversity; figure 1A) most likely due to the loss of the most representative SFB members, although the overall community diversity was similar between environments (beta-diversity, which evaluates the similarity or dissimilarity between two communities; figure 1B). Sequences of five genera were significantly altered in the ileum-associated mucosa of BMAA-treated mice with the most significant decreases of '*Candidatus Arthromitus*' (approximately 17-fold decrease) and *Turicibacter* (approximately 13-fold decrease) and increases of *Colidextribacter* (approximately 6-fold increase), *Bacteroides* (approximately 7-fold increase) and Lachnospiraceae_NK4A136 (approximately 11-fold increase) (figure 1C–I). Most microbiome studies have been focused on the transient stools' microbiota, which may not translate the real impact of different disease states. In the faecal material, no significant changes were detected in the richness of microbiota between BMAA-treated and untreated mice at any taxonomic level (alpha diversity; figure 2A) and some overlap in the microbial composition of both communities was observed (beta diversity; figure 2B). We found that 11 genera were significantly altered in faecal samples of BMAA-treated mice: *Olsenella*, *Mucispirillum*, Eggerthellaceae_unclassified and *Roseburia* were enriched while RF39_ge, *Monoglobus*, Family_XIII_AD3011_group, *Akkermansia*, *Tyzzerella*, *Turicibacter* and Clostridia_UCG_014_ge were depleted (figure 2C–I). Although *Roseburia* (figure 2G) have been associated with short chain fatty acids (SCFAs) production and were found to be depleted in patients with PD,⁴⁵ they were enriched in the stools of BMAA-treated mice. On the other hand, the abundance of *Akkermansia* known to improve gut barrier function by modulating the mucus layer with beneficial impact in the immune response, was lower in BMAA-treated mice stools (figure 2H).⁴⁵ Interestingly, BMAA treatment elicited significant depletion of members of the *Turicibacter* and of members of the cryptic Clostridia_UCG_014_ge (figure 2F,I). Our results link the erosion of the ileum-associated *Turicibacter* with that observed in the transient faecal microbiota and in the cecum microbiota, the latter showing overall decreased diversity and a distinct microbial community composition (online supplemental figure S2A,B). As observed in the faecal material, the abundances of *Turicibacter* and Clostridia_UCG_014_ge were also depleted in the cecum after BMAA treatment (online supplemental figure S2C–F, I). *Bacteroides* were enriched in both the ileum and cecum of BMAA-treated mice (figure 1H, online supplemental figure S2C–E, G). Of notice was the near disappearance of cecum *Bifidobacterium* levels (online supplemental figure S2H), whose members are thought to promote health and restore mucus growth via SCFAs production.

BMAA-induced gut dysbiosis impairs GI function and exacerbates inflammation

BMAA-induced erosion of '*Candidatus Arthromitus*' from the ileum mucosa (figure 1E–F) was accompanied by an increase of taxa that may signal intestinal epithelial cells and CD11b+ positive macrophages or dendritic cells to promote tissue inflammation, which we also found to be increased in the ileum of BMAA-treated mice (figure 3A–B). Activation of resident immune cells or amplified diffusion of noxious molecules by pathobionts proliferating in the ileum mucosa indeed induced inflammation, as inferred from the increased levels of proinflammatory mediators such as tumour necrosis factor (TNF)- α (figure 3C), IL-8 (figure 3D) and IL-17 (figure 3E) and from the lower levels of IL-10, an anti-inflammatory cytokine (figure 3F). Ileum SFB are known to actively stimulate gut immunity and induce homeostatic Th17 cell response and promote intestinal barrier integrity.¹⁵ Our results indicate that the increase in IL-17 levels were not due to SFB-induced tissue-resident homeostatic Th17 cells, but instead to the stimulation of inflammatory Th17 cells.¹⁴ The loss of SFB in BMAA-treated mice may have hypothetically allowed overgrowth of pathobionts able to stimulate inflammatory Th17 cells. Under these inflammatory conditions NOD-like receptor (NLRP) 3 inflammasome is activated due to the translocation of nuclear factor kappa-B (NF- κ B) p65 into the nucleus (figure 3G) allowing the activation of caspase 1 and the production of IL-1 β (figure 3H–I). This seems to be a specific effect of '*Candidatus Arthromitus*' depletion in the ileum since BMAA effects in the cecum are not consistent with a major inflammatory profile (online supplemental figure S3A–D). Increased levels of TNF- α and IL-1 β are known to enhance intestinal permeability due to the internalisation of tight junction proteins, namely ZO-1 and occludin.⁴⁶ We observed a significant decrease in ZO-1 (figure 3J,K) and occludin (figure 3J,L) levels in the ileum of BMAA-treated mice, which indicates disruption of intestinal barrier integrity. Since infiltrated T cells are mostly CD4-expressing 'helper' T cells, we tackled the levels of CD4+ cells in ileum lamina propria of BMAA-treated mice but did not detect relevant differences (figure 3M,N). Nevertheless, we observed an alteration in CD4/CD8 ratios in the blood of BMAA-treated mice (figure 3O,P), due to a decrease in CD4 cells (online supplemental figure S3E,F), which might indicate systemic inflammation and concomitantly increased permeabilisation of the BBB. Indeed, we observed an increase in IFN γ and IL-6 levels in the plasma of BMAA-treated mice (figure 3Q), similarly to what is observed in patients with PD⁴⁷ and that may impact the BBB integrity.⁴⁸ We observed a positive microvascular leak in SN in BMAA-treated mice (figure 3R,S), but no alterations in the striatum or cortex (online supplemental figure S3G–J). Additionally, we did not observe CD4+ cellular infiltration in SN at this time point, despite the increased BBB permeability (online supplemental figure S3K). Noteworthy, the cortex and striatum have increased permeability to blood-borne components compared with midbrain areas,⁴⁹ leaving the question of why we see BBB permeability alteration in the SN, but not in the cortex or striatum unanswered.

BMAA targets mesencephalic mitochondria

Our findings support the notion that BMAA, a non-proteinogenic amino acid of microbial origin, may target their ancient relatives, the mitochondria. After BMAA treatment we isolated mice mesencephalic mitochondria and observed a reduction in mitochondrial function, namely a decrease in basal and maximal respiration and a decrease in ATP synthesis (figure 4A–D),

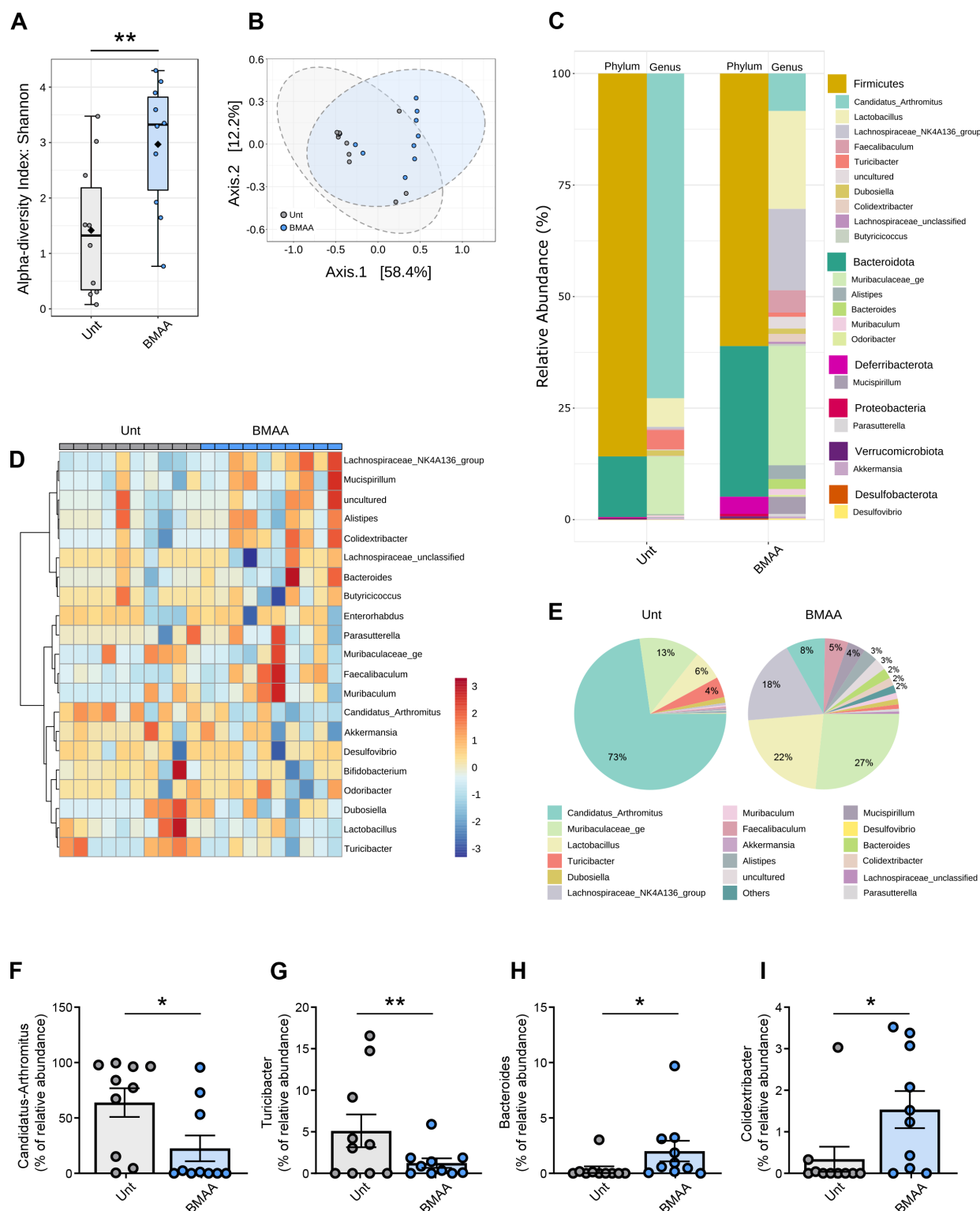


Figure 1 Ileum mucosa-associated microbiome diversity in β -N-methylamino-L-alanine (BMAA)-treated mice. (A) Alpha diversity measured using the Shannon index at operational taxonomic unit (OTU) level derived from 16S rDNA sequencing of ileum intestinal samples from untreated (Unt) or BMAA-treated mice (n values for Unt=10 and BMAA=10, Unt vs BMAA, Mann-Whitney test, **p=0.0089). (B) Beta diversity evaluated by principal coordinate analysis (PCoA) based on Bray-Curtis index of OTUs derived from 16S rDNA sequencing of ileum intestinal samples from Unt or BMAA-treated mice (n values for Unt=10 and BMAA=10; PERMANOVA: $r^2=0.205$, *p<0.015; PERMDISP: F=0.773, p=0.391). (C) Taxonomic diversity of ileum intestinal samples from Unt or BMAA-treated mice at the phylum and genus levels. (D) Heatmap of the relative abundances of genera detected in ileum intestinal samples from 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 the proportional taxonomic composition at the genus level of ileum intestinal microbiota samples from Unt or BMAA-treated mice. (F–I) Differential abundance of selected genera in ileum intestinal samples from Unt or BMAA-treated mice (n values for Unt=10 and BMAA=10, Unt vs BMAA, DESeq2 statistical analysis). (F) *Candidatus Arthromitus* (*padj=0.0296). (G) *Turicibacter* (**padj=0.0027). (H) *Bacteroides* (*padj=0.0150). (I) *Colidextribacter* (*padj=0.0103).

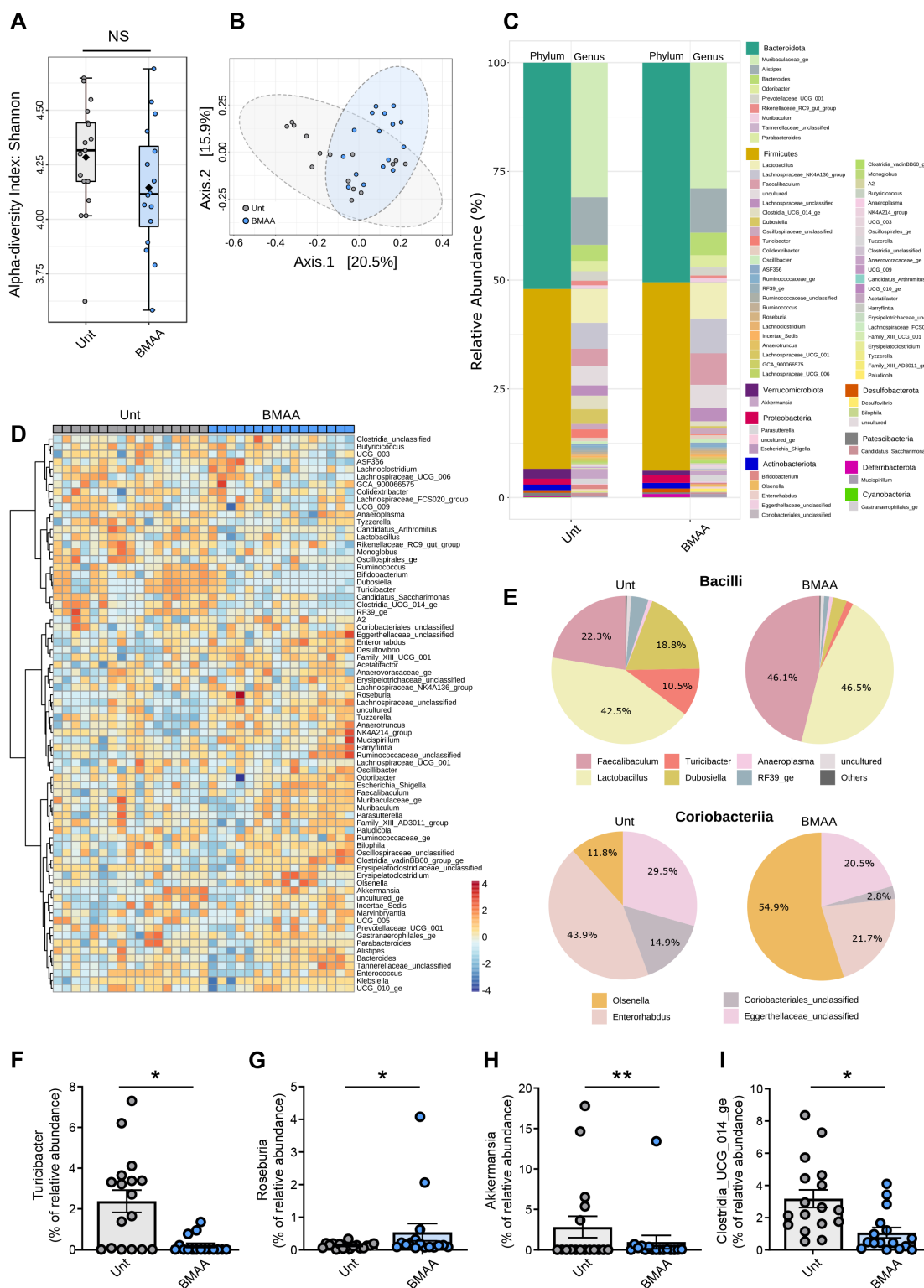


Figure 2 Microbiome diversity in the stools of β -N-methylamino-L-alanine (BMAA)-treated mice. (A) Alpha diversity measured using the Shannon index at operational taxonomic unit (OTU) level derived from 16S rDNA sequences obtained from faecal samples from untreated (Unt) or BMAA-treated mice (n values for Unt=17 and BMAA=16, Unt versus BMAA, Mann-Whitney test, $p=0.136$). (B) Beta diversity evaluated by principal coordinate analysis (PCoA) based on Bray-Curtis index of OTUs derived from 16S rDNA sequencing of faecal samples from Unt or BMAA-treated mice (n values for Unt=16 and BMAA=16; PERMANOVA: $r^2=0.108$, *** $p<0.001$; PERMDISP: $F=2.378$, $p=0.134$). (C) Taxonomic diversity of faecal microbiota from Unt or BMAA-treated mice at the phylum and genus levels. (D) Heatmap of relative abundances of the genera detected in the faeces from 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 the proportional taxonomic composition at the genus level of faecal microbiota samples from Unt or BMAA-treated mice for two selected class level taxa affected by BMAA treatment, Bacilli and Coriobacteriia. (F–I) Differential abundance of selected genera in faecal samples from Unt or BMAA-treated mice (n values for Unt=17 and BMAA=16, Unt vs BMAA, DESeq2 statistical analysis). (F) *Turicibacter* (* $\text{padj}=0.0169$). (G) *Roseburia* (* $\text{padj}=0.0242$). (H) *Akkermansia* (** $\text{padj}=0.0078$). (I) *Clostridia_UCG_014_ge* (* $\text{padj}=0.0190$).

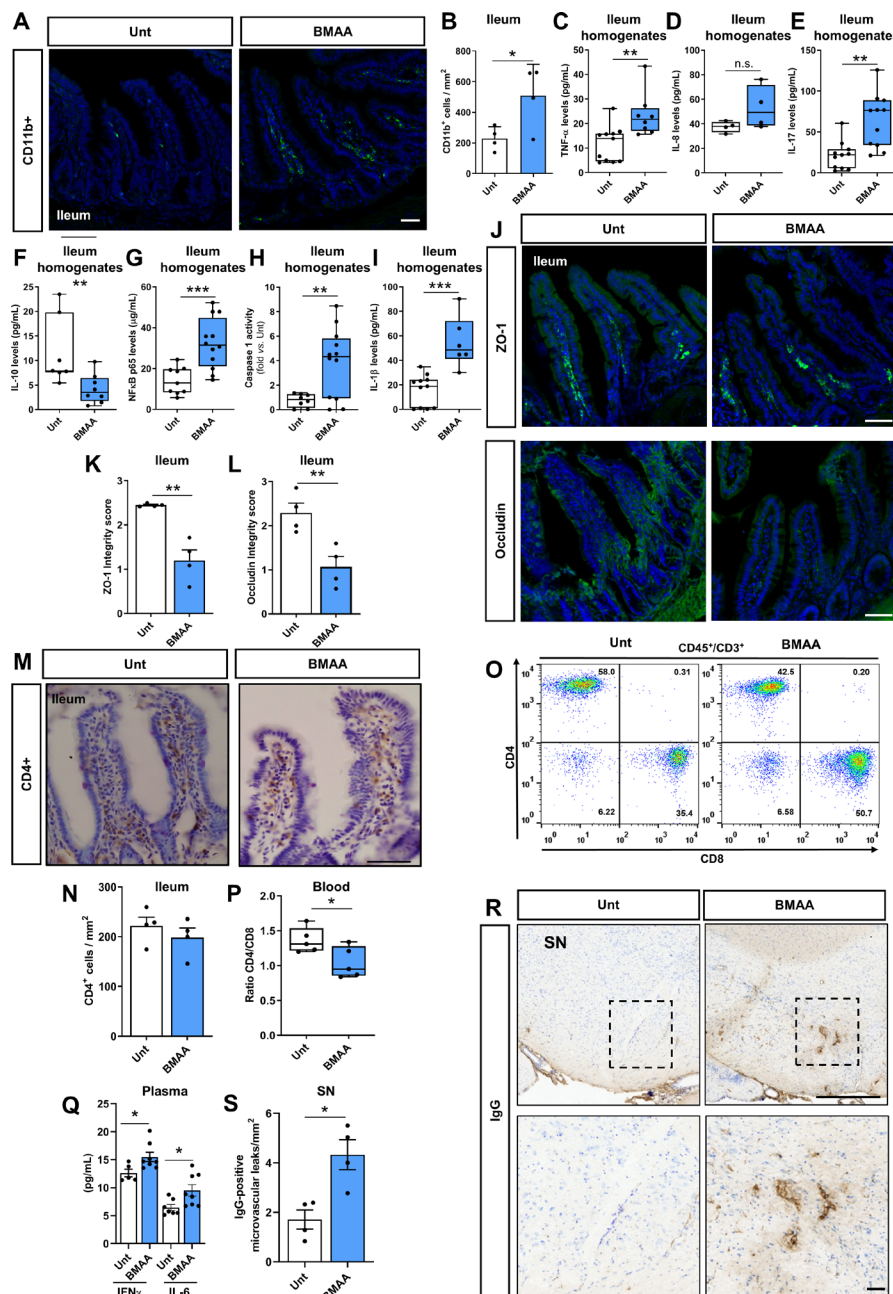


Figure 3 β -N-methylamino-L-alanine (BMAA) induces ileum inflammation and substantia nigra (SN) microvascular leaks. (A) Representative images of transversal ileum sections stained with anti-CD11b in untreated (Unt) and BMAA-treated mice. (B) Quantification of CD11b⁺ cells per mm² in the ileum (n values for all conditions=4, Unt vs BMAA, * $p=0.0435$). (C–I) Measurement of specific mediators involved in the inflammatory response in ileum intestinal samples from Unt or BMAA-treated mice. (C) Tumour necrosis factor measured by ELISA (n values for Unt=11 and BMAA=8, Unt vs BMAA, ** $p=0.0036$), (D) Interleukin (IL)-8 measured by ELISA (n values for Unt=4 and BMAA=4, Unt vs BMAA $p=0.135$), (E) IL-17 measured by ELISA (n values for Unt=11 and BMAA=11, Unt vs BMAA, ** $p=0.0011$), (F) IL-10 measured by ELISA (n values for Unt=7 and BMAA=8, Unt vs BMAA, ** $p=0.009$), (G) NF- κ B measured by ELISA (n values for Unt=9 and BMAA=12, Unt vs BMAA, *** $p=0.0008$), (H) Caspase-1 activity. Values were subtracted to Unt (n values for Unt=8 and BMAA=12, Unt vs BMAA, ** $p=0.0068$) and (I) IL-1 β measured by ELISA (n values for Unt=7 and BMAA=6, Unt vs BMAA *** $p=0.0002$). (J–L) Assessment of intestinal barrier integrity. (J) Immunofluorescence staining for zonula occludens-1 (ZO-1) and occludin. (K) ZO-1 integrity score (n values for all conditions=4, Unt vs BMAA, ** $p=0.0019$). (L) Occludin integrity score (n values for all conditions=4, Unt vs BMAA, ** $p=0.0095$). (M) Representative images of transversal ileum sections stained with anti-CD4 in Unt and BMAA-treated mice. (N) Quantification of CD4⁺ cells per mm² in ileum (n values for all conditions=4, Unt vs BMAA, $p=0.411$). (O–P), Determination of lymphocyte populations in the blood by flow cytometry. (O) Representative plots of lymphocytes (CD45⁺CD3⁺) CD4 and CD8 populations in Unt and BMAA-treated mice. (P) Quantification of the CD4/CD8 ratio (n values for all conditions=5, Unt vs BMAA, * $p=0.036$). (Q) Interferon (IFN) γ and IL-6 levels in the plasma were measured by ELISA (n values for Unt=5–7 and BMAA=8, Unt vs BMAA * $p=0.0346$ for IFN γ and * $p=0.0238$ for IL-6). (R–S) Assessment of IgG-positive microvascular leaks in the SN of Unt and BMAA-treated mice. (R) Representative images of SN coronal sections stained with IgG. (S) Quantification of IgG-positive microvascular leaks per mm² in SN (n values for all conditions=4, Unt vs BMAA, * $p=0.0108$). Scale bars are 50 μ m except in R (upper box)=500 μ m. Data represent mean \pm SEM. Statistical analysis: Unpaired Student's t-test was performed in B, D–E, G–I, K–L, N, P–Q and S. Mann-Whitney test was performed in C and F.

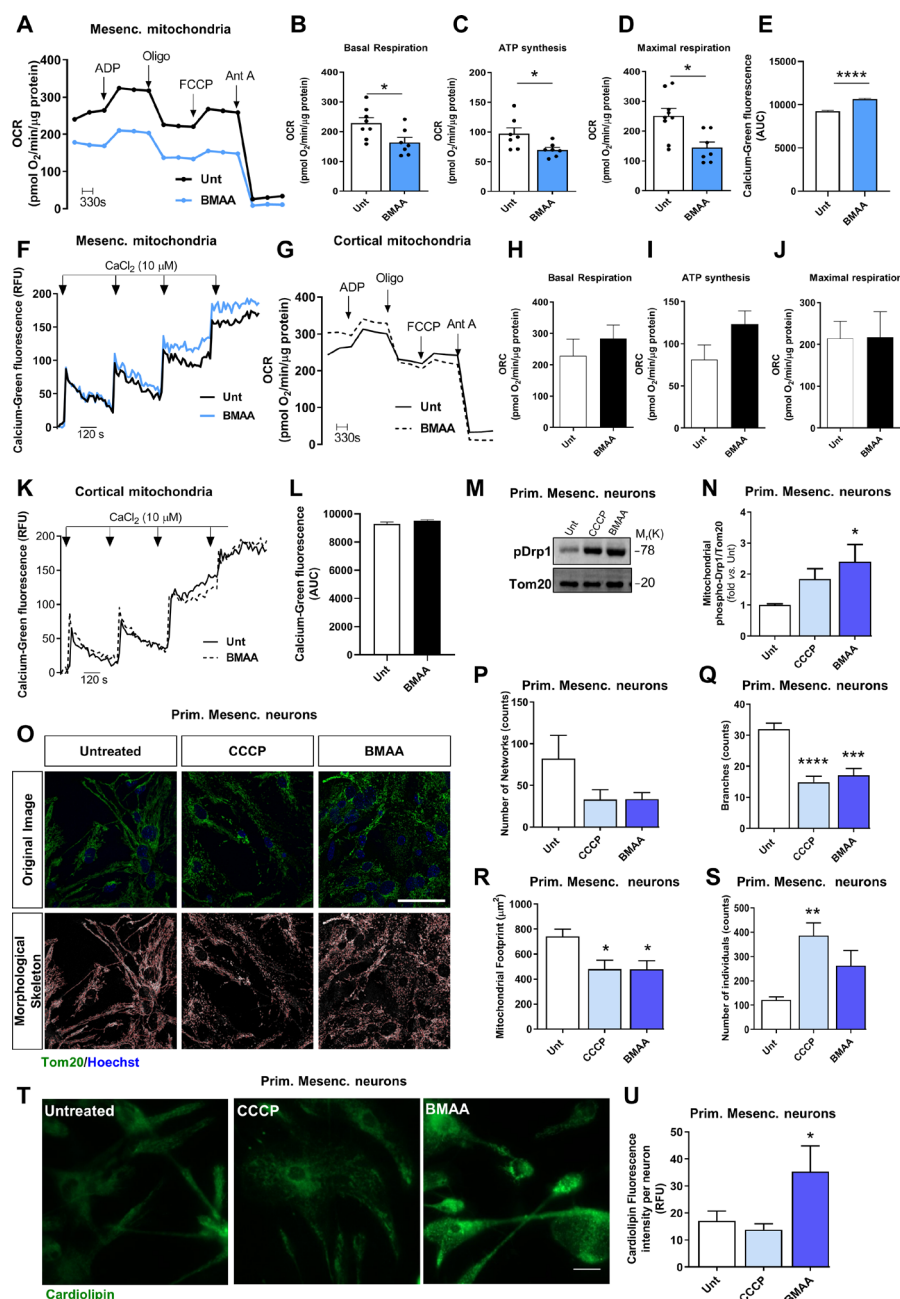


Figure 4 β -N-methylamino-L-alanine (BMAA) induces neuronal mitochondrial dysfunction, fragmentation and cardiolipin exposure. Isolated mesencephalic mitochondria from untreated (Unt) and BMAA-treated mice were examined. (A) Representative graph showing oxygen consumption rate (OCR); (B) basal respiration; (C) ATP synthesis; (D) maximal respiration. Values are pmol O₂/min/μg protein. (B–D, n values for all conditions=7, Unt vs BMAA, B, *p=0.0125, C *p=0.0286 and D, *p=0.020). (E–F) Mesencephalic mitochondria's ability to uptake calcium was evaluated with the fluorescent probe Calcium-green (n values for all conditions=4, Unt vs BMAA, ****p<0.0001). Isolated cortical mitochondria from Unt and BMAA-treated mice were examined. (G) Representative graph showing OCR in the cortex; (H) basal respiration; (I) ATP synthesis; (J) maximal respiration. Values are pmol O₂/min/μg protein. (H–J) n values for all conditions=4, Unt vs BMAA; H, p=0.4456; I, p=0.1246 and J, p=0.9689). (K–L) Cortical mitochondria's ability to uptake calcium was evaluated with the fluorescent probe calcium-green (n values for all conditions=4, Unt vs BMAA, p=0.1870). (M–U) Primary mesencephalic neuronal cultures were treated with 3 mM BMAA and 1 μM CCCP for 2 hours. (M) Representative immunoblot for phospho-Drp1 levels and (N) respective densitometric analysis. The blots were re-probed for TOM20 to confirm equal protein loading and mitochondrial fraction purity (n values for Unt and CCCP=4, BMAA=3. Unt vs CCCP, p=0.0711; Unt vs BMAA, *p=0.0375). (O) Primary mesencephalic neurons were immunostained with Tom20. (P–S) Alterations in mitochondrial network were calculated with an ImageJ Macro tool (n values for all conditions=6). (P) Number of mitochondrial networks (Unt vs CCCP, p=0.2674; Unt vs BMAA, p=0.3169). (Q) Number of mitochondrial branches (Unt vs CCCP, ****p<0.0001; Unt vs BMAA, ***p=0.0002). (R) Mitochondrial footprints (Unt vs CCCP, *p=0.0247; Unt vs BMAA, *p=0.0241). (S) Number of mitochondrial individuals (Unt vs CCCP, **p=0.026; Unt vs BMAA, p=0.0968). (T) Representative images of cardiolipin exposure which was visualised with the fluorescent dye 10-N-nonyl acridine orange. (U) Cardiolipin fluorescence was calculated with ImageJ. Data is reported as absolute values (n values for all conditions=6, Unt vs CCCP, p=0.676; Unt vs BMAA, *p=0.0255). Scale bars in O=50 μm and T=33 μm. Data represents mean±SEM. Statistical analysis: Unpaired Student's t-test was performed in B–E, H–J and L. One-way analysis of variance followed by Dunnett's test was performed in N, Q–S and U. Kruskal-Wallis test followed by Dunn's test was performed in P.

which reduced mitochondrial pool calcium-buffering capacity (figure 4E–F). However, cortical mitochondria isolated from BMAA-treated mice were not affected (figure 4G–L). To investigate if BMAA contributes to neurodegeneration by specifically targeting neuronal mitochondria, we performed *in vitro* experiments using pure isolated mitochondria, to find that acute BMAA administration decreased oxidative phosphorylation both in isolated mesencephalic mitochondria (online supplemental figure S4A–G) and in cortical mitochondria of WT mice.²¹ Our results indeed confirm that both brain areas are differentially affected, not due to differences in the mitochondrial pools between mesencephalon and cortex, but as a consequence of blood-borne translocation or vagal rostral trajectory of BMAA or of other unknown effectors that could not reach cortical areas at the time point defined in this work. Additionally, we also determined OCR in primary mesencephalic neurons exposed to BMAA and observed a decrease in basal mitochondrial respiration and ATP synthesis, similar to those elicited by CCCP (online supplemental figure S4H–L). Moreover, we observed a BMAA-induced decrease in glycolysis, glycolytic capacity rate and spare glycolytic capacity in primary mesencephalic neurons, effects comparable to those caused by CCCP (online supplemental figure S4M–P). Accordingly, and due to the observed mitochondrial dysfunction in BMAA-treated primary mesencephalic neurons, we detected a decrease in the mitochondrial membrane potential (online supplemental figure S5A). Interestingly, BMAA did not further decrease the mitochondrial membrane potential in Rho0 cells (mitochondrial-deficient cells that lack mitochondrial DNA) (online supplemental figure S5B). While a dysfunctional mitochondrial pool needs fragmentation to be degraded by mitophagy and avoid mitochondrial-mediated death,⁵⁰ we found that BMAA increased the levels of the fission protein phosphoDrp1 in the mitochondria of primary mesencephalic neurons (figure 4M,N) thus leading to a fragmented mitochondrial network (figure 4O–S). Remarkably, the toxin was unable to further fragment the mitochondrial pool in cells devoid of functional mitochondria (online supplemental figure S5C–E). A decreased mitochondrial function with increased fragmentation leads to the exposure of cardiolipin, a lipid occurring both in the mitochondrial inner membrane and in bacteria.⁵¹ Indeed, we confirmed that treatment of primary mesencephalic neurons with BMAA triggered cardiolipin exposure (figure 4T,U). To overcome the exposure of this danger-associated molecular pattern (DAMP), dysfunctional and fragmented mitochondria are expected to be removed by mitophagy.⁵² We examined mitochondrial movement and macroautophagy in primary mesencephalic neurons and observed a reduction in mitochondrial average velocity induced by BMAA (online supplemental figure S6A,B). Moreover, we also observed a decrease in the autophagic flux in primary mesencephalic neurons treated with BMAA (online supplemental figure S6C–E), which indicates a decreased turnover of dysfunctional mitochondria. We further showed that mitochondria were located in autophagosomes (online supplemental figure S6F–H) but not co-localised with autolysosomes (online supplemental figure S6I–K), which points to an accumulation of dysfunctional mitochondria in BMAA-treated primary mesencephalic neurons with the undesirable overexposure of cardiolipin.

Mitochondrial exposure of cardiolipin drives innate immunity activation and neuroinflammation

Dysfunctional mitochondria exposing cardiolipin, a recognised DAMP,⁵² activate innate immune responses in a self-amplified

loop that culminates in progressive neurodegeneration. To tackle neuronal contribution to innate immunity activation we confirmed a low level of glial cell contamination in primary mesencephalic neuronal cultures (less than 1% of Iba1+, Trem2+, CD11b+ cells and less than 20% of glial fibrillary acidic protein, GFAP-positive cells) (data not shown), which led us to propose that neurons can mount an innate immune response mediated by BMAA-induced mitochondrial dysfunction. We observed that BMAA induced an increased expression of toll-like receptor (TLR) 4 in primary mesencephalic neurons (figure 5A–B). TLR signalling leads to NF- κ B activation and translocation into the nucleus, where it binds to the promoter region of IL-1 β gene inducing its transcription.⁵³ We also observed NF- κ B activation after BMAA treatment (figure 5C) and the expected increase in pro-IL-1 β levels (figure 5A,D). Caspase 1 that cleaves pro-IL-1 β was also found to be activated in neurons after BMAA treatment (figure 5E), leading to an increase in mature IL-1 β levels (figure 5F).

To demonstrate that BMAA treatment selectively induces neuroinflammation through mitochondria-dependent neuronal innate immunity activation, we isolated mice mesencephalon to tackle NLRP3 inflammasome activation and observed that BMAA induced an increased expression of TLR7 and TLR4 receptors *in vivo* (figure 5G–I). Both TLR7, located in endosomes, and TLR4 in the plasma membrane, are able to trigger innate immune responses in neurons.⁵⁴ These receptors signal NF- κ B (figure 5J), which will activate NLRP3 inflammasome. Indeed, we observed an increase in pro-IL-1 β levels (figure 5G and K) and the activation of caspase 1 (figure 5L), which allowed the release of the pro-inflammatory IL-1 β (figure 5M). Taken together, our results strongly suggest that orally-administered BMAA activated mice brain innate immunity probably as a result of mitochondrial exposure of DAMPs. While recent studies link the production of IL-17 in the brain with BBB leakage and inflammatory disease progression,⁵⁵ our mouse model fails to reveal significant increase in mesencephalic levels of IL-17 (figure 5N) or statistically significant alterations in the levels of anti-inflammatory IL-10 (figure 5O). We observed the activation of microglia in the SN with enlarged cell bodies and processes visualised with Iba1 (figure 5P–Q), and detected increased localisation of Trem2-positive disease-associated microglia (figure 5P–R). Activated microglia also releases pro-inflammatory cytokines, namely TNF- α and IL-1 β that may favour BBB permeabilisation and subsequent infiltration of peripheral leucocytes into the CNS.⁵⁵ Nevertheless, at the selected time point we did not observe CD4+ cell infiltration in the SN of BMAA-treated mice (online supplemental figure S3K).

BMAA treatment elicits propagation of aSyn aggregates from the gut to the nigrostriatal regions of the brain likely via the vagus nerve

aSyn aggregates are major histopathological hallmarks of PD. Recent data from humans and PD mouse models suggest that the upregulation of aSyn expression and the subsequent oligomerisation and aggregation occurs initially in gut enteric neurons and later in SN dopaminergic neurons.^{3,5} Indeed, we observed aSyn aggregates and p-aSyn (S129P) in ileum samples of BMAA-treated mice (figure 6A–E). Ileum samples from BMAA-treated mice revealed increased levels of aSyn aggregates and/or oligomers by immunohistochemistry (figure 6A,B), ELISA (figure 6C) and western blot (figure 6D–E). These differences were significant in the ileum, but not in the cecum (online supplemental figure S7A). Furthermore, and in agreement with Braak's hypothesis

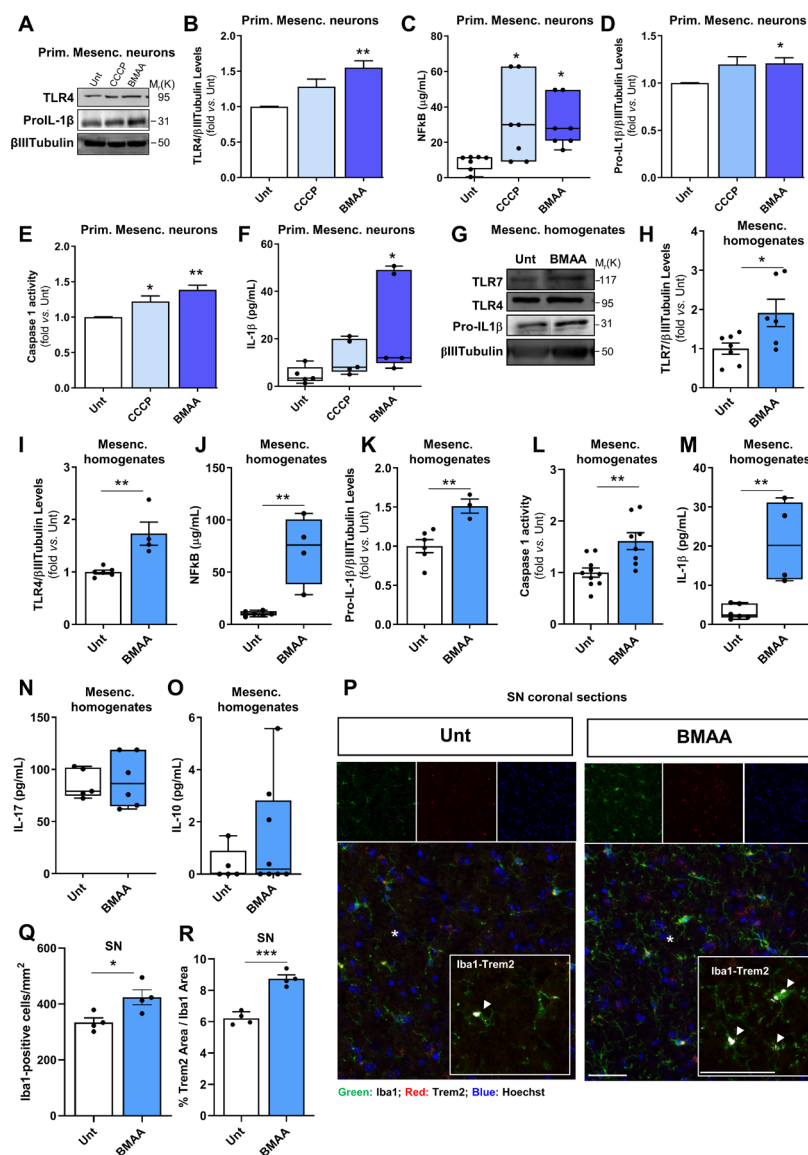


Figure 5 β -N-methylamino-L-alanine (BMAA) activates brain neuronal innate immunity *in vitro* and *in vivo*. Primary mesencephalic neuronal cultures from naive mice were treated with 1 μ M CCCP for 2 hours and 3 mM BMAA for 48 hours. (A) Representative immunoblot for toll-like receptor (TLR)4 and pro-interleukin (IL)-1 β levels. Blots were re-probed for β III-Tubulin to confirm equal protein loading. (B) Densitometric analysis of the levels of TLR4 was normalised with β III-Tubulin (n values for all conditions=4, untreated (Unt) vs CCCP, $p=0.076$; Unt vs BMAA, $**p=0.0023$). (C) Nuclear factor kappa-B (NF- κ B) levels were calculated using NF- κ B p65 ELISA kit. Values are μ g/mL (n values for all conditions=7, Unt vs CCCP, $*p=0.0039$, Unt vs BMAA, $*p=0.013$). (D) Densitometric analyses of the levels of pro-IL-1 β were normalised with β III-Tubulin (n values for all conditions=5, except BMAA=4, Unt vs CCCP, $p=0.1213$; Unt vs BMAA, $*p=0.044$). (E) Caspase-1 activation (n values for all conditions=4, Unt vs CCCP, $*p=0.047$, Unt vs BMAA, $**p=0.002$). (F) IL-1 β levels in the isolated cytosolic fraction was determined using an IL-1 β ELISA kit. Values are pg/mL (n values for all conditions=5, Unt vs CCCP, $p=0.5928$; Unt vs BMAA, $*p=0.018$). Homogenates from the mesencephalon of mice treated with or without BMAA were examined. (G) Representative immunoblot for TLR7, TLR4 and pro-IL-1 β levels. The blots were re-probed for β III-tubulin to confirm equal protein loading. (H) Densitometric analyses of TLR7 levels normalised against β III-tubulin (n values for Unt=7 and BMAA=6, Unt vs BMAA, $*p=0.027$). (I) Densitometric analyses of TLR4 levels normalised against β III-tubulin (n values for Unt=6 and BMAA=4, Unt vs BMAA, $**p=0.004$). (J) NF- κ B levels were calculated using NF- κ B p65 ELISA kit. Values are μ g/mL (n values for Unt=6 and BMAA=4, Unt vs BMAA, $**p=0.002$). (K) Densitometric analyses of pro-IL-1 β levels normalised against β III-tubulin (n values for Unt=6 and BMAA=3, Unt vs BMAA, $**p=0.007$). (L) Caspase-1 activation (n values for Unt=10 and BMAA=8, Unt vs BMAA, $**p=0.003$). (M) IL-1 β levels were determined using an IL-1 β ELISA kit. Values are pg/mL (n values for Unt=6 and BMAA=4, Unt vs BMAA, $**p=0.003$). (N) IL-17 levels were determined using an IL-17 ELISA kit. Values are pg/mL (n values for Unt=5 and BMAA=6, Unt vs BMAA, $p=0.8029$). (O) IL-10 levels were determined using an IL-10 ELISA kit. Values are pg/mL (n values for Unt=5 and BMAA=8, Unt vs BMAA, $p=0.483$). (P–R), Iba1 and Trem2 expression in SN from Unt and BMAA-treated mice by immunofluorescence. (P) Representative images of brain coronal sections stained with Iba1 (microglial and macrophage-specific calcium-binding protein), Trem2 (triggering receptor expressed on myeloid cells 2) and Hoechst 33342 as nuclei marker in SN. Enlarged boxes show the area of Trem2 (white pixels) contained in Iba1 signal. (Q) Quantification of the number of Iba1 $^{+}$ cells per mm 2 (n values for all conditions=4, Unt vs BMAA, $*p=0.028$). (R) Percentage of Trem2 area contained in Iba1 expression (n values for all conditions=4, Unt vs BMAA, $**p=0.0002$). Scale bars are 50 μ m. Data represent mean \pm SEM. Statistical analysis: One-way analysis of variance followed by Dunnett's test was performed in B, D–E. Kruskal-Wallis test followed by Dunn's test was performed in C and F. Unpaired Student's t-test was performed in H–N and Q–R and Mann-Whitney test in O.

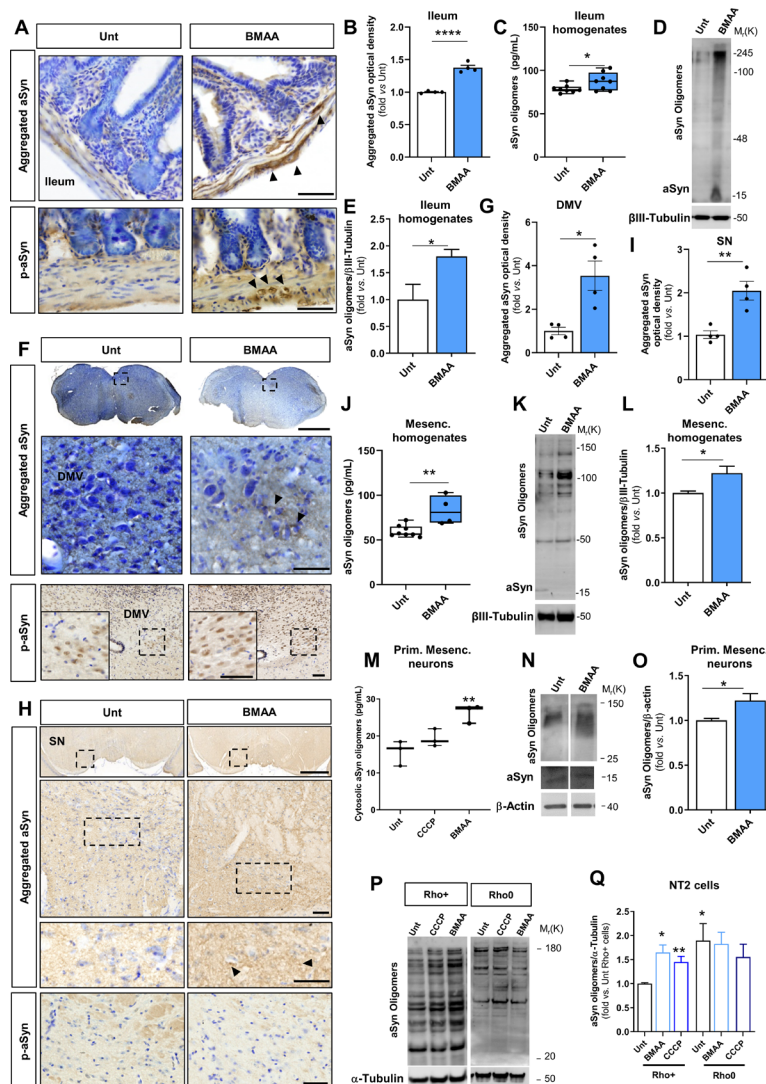


Figure 6 β -N-methylamino-L-alanine (BMAA) induces caudo-rostral alpha-synuclein (aSyn) aggregation. (A) Photomicrographs represent histology for aSyn aggregates and phosphorylated-aSyn (p-aSyn) (S129P) immunoreactivity in the ileum from untreated (Unt) and BMAA-treated mice. (B) Quantitative analysis of optical density (OD) for aSyn aggregates immunoreactivity in myenteric plexuses. Data was normalised to Unt group (n values for all conditions=4, Unt vs BMAA, **** $p < 0.0001$). (C) aSyn oligomers were assessed in ileum homogenates with an ELISA kit. Values are pg/mL (n values for all conditions=8, Unt vs BMAA, * $p = 0.0425$). (D) Representative immunoblot showing aSyn monomer and oligomers in ileum homogenates. The blots were re-probed for β III-Tubulin to confirm equal protein loading. (E) Densitometric analyses of the levels of aSyn normalised against β III-Tubulin. Data are expressed relatively to Unt group (n values for all conditions=4, Unt vs BMAA * $p = 0.0286$). (F) aSyn aggregates and p-aSyn (S129P) histological immunoreactivity in the dorsal motor nucleus of the vagus (DMV). (G) Quantitative analysis of OD for aSyn aggregates immunoreactivity in DMV. Data normalised to Unt group (n values for all conditions=4, Unt vs BMAA, * $p = 0.0109$). (H) aSyn aggregates and p-aSyn (S129P) histological immunoreactivity in substantia nigra (SN). (I) Quantitative analysis of OD for aSyn aggregates immunoreactivity in the SN. Data normalised to Unt group (n values for all conditions=4, Unt vs BMAA, ** $p < 0.0047$). (J) aSyn oligomeric levels were calculated using an ELISA kit in mesencephalic homogenates of Unt and BMAA-treated mice. Values are pg/mL (n values for Unt=8 and BMAA=4, Unt vs BMAA, ** $p = 0.0042$). (K) Representative immunoblot showing aSyn monomer and oligomers in mesencephalic homogenates. The blots were re-probed for β III-Tubulin to confirm equal protein loading. (L) Densitometric analyses of the levels of aSyn normalised against β III-Tubulin. Data are expressed relatively to Unt group (n values for all conditions=4, Unt vs BMAA * $p = 0.0286$). (M) Cytosolic aSyn oligomeric levels from primary mesencephalic neuronal cultures treated with 1 μ M CCCP for 2 hours and 3 mM BMAA for 48 hours were calculated using an ELISA kit. Values are pg/mL (n values for all conditions=3, Unt vs CCCP, $p = 0.2615$; Unt vs BMAA, ** $p = 0.0063$). (N) Representative immunoblot showing aSyn monomer and oligomers in mesencephalic neuronal cultures treated with 3 mM BMAA for 48 hours. The blots were re-probed for β -Actin to confirm equal protein loading. (O) Densitometric analyses of the levels of aSyn normalised against β -Actin. Data are expressed relatively to Unt neurons (n values for all conditions=4, Unt vs BMAA * $p = 0.0286$). (P) NT2-Rho+ and Rho0 cells treated with 5 μ M CCCP for 2 hours and 3 mM BMAA for 48 hours were evaluated by western blot. Representative immunoblot showing aSyn oligomers. The blots were re-probed for α - β III-Tubulin to confirm equal protein loading. (Q) Densitometric analyses of the levels of aSyn normalised against α - β III-Tubulin in Rho+ cells (n values for all conditions=6, Unt vs CCCP, * $p = 0.0236$, Unt vs BMAA, ** $p = 0.0018$) and Rho0 cells (n values for all conditions=4, $p > 0.5$). Histology samples were counterstained with cresyl violet. Scale bars are 50 μ m (enlarged inner square) and 1 mm. Data represent mean \pm SEM. Statistical analysis: Unpaired Student's t-test was performed in B–J and Mann-Whitney test in L and O. One-way analysis of variance (ANOVA) followed by Dunnett's test was performed in M. In Q 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 Rho+ vs Rho0 cells.

proposing that a pathogen or a toxin may travel from the gut to the brain via the vagus nerve,⁵ we could detect aSyn aggregates and p-aSyn (S129P) forms in the DMV (figure 6F–G), showing significant high levels of aSyn aggregates in BMAA-treated mice (figure 6G). Finally, we detected aSyn aggregates in the mesencephalon of BMAA-treated mice, but not p-aSyn (S129P) forms (figure 6H–I). Indeed, it was proposed that only after the accumulation of degradation-resistant aSyn aggregates we would see extensive phosphorylation in the aggregates.⁵⁶ In agreement with Braak's stages, it is possible that insufficient degradation-resistant aggregates are present to induce extensive phosphorylation in SN at the time point of our experiment. Accordingly, our data did not show an increase in aSyn aggregates immunoreactivity in the cortex of BMAA-treated mice (online supplemental figure S7B). Herein, we showed that BMAA promoted overexpression and oligomerisation of aSyn probably due to its effect on neuronal mitochondria, and essentially because BMAA promoted aSyn oligomerisation in primary mesencephalic neurons (figure 6M–O) and in Rho+ cells, but failed to increase aSyn aggregation in Rho0 cells (figure 6P–Q). Indeed, data from the literature indicate that different cellular and animal models of PD can be obtained by targeting mitochondrial function, as is the case of PD cybrids and of the MPTP-mice model,⁵⁷ both developing PD-related neuropathological hallmarks including aSyn oligomerisation. In fact, we also observed aSyn oligomers' accumulation in the mesencephalic mitochondrial fraction of BMAA-treated mice (online supplemental figure S7C,D).

Mice treated orally with BMAA developed nigrostriatal degeneration and motor dysfunction

BMAA-induced erosion of '*Candidatus* Arthromitus' from the ileum mucosa (figure 1E–F) led to GI inflammation and aSyn caudo-rostral aggregation, which may have contributed to the loss of TH-positive neurons. Our study revealed that the levels of TH-positive neurons decreased in BMAA-treated animals, but not of ChAT-positive neurons in the DMV (figure 7A–C). This reflects an increased susceptibility of dopaminergic neurons to BMAA that can be propagated to the SN through vagal catecholaminergic fibres located in the abdominal vagus nerve where roughly 70% of the neurons are TH-positive.⁵⁸ To further examine whether BMAA-induced neuroinflammation and aSyn oligomerisation led to dopaminergic neuronal damage we analysed TH levels in mesencephalon and STR of BMAA-treated mice. Interestingly, we observed a loss of dopaminergic neurons in the mesencephalon as determined by the decrease in TH levels (figure 7D–E), without any detectable alteration of other synaptic markers, such as PSD95 and synaptophysin (figure 7D and F–G). We also observed depletion of TH-positive fibres in the STR (figure 7H–I) and a decrease of the total number of nigral TH-positive cells in the SN (figure 7H and J) of BMAA-treated mice, whose loss led to lower dopamine levels in the STR (figure 7K). Correlational analysis indicates that the levels of aggregated aSyn in the DMV and SN contributes to depletion of mesencephalic TH-positive neurons, although we observed that SN IgG leakage also contributes to the increase of aSyn aggregates levels in the SN, an effect that may not only be caudo-rostral, since SN IgG leakage does not correlate with aSyn aggregated levels in the DMV (online supplemental figure S7E–H).

To test if the BMAA-induced dopaminergic neuronal loss, potentiated by systemic low-grade inflammation (figure 3Q), could affect motor performance, we evaluated mice motor performance using different motor behavioural tests. We found

that motor performance in the beam walking test (8 mm) was significantly impaired in BMAA-treated mice (figure 8A). We also observed that BMAA increased the hind clasping score (figure 8B) and decreased the latency in the inverted grip test (figure 8C). These results show that BMAA-induced motor impairment but had no apparent impact on the memory function as evaluated by the T-maze test at the selected time point (figure 8D–E). The locomotor activity in the open field test was also significantly decreased (figure 8F–I). Additionally, we also observed that BMAA treatment induced odour discrimination alterations, which agrees with PD symptomatology (data not shown).

DISCUSSION

Idiopathic PD represents approximately 90% of the disease cases but the aetiological factors remain elusive.¹ It is currently accepted that motor symptoms are preceded by non-motor features such as GI dysfunction.² Indeed, aSyn aggregates, a major PD pathological hallmark, were found to accumulate in some patients' peripheral organs such as the gut, early in the prodromal phase.^{3–5} Accordingly, it was postulated that environmental factors, namely certain gut microbes or their products, could act as environmental triggers of PD.⁶ Understanding the impact of gut dysbiosis on the CNS has become critical for developing new diagnostic and therapeutic tools that in the future may arrest disease progression. This work shows that BMAA, an environmental microbial metabolite with known neurotoxic properties, depletes specific bacterial groups that essentially regulate ileum mucosal immunity and possibly gut motility. Such narrow-spectrum erosion was accompanied by exacerbated GI inflammation, barrier disruption and aSyn aggregation, and it was also further possible to track the progression of the pathology to the mesencephalon, where the mitochondria became dysfunctional. Consequently, mitochondrial network fragmentation and exposure of DAMPs activated neuronal innate immunity promoting aSyn aggregation, neuroinflammation, loss of dopaminergic neurons and motor alterations in WT mice. These observations support the idea that certain microbial metabolites can trigger 'gut-first' PD but they also reinforce the role of mitochondria as key players in disease progression.

Changes detected in the intestinal microbiota of patients with PD include increased proportions of bacterial groups with pro-inflammatory properties and concomitant decrease in the relative abundance of groups with anti-inflammatory action.⁴⁵ Gut dysbiosis was also detected in individuals with rapid eye movement sleep behaviour disorder⁵⁹ suggesting that these alterations occur early in the prodromal phase of 'body-first' PD cases.⁶⁰ Here we show that BMAA exposure significantly alters the relative levels of key bacterial populations in the ileum and cecum mucosa, as well as in the faecal material, although the effects are more conspicuous in the ileum mucosa. *Turicibacter* are filamentous spore-forming bacteria that were significantly depleted after BMAA treatment. These bacteria were recently found to stimulate the release of serotonin from enterochromaffin cells, a neurotransmitter that regulates intestinal motility.⁶¹ Since constipation is a common PD symptom, we speculate that the extensive erosion of *Turicibacter* in the ileum and cecum mucosa and in the faecal material may contribute to impaired serotonin signalling which may hypothetically lead to impaired gut motility. Relevantly, BMAA treatment also significantly depleted '*Candidatus* Arthromitus' the most prominent bacterial genus in the mice ileum mucosa. Since SFB have fundamental roles in mice gut homeostasis,^{14 15} their depletion could justify enhanced gut inflammation¹³ and the observed loss of intestinal barrier integrity. Indeed,

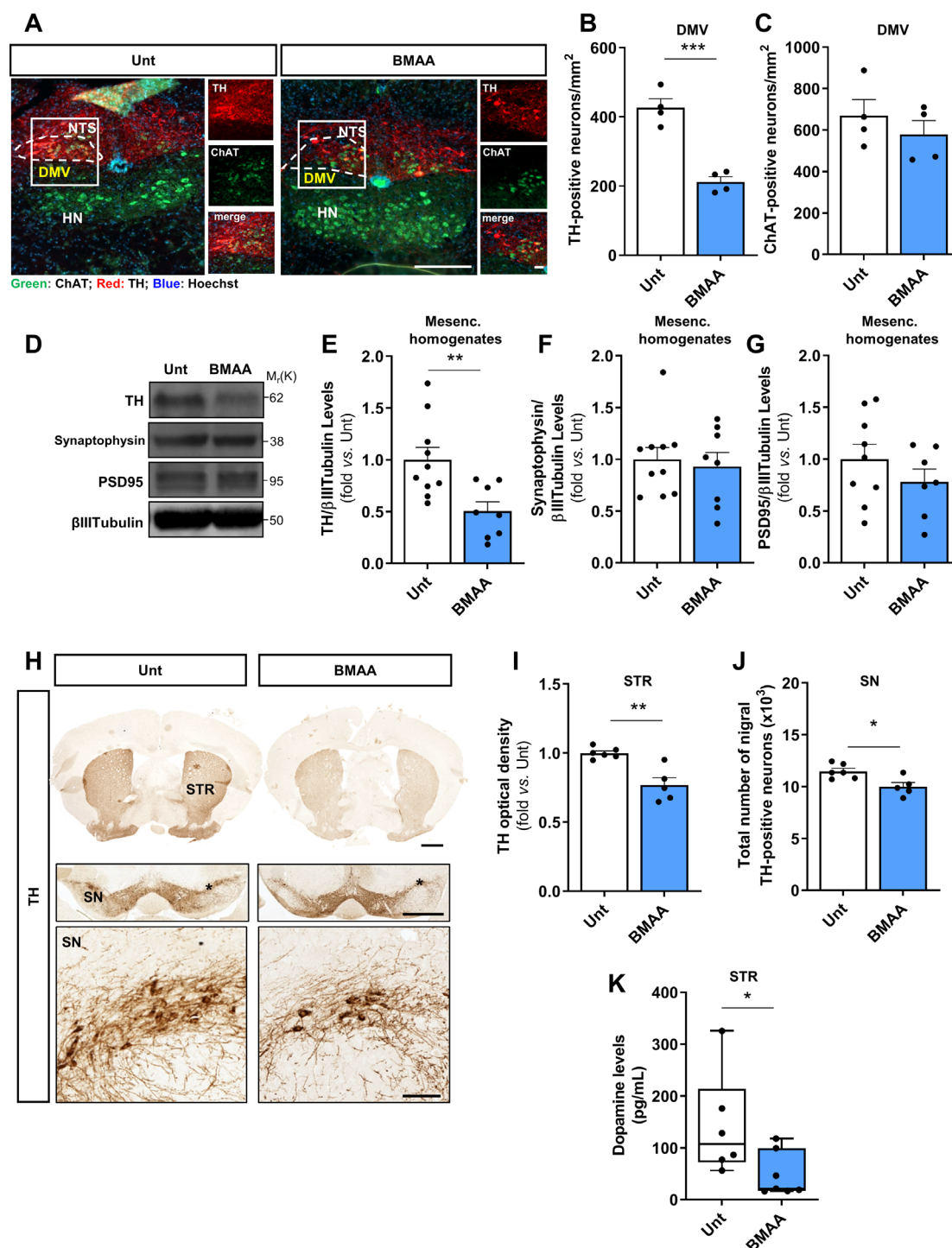


Figure 7 β -N-methylamino-L-alanine (BMAA) induces dopaminergic neurodegeneration *in vivo*. (A) Representative immunofluorescence photomicrographs of the localisation of tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT)-positive neurons in the dorsal motor nucleus of the vagus (DMV) from untreated (Unt) and BMAA-treated mice. (B) Quantification of the number of TH-positive neurons per mm² in the DMV region (n values for all conditions=4, Unt vs BMAA, ***p=0.004). (C) Quantification of the number of ChAT-positive neurons per mm² in the DMV region (n values for all conditions=4, Unt vs BMAA, p=0.41). (D) Representative immunoblot for TH, synaptophysin and PSD95 proteins. The blots were re-probed for β III-tubulin to confirm equal protein loading. (E) Densitometric analysis of TH, (F) synaptophysin and (G) PSD95. Data were normalised with β III-tubulin and expressed relatively to Unt mice (E–F, n values for Unt=10 and BMAA=8, G, Unt=9 and BMAA=7, Unt vs BMAA; E, **p=0.006, F, p=0.8286 and G, p=0.2799). (H) Representative photomicrographs of brain coronal sections immunostained with TH in striatum (STR) and substantia nigra (SN) from Unt and BMAA-treated mice. (I) Optical density analysis of the TH-positive fibres in the STR normalised to Unt group (n values for Unt=6 and BMAA=5, Unt vs BMAA, **p=0.0016). (J) Total number of nigral TH-positive neurons in SN assessed by stereological analysis (n values for Unt=6 and BMAA=5, Unt vs BMAA, *p=0.0135). (K) Dopamine levels were assessed in STR homogenates from Unt and BMAA-treated mice by Dopamine ELISA kit. Values are pg/mL (n values for Unt=6 and BMAA=7, Unt vs BMAA, *p=0.035). Scale bars are 50 μ m (A) and 100 μ m (H) (enlarged inner square) and 200 μ m (A) and 1 mm (H). Data represents mean+SEM. Statistical analysis: Unpaired Student's t-test was performed in B–C, E, G, I–J and Mann-Whitney test in F and K.

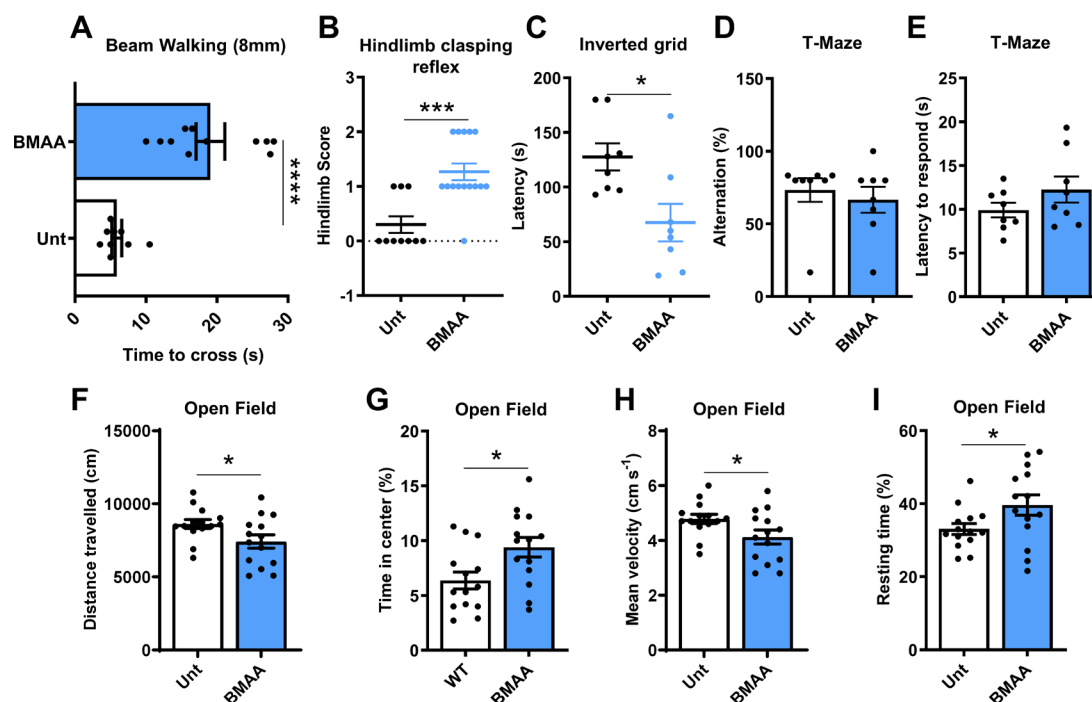


Figure 8 β -N-methylamino-L-alanine (BMAA) *in vivo* administration induces motor behavioural changes. (A) Balance and motor coordination performance was assessed with the beam walking test (n values for untreated (Unt)=9 and BMAA=11, Unt vs BMAA, ****p<0.0001). (B) Hindlimb clasp reflex was monitored, as a quick phenotypic neurological scoring system for evaluating disease progression (n values for Unt=10 and BMAA=15, Unt vs BMAA, ***p=0.0008). (C) Inverted grip test was used to evaluate muscular strength of limb muscles (n values for all conditions=8, Unt vs BMAA, *p=0.0132). (D–E) Cognitive and memory ability was assessed using a T-maze. (D) Percentage of alternation between arms and (E) latency to respond (s) was assessed (n values for all conditions=8, Unt vs BMAA, D, p=0.2165; E, p=0.192). (F–I) Locomotor activity was evaluated in an open field arena. (F) Distance travelled (cm), (G) % time spent at the centre of the arena, (H) mean velocity (cm.s⁻¹) and (I) % resting time (n values for all conditions=14, Unt vs BMAA, F, *p=0.0384; G, *p=0.0165; H, *p=0.0383 and I, *p=0.0495). Data represents mean+SEM. Statistical analysis: Unpaired Student's t-test was performed in A, C and E–I, and Mann-Whitney test in B and D.

depletion of SFB activated CD11b+ gut resident cells that released TNF- α and IL-1 β , and may have also activated non-resident pro-inflammatory Th17 cells driving the production of IL-17 with effects in the periphery.¹⁴ Interestingly, circulating Th17 cells are increased in early-stage PD.⁶² Under inflammatory conditions, the intestinal epithelium is exposed to multiple cytokines that may synergistically impair intestinal barrier through reduction in occludin and ZO1 levels as well as cytoskeletal rearrangement.⁴⁶ Additionally, we observed a decrease in CD4+ lymphocytes and an increase in the levels of IFN γ and IL-6 in the blood of BMAA-treated mice, resembling what has been described in patients with PD.^{48,63} The decline of CD4+ T cells and the increase of pro-inflammatory cytokines in PD patients' blood reported in previous studies might suggest impairment of peripheral immunity with propensity to systemic inflammation.⁶⁴ The increased mesencephalic IgG infiltration detected in our study suggests that BMAA promoted BBB permeability in the SN although no detectable effects were observed in the striatum or the cortex. BMAA has been shown to cross the BBB and has been detected in the brain of patients with ALS/PDC.²⁰ Herein, we show that *in vivo* BMAA treatment impaired the OCR and calcium uptake by mesencephalic mitochondria but not in cortical mitochondria, despite the fact that direct acute *in vitro* treatment of isolated mesencephalic or cortical mitochondria with BMAA induced a decrease in OCR.²¹ BMAA treatment led to mitochondria dysfunction and fragmentation in primary mesencephalic neurons, which due to mitophagy impairment were not degraded and consequently exposed cardiolipin, a DAMP able to activate neuronal innate immunity.⁵² Interestingly, when the experiments were conducted in Rho0 cells that lack functional mitochondria, BMAA had no effect on the

membrane potential or on the fragmentation of the mitochondrial net. We also observed that BMAA treatment activated the NLRP3 inflammasome accompanied by the release of IL-1 β in enriched mesencephalic neuronal cultures. Although in the *in vivo* context we cannot affirm that NLRP3 activation and IL-1 β release are restricted to neurons, the activation of the microglia in SN and the more intense co-localisation of Trem2-positive monocytes strongly indicate the involvement of systemic immunity.⁵⁴ Since neurons express TLRs and major histocompatibility complex class I proteins and are able to release pro-inflammatory mediators,⁶⁵ we propose that in BMAA-treated mice, neurons are the ones to be initially affected and only later signal microglia.

One well described consequence of innate immunity activation is the production of antimicrobial peptides.⁶⁶ Recent data suggest that, in the enteric nervous system (ENS), aSyn plays a role in innate immune defences of the GI tract. Indeed, increased expression of aSyn in the enteric neurites of the upper GI tract of paediatric patients positively correlated with the degree of acute and chronic inflammation induced during norovirus infection in the intestinal wall.⁶⁷ It was also demonstrated that aSyn exhibits antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, which suggests that, in addition to a role in neurotransmitter release, aSyn may also function as a natural bacteriostatic protein.⁶⁸ In BMAA-treated mice, we observed an evident increase in aSyn aggregation in the ileum and a caudo-rostral progression through the DMV nucleus into the SN. Our findings are in line with studies conducted in germ-free mice colonised with PD gut microbiota or with curli-producing *E. coli*, which showed increased aggregation of aSyn in the gut and in the brain,^{9,69} and that suggest an aSyn-dependent mechanism in the

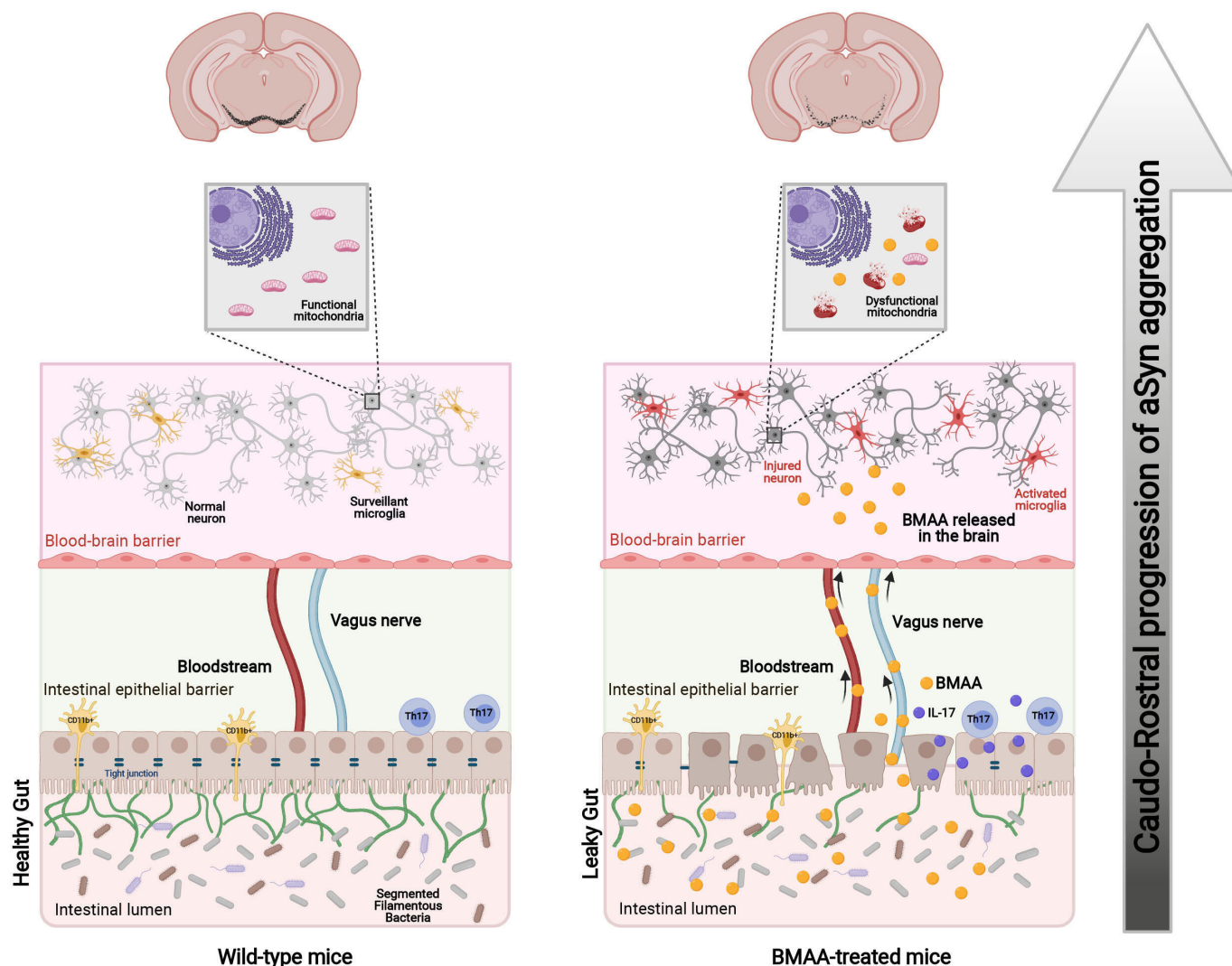


Figure 9 Schematic diagram of 'Gut-first' PD. Environmental microbial toxins lead to the erosion of segmented filamentous bacteria (SFB, green) in the ileum, which potentiates a Th17 proinflammatory response and the loss of intestinal barrier integrity. These events in the gut allow the progression of the disease into the brain either through the blood or the vagus nerve. Microbial toxins target mesencephalic mitochondria and activate neuronal innate immunity followed by aSyn expression, microglial activation and ultimately PD. aSyn, alpha-synuclein; BMAA, β -N-methylamino-L-alanine; IL, interleukin; PD, Parkinson's disease; Th17, T helper 17. (This image was created at BioRender.com).

aetiology of some PD 'gut-first' cases.⁷⁰⁻⁷¹ The proposal that a bacterial toxin such as BMAA can stimulate the expression and spread of aSyn through enteroendocrine cells that synapse with enteric nerves in the GI tract³ is innovative and may reveal important new targets to address prodromal, peripheral synucleinopathy. Regarding the ENS, a very interesting study performed in patients with PD⁷ duodenal biopsies failed to demonstrate alterations in the number of submucosal neurons as well as alterations in mitochondrial membrane potential and aSyn levels.⁷² Nevertheless, this data agrees with our study, since we did not observe an increase in duodenal aSyn aggregates in BMAA-treated mice (data not shown). Additionally, aSyn has been proposed to behave as prion-like protein spreading through a caudo-rostral trajectory from the gut to the brain via the vagus nerve.⁷⁰⁻⁷³⁻⁷⁶ We also observed that aSyn accumulates within brain mitochondria, which may indicate a positive feedback mechanism further contributing to their dysfunction. Several *in vitro* studies showed that aSyn targets the mitochondria inducing morphological and functional alterations.⁷⁷ Interestingly, rotenone, a complex I inhibitor that leads to mitochondrial dysfunction and fragmentation, also induces aSyn accumulation.⁷⁸ We therefore

propose that the PD neurodegenerative process is intimately associated to the role of mitochondria in the activation of innate immunity. This work further reinforces the crucial role of mitochondria in the aetiology of PD, essentially because BMAA treatment did not increase aSyn aggregation in Rho0 cells. The observation that BBB permeabilisation and mitochondrial dysfunction occur in the SN but not in the cortex led us to hypothesise the vital impact of the caudo-rostral progression of aSyn pathology through the vagus nerve. Indeed, and despite previous observations showing increased permeability of cortical and STR regions to blood-borne components,⁴⁹ we observed a positive correlation between BBB permeabilisation and aSyn aggregates levels in the midbrain, which indicates that both the blood and the vagal routes are likely involved in the full deleterious effect of BMAA on the mitochondria. This will in turn fully activate innate immune responses and neuroinflammation, which will synergistically induce dopaminergic neurodegeneration and impair motor function. In summary, our findings indicate that in genetically susceptible PD patients, the pathology may start in the gut when triggered by an environmental toxin such as BMAA, a microbial product commonly found in seafood, shellfish and

fish. From the observed effect of BMAA on specific members of the mice gut microbiome namely on ‘*Candidatus* Arthromitus’, we propose that this toxin may have initially evolved as an antimicrobial compound, possibly to provide some competitive advantage to its producers in a shared ecosystem, and accidentally targeted the mitochondria, acknowledged relatives of an ancient endosymbiotic proteobacteria. In support of the gut-to-brain paradigm, we demonstrate that gut inflammation is correlated with aSyn aggregation and highlight the key role of mitochondria in the downstream activation of neuronal innate immunity, aSyn aggregation and loss of DMV and SN TH-positive neurons. Although we did not determine ENS neuronal mitochondrial function, we share the view that the gut and the ENS act as a gateway through which BMAA and aSyn reach the DMV and the SN, target the mitochondria and gradually inflict damage to the most vulnerable TH-positive neurons.⁷⁹ However, we cannot discard completely the possibility of peripheral inflammation and consequent BBB disruption being involved in the access of BMAA to the brain parenchyma, an important question that must be addressed in a future study.

We theorise that chronic exposure to microbial BMAA via dietary sources impacts the host and may elicit phenotypic alterations that might trigger PD. Our results suggest that BMAA possesses narrow-spectrum antibiotic activity against some bacterial ‘sentinels’ responsible for gut mucosal immune homeostasis, the SFB. Notwithstanding the role of host genetic susceptibility and of other contributors, namely those driven by gut dysbiosis as probable drivers of pathology progression, this work is important to alert health authorities to the pressing need to implement routine protocols to monitor the levels of this (and other) toxin in foods and food supplements. In conclusion, our work sheds additional light into a long-lasting discussion⁸⁰ by providing the first proof-of-concept for the possible involvement of a microbial toxin in the aetiology of PD (figure 9), which stands as an important step towards development of innovative therapies that may successfully thwart the onset and chronic course of this neurodegenerative disease.

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Contributors NE and SMC conceptualised and designed the study and the research plan. NE supervised the microbiome work and SMC supervised the rest of the work. SA, IT and DN-C generated the microbiome data. DFS and ARE performed all the *in vitro* experiments. EC and MFM-P performed animal surgeries, tissue clearing. MFM-P and JDM performed histology analyses. MFM-P performed FACS analyses. EC, ARE and DFS performed cytokine analyses. EC, ARE, JDM, MFM-P and ARP-S performed behaviour experiments. ILF, ARE and DFS performed Seahorse technique. ARE and DFS performed protein analysis. ARE, DFS and MFM-P performed confocal imaging. MFM-P and DN-C performed data analysis. NE and SMC wrote the manuscript. All authors contributed to the material and methods section, results section and figure legends. SMC and NE are guarantors of this work.

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Supplemental material for:

“Footprints of a microbial toxin from the gut microbiome to mesencephalic mitochondria”

Table of Contents

Table S1. List of Reagents

Detailed Methods

Supplemental Figures and Figure legends (S1-S7)

Whole representative WB membranes

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)

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- β 3-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-1 β	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	
NF κ B 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 IFN γ Quantikine ELISA	R&D Systems (Cat. No. MIF00)
Mouse IL-6 Quantikine ELISA	R&D Systems (Cat. No. M6000D)
Mouse IL-1 β 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 ₄ Cl)	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).

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

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

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 (mm²).

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 (mm²).

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 (mm²).

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 (mm²) 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 °C. 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 °C in a humidified chamber.

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 °C 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 °C for 10 min. The cell suspension was washed with PBS,

centrifuged at 250×g at 4 °C for 10 min. the pellet was fixed with 2% PFA solution for 10 min at 4 °C and washed with PBS. Finally, cells were centrifuged at 250×g at 4 °C 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 MgCl₂, 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 MgCl₂; 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 °C. 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-

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 MgCl₂, 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 °C. 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 cm² 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-p0 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% CO₂. 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.

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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 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 $140\times g$ 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 μ M 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 NH₄Cl and/or 20 μ M Leupeptin (Sigma, St. Louis, MO, USA) were added for 4 h to the culture medium. The combination of NH₄Cl 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 \times) and lysed in 1% Triton X-100 containing hypotonic lysis buffer (25 mM HEPES, 2 mM MgCl₂, 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 \times 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 \times) 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 \times g for 12 min at 4 °C and the resulting supernatant was further centrifuged at 11431 \times 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 MgCl₂; 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 °C 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 MgCl₂, 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.

Analysis of Oxygen Consumption (OCR)

Mesencephalic neurons

Neurons were seeded in 24-well XF culture plates and placed in a 37 °C, 5% CO₂ 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-CO₂ 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 °C. 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 KH₂PO₄, 5 mM MgCl₂, 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 μ M) caused an increase in OCR reflecting the maximal respiratory chain activity as well as the maximal substrate oxidation rate. Finally, antimycin A (4 μ M; 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% CO₂ 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-CO₂ 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 μ M 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 $\times 100$).

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 Ca²⁺ handling capacity

Mitochondrial Ca²⁺ uptake was measured fluorometrically in fresh mesencephalic or cortical mitochondria, in the presence of the Ca²⁺-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 μ g of protein per well) which were incubated with 0.5, 1 or 3 mM BMAA for 30 min at 30 °C. Calcium Green is a cell-impermeant visible light-excitable Ca²⁺ indicator that exhibits an increase in fluorescence emission intensity upon binding to Ca²⁺; thus, a decrease in the Calcium Green fluorescence is function of external Ca²⁺ concentration which indicates the capacity of mitochondria to handle Ca²⁺. Briefly, 5 μ g of isolated mitochondria were added to the standard incubation medium containing 125 mM KCl,

0.5 mM MgCl₂, 3 mM KH₂PO₄, 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 CaCl₂, were added every 4 min and Ca²⁺ 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 $\mu\text{g}/\mu\text{L}$ 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 \times 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_2PO_4 , 137 mM NaCl, 4.16 mM NaHCO_3 , 0.34 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 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 \times lens on an Axio Observer.Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software with a stage-based chamber (5% CO_2 , 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 \times magnification (Zeiss Plan-ApoChromat 63 \times , 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 ($\Delta\psi_m$) 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 $\Delta\psi_m$. 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 MgCl₂, 6 mM glucose, 10 mM HEPES, 10 mM NaHCO₃, and 1 mM CaCl₂. Basal fluorescence was recorded for 5 min at 37 °C (λ_{ex} = 540 nm and λ_{em} = 590 nm). Subsequently, 1 μ M FCCP (protonophore) and 2 μ g/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 \pm 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.

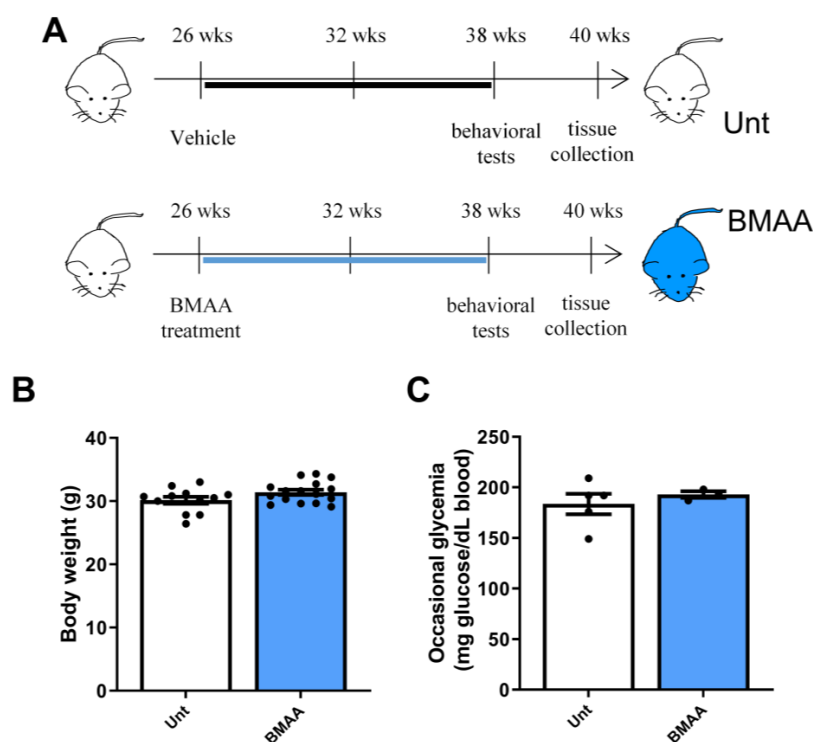
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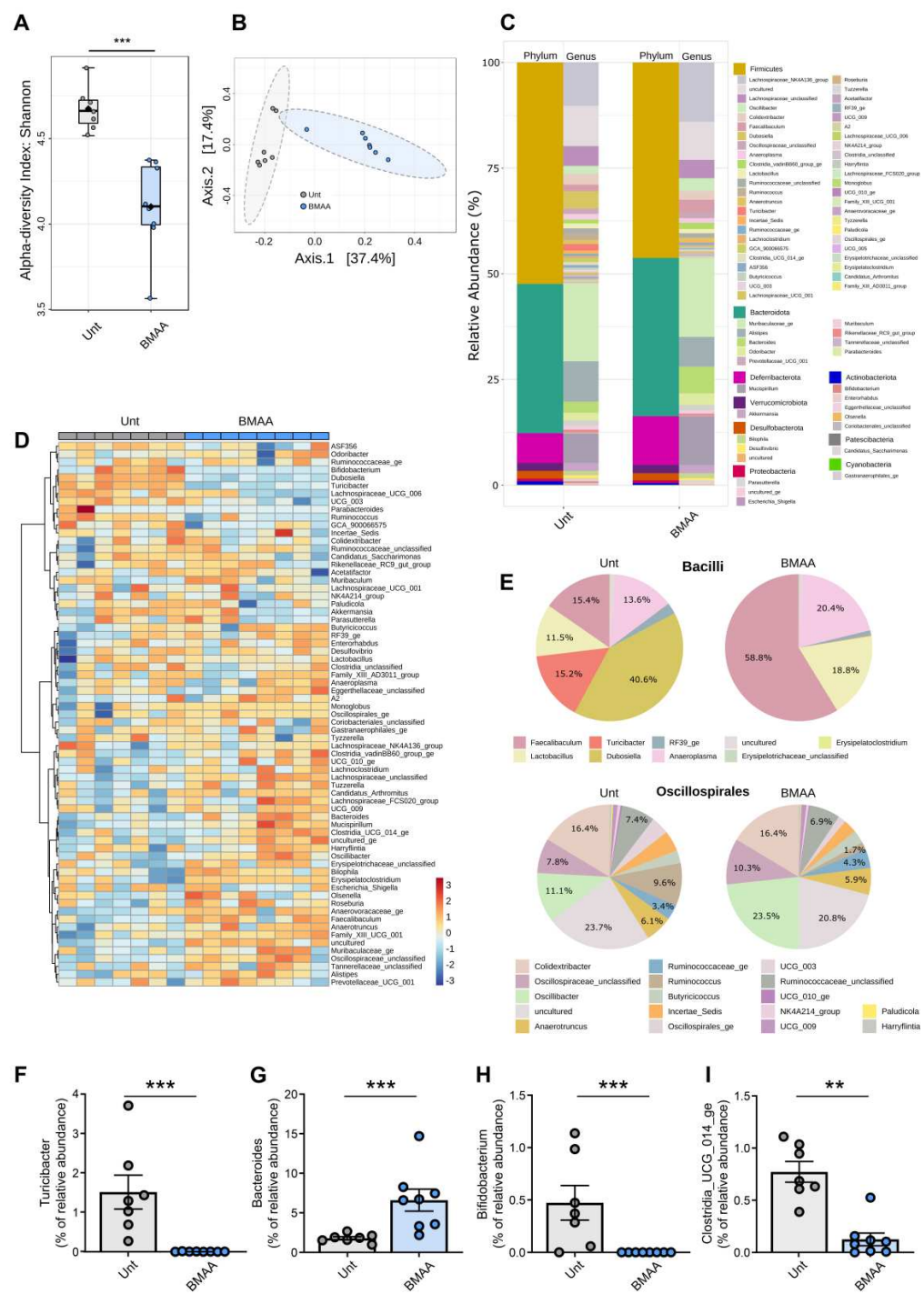
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- 25 Scaduto RC, Grotyohann LW. Measurement of Mitochondrial Membrane Potential Using Fluorescent Rhodamine Derivatives. *Biophys J* 1999;76:469–77.
- 26 Hsu Y-H, Dumlao DS, Cao J, *et al.* Assessing Phospholipase A2 Activity toward Cardiolipin by Mass Spectrometry. *PLoS One* 2013;8:e59267.

Supplemental figures and respective legends

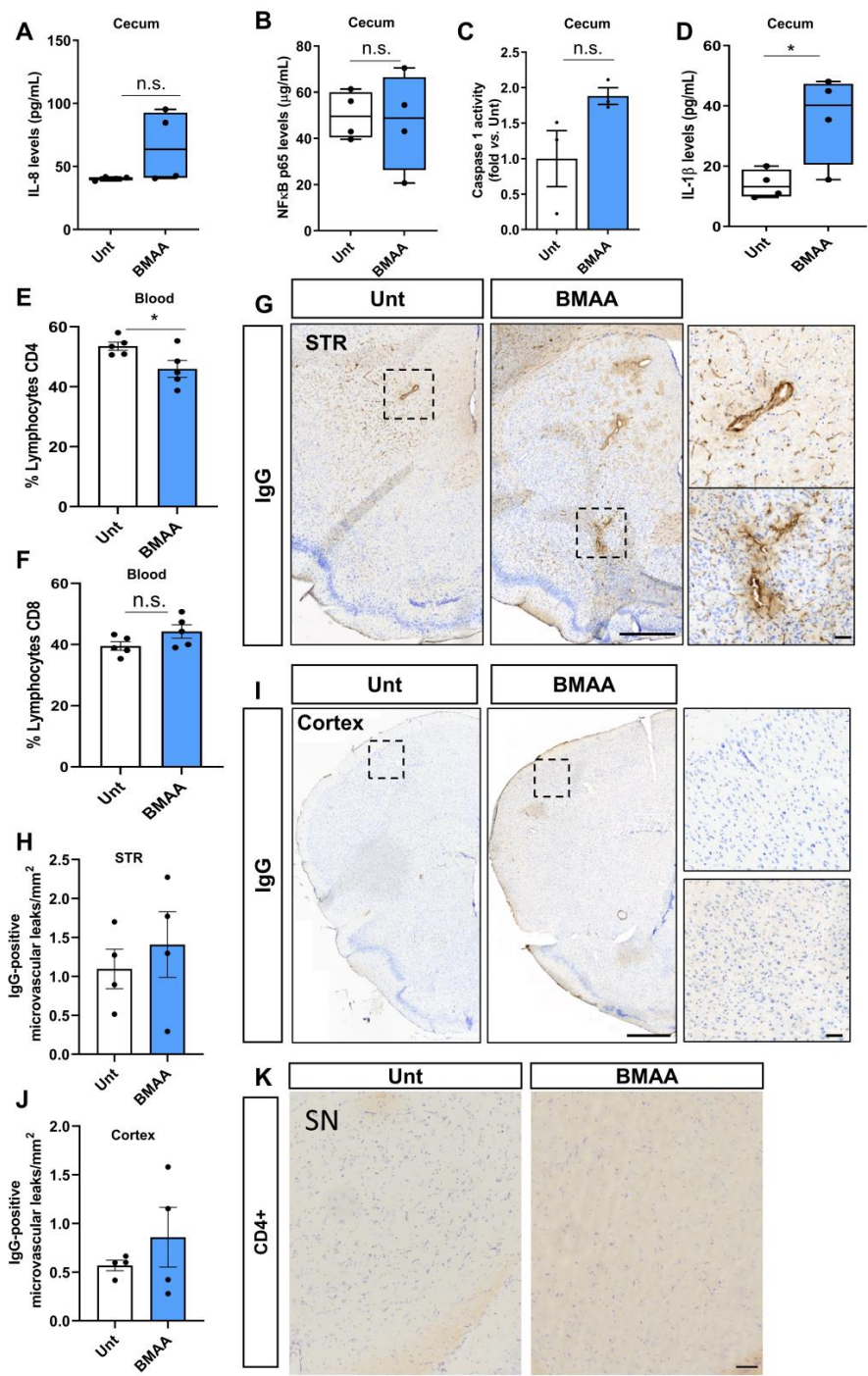


Supplemental Fig. S1. Mice body weight and glycemia were unaltered during the treatment. (A) Schematic representation of experimental design. (B) The body weights of mice were measured twice a week during the treatment. (C) Blood glucose was measured at the end of treatment and occasional glycemia were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



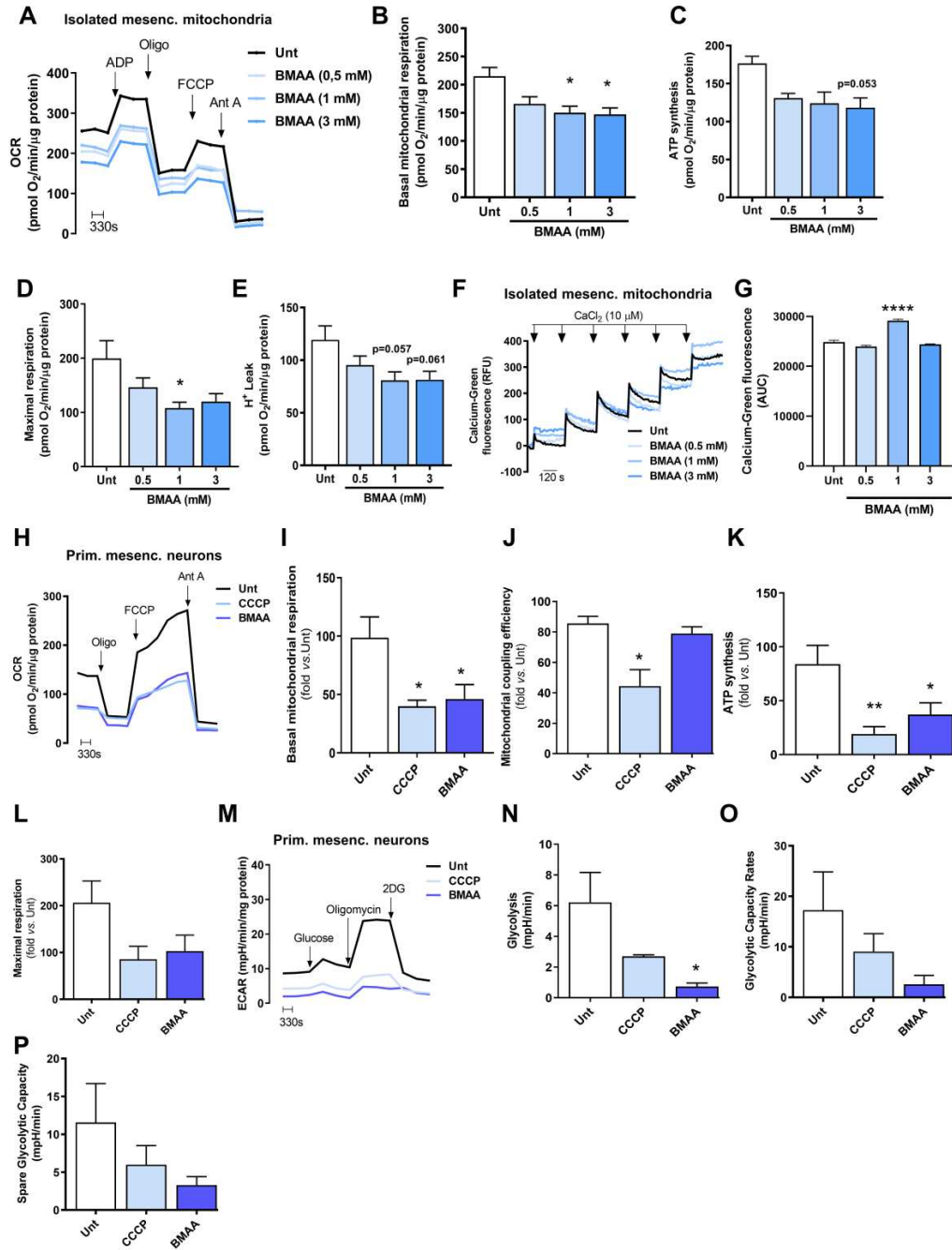
Supplemental Fig. S2. Cecum mucosa-associated microbiota diversity in BMAA-treated mice.

(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 Oscillospirales. (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).



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.

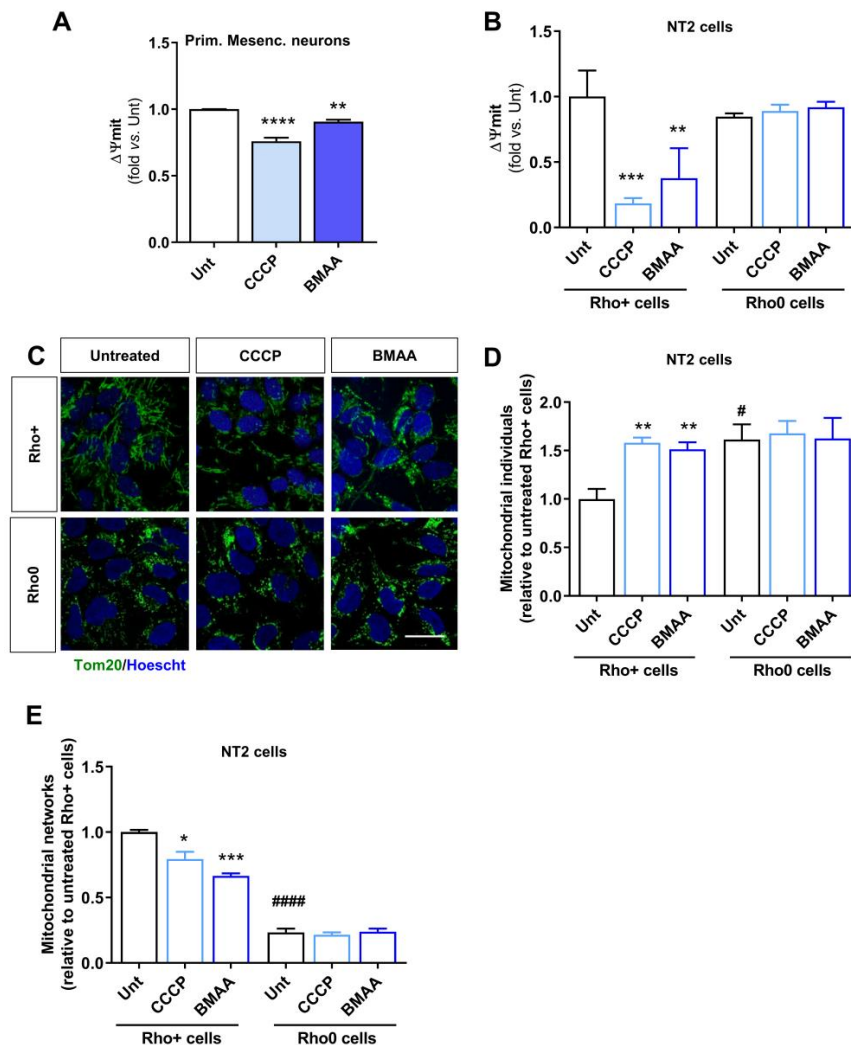
(A-D) Measurement of specific mediators involved in the inflammatory response in cecum intestinal samples from untreated (Unt) or BMAA-treated mice measured by ELISA. **(A)** IL-8 (n values for all conditions = 4, Unt vs BMAA, $p = 0.123$), **(B)** NF κ B (n values for all conditions = 4, Unt vs BMAA, $p = 0.8162$), **(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-1 β (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 mm² 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 μ m (enlarged inner boxes) and 500 μ m. 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);

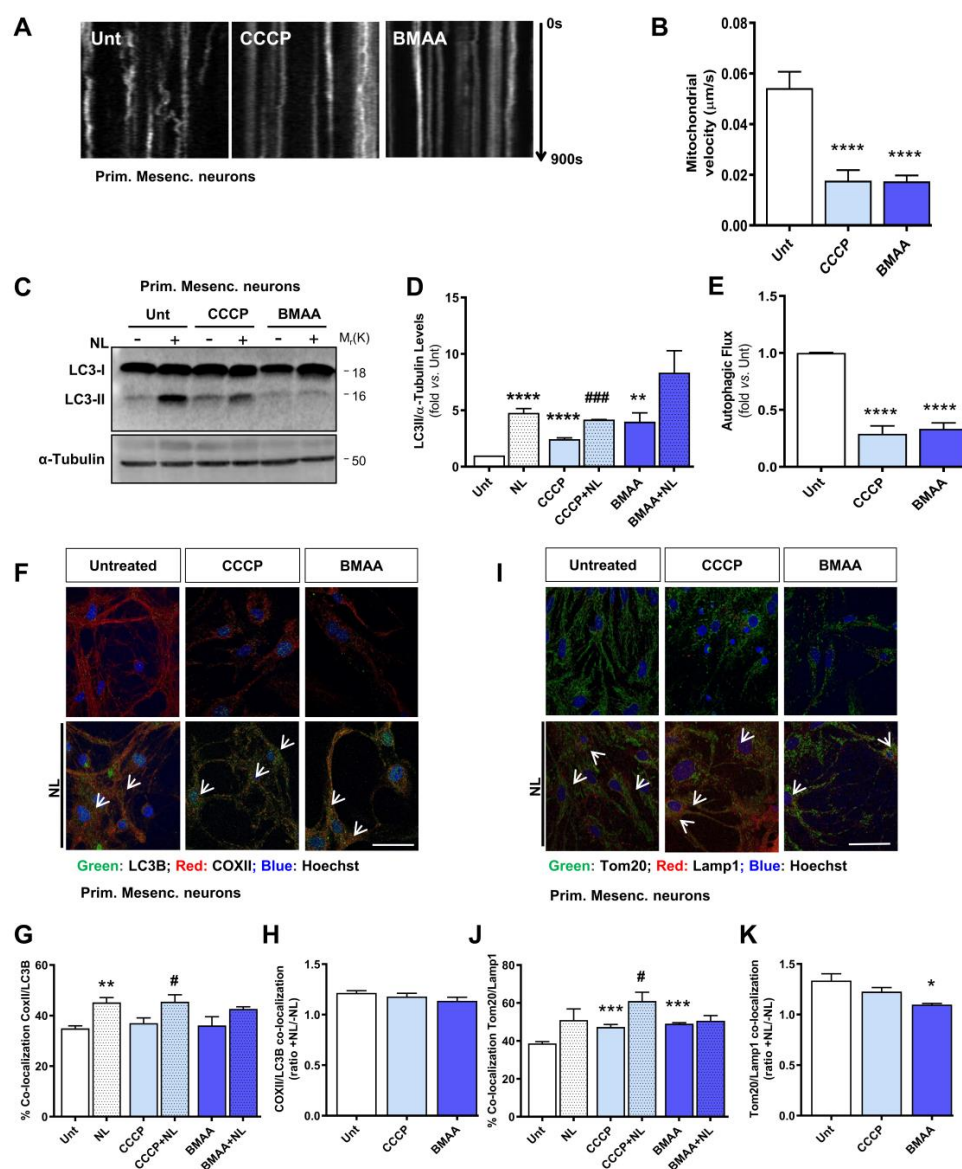
(C) ATP synthesis; (D) maximal respiration (Unt vs 1 mM BMAA, * $p = 0.0343$); (E) proton Leak. Values are pmol O₂/min/μg protein. n values for all conditions = 3, (F-G) Isolated mesencephalic mitochondria's ability to uptake calcium was evaluated with the fluorescent probe Calcium-green (n values for 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.

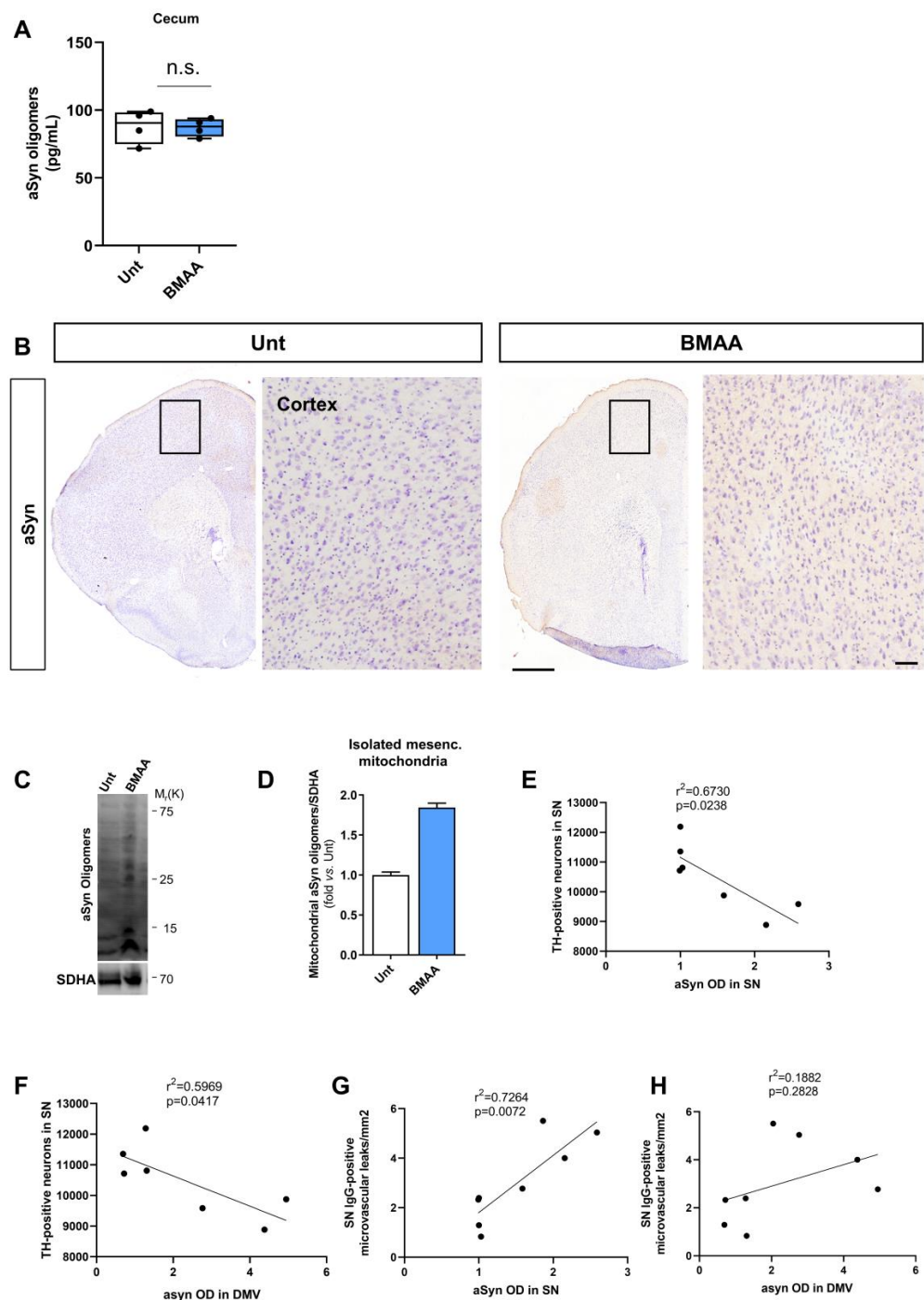
(A) Primary mice mesencephalic neurons treated with 1 μ M CCCP for 2 h and 3 mM BMAA for 48 h. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were assessed using the fluorescent cationic dye TMRM. Values are mean \pm S.E.M. (n values for Unt = 5, CCCP = 4 and BMAA = 5, Unt vs CCCP, ****p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

assessed using the fluorescent cationic dye TMRM. Values are mean \pm 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 \pm 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 \pm 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. aSyn 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

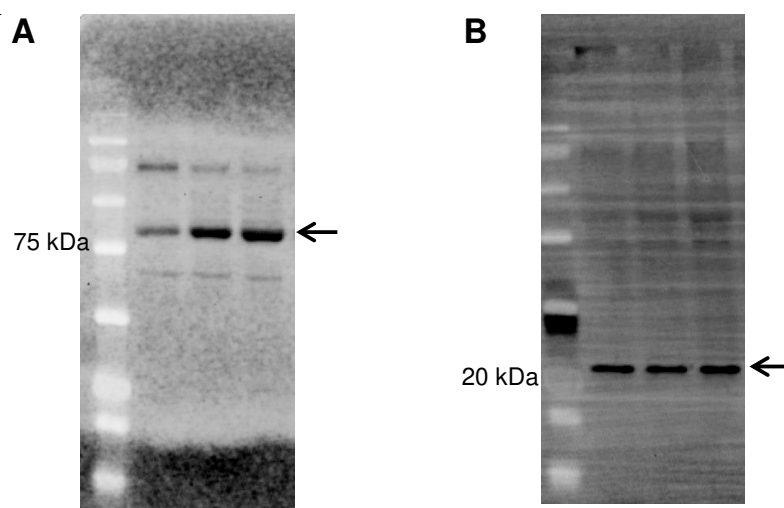


Figure 1. Whole representative western blots of Figure 4M showing phospho-Drp1 at 78 kDa (A) and Tom20 at 20 kDa (B).

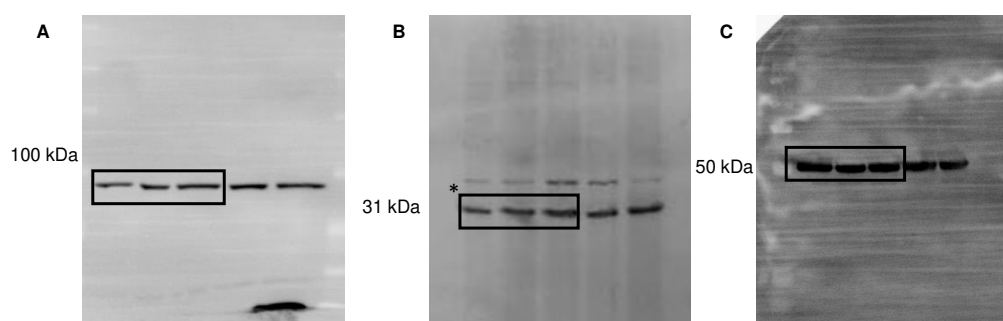


Figure 2. Whole representative western blots of Figure 5A showing TLR4 at 95 kDa (A), ProIL1 β at 31 kDa (B) and β III-Tubulin at 50 kDa (C). * This representative blot of ProIL1 β those not match the inset in figure 5B, which was lost.

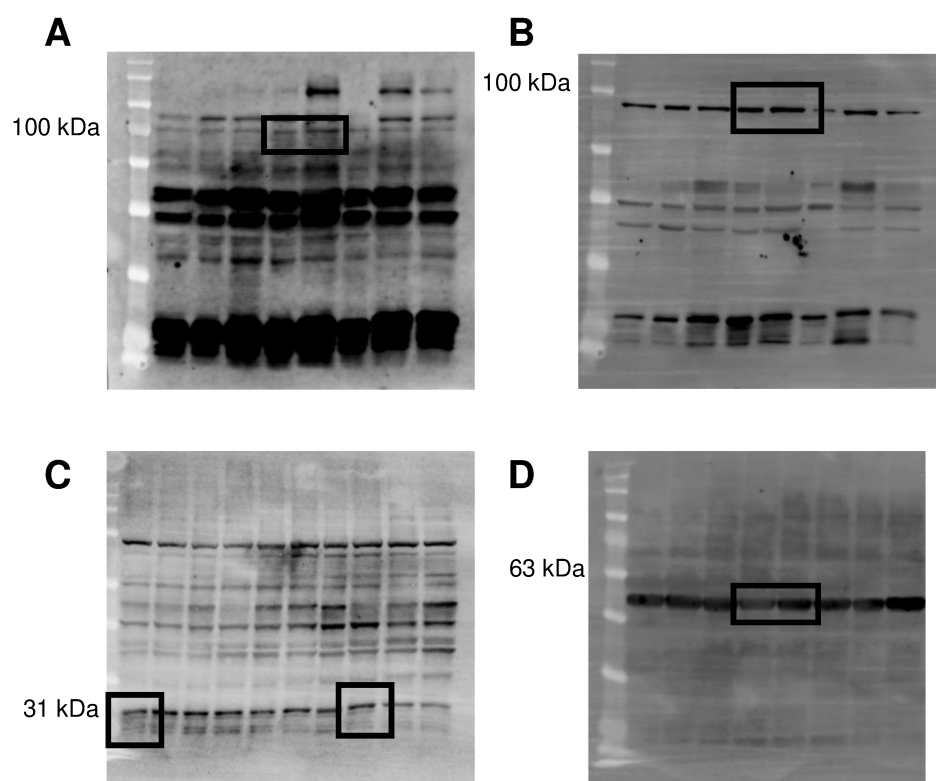


Figure 3. Whole representative western blots of Figure 5G showing TLR7 at 116 kDa (A), TLR4 at 95 kDa (B), ProIL1 β at 31 kDa (C) and respective β III-Tubulin at 50 kDa (D).

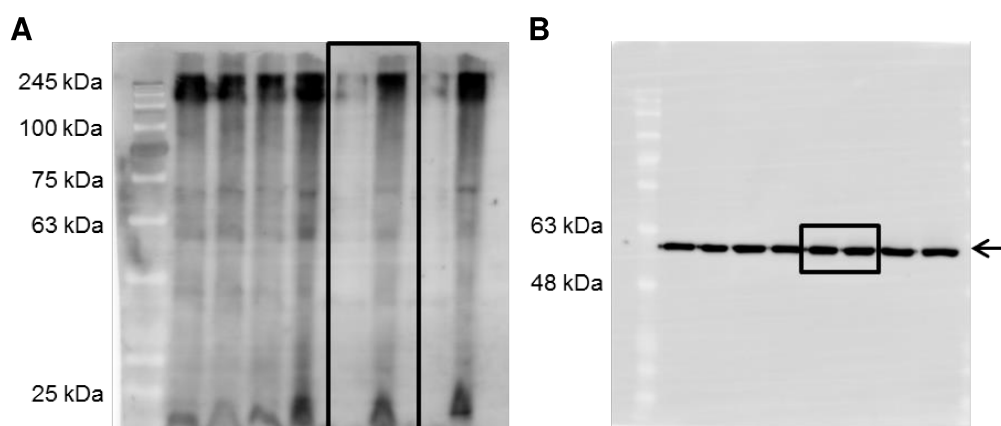


Figure 4. Whole representative western blots of Figure 6D showing aSyn Oligomers (A) and β III-Tubulin at 50 kDa (B).

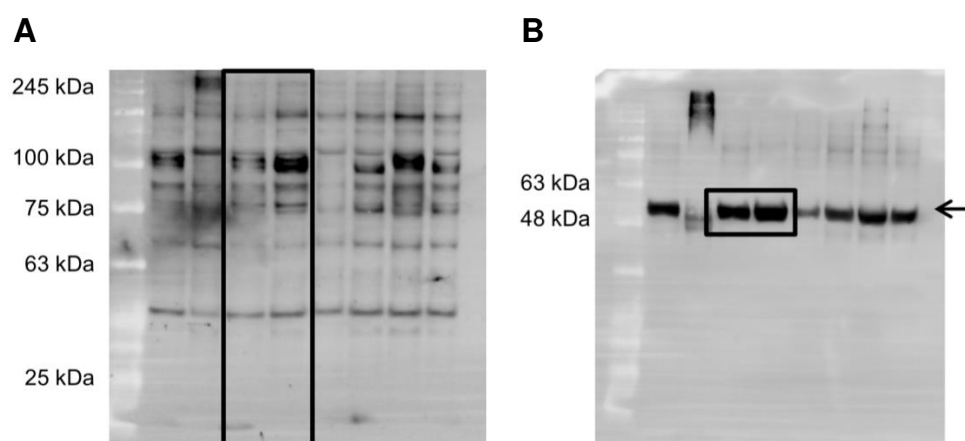


Figure 5. Whole representative western blots of Figure 6K showing aSyn Oligomers (A) and βIII-Tubulin at 50 kDa (B).

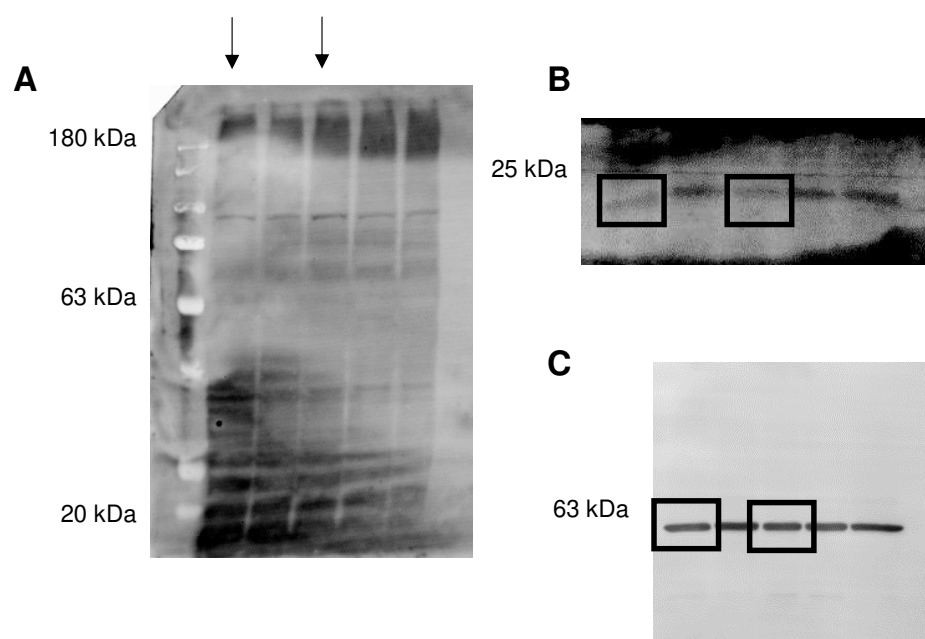


Figure 6. Whole representative western blots of Figure 6N showing aSyn Oligomers (A), aSyn monomer (B) and β-actin at 40 kDa (C).

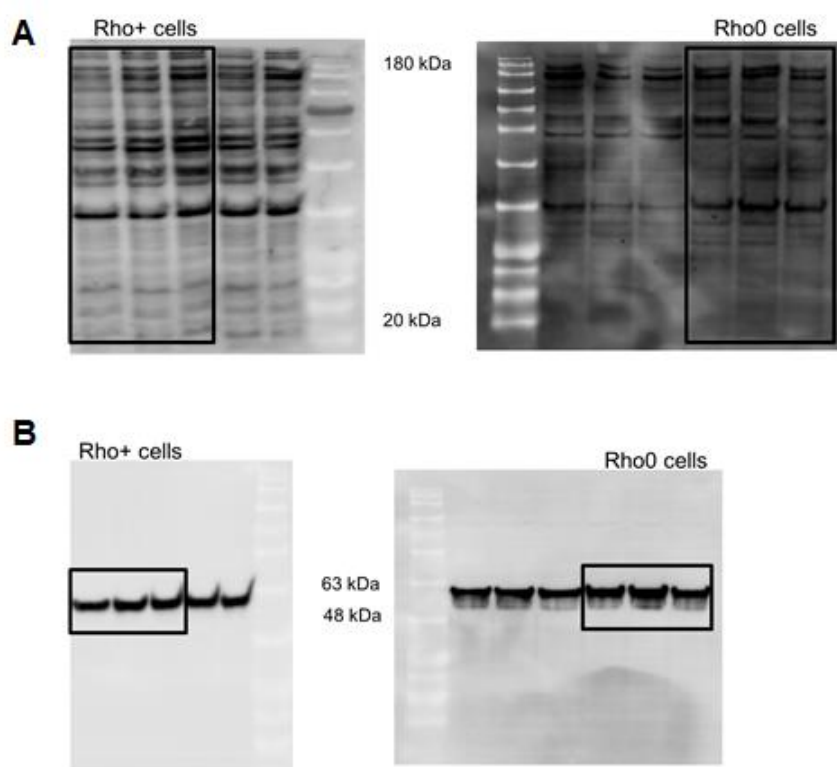


Figure 7. Whole representative western blots of Figure 6P showing aSyn Oligomers (A) and βIII-Tubulin at 50 kDa (B).

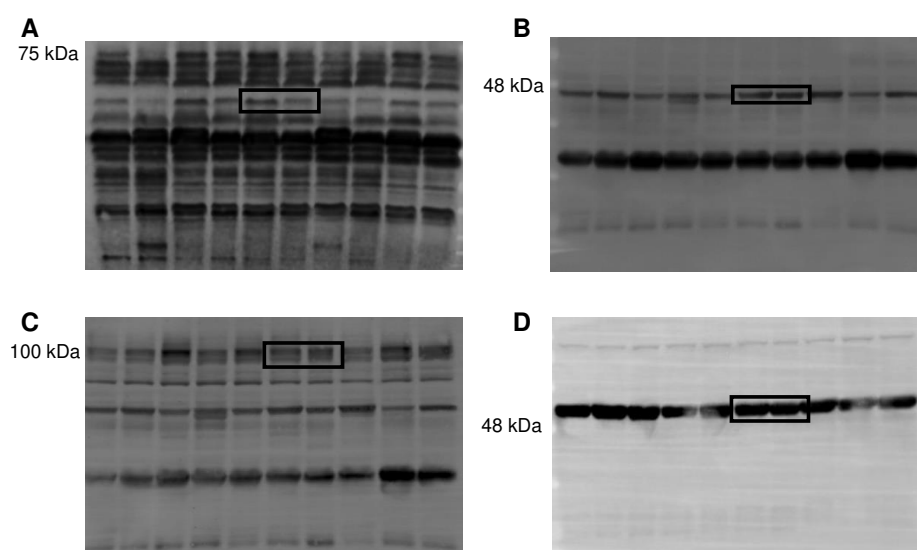


Figure 8. Whole representative western blots of Figure 7D showing TH at 62kDa (A), Synaptophysin at 38 kDa (B), PSD95 at 95 kDa (C) and βIII-Tubulin at 50 kDa (D).

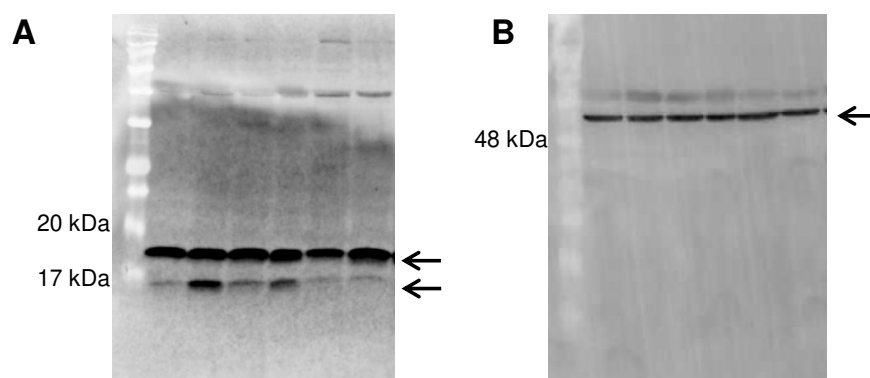


Figure 9. Whole representative western blots of Supplementary Figure S6C showing LC3B at 16 and 18 kDa (**A**) and α -Tubulin at 50 kDa (**B**).

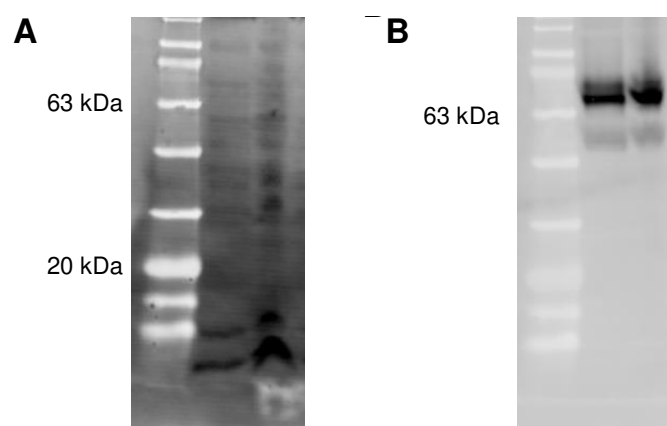


Figure 10. Whole representative western blots of Supplementary Figure S7C showing mitochondrial aSyn Oligomers (**A**) and SDHA at 70 kDa (**B**).

Supplemental material for:

“Footprints of a microbial toxin from the gut microbiome to mesencephalic mitochondria”

Table of Contents

Table S1. List of Reagents

Detailed Methods

Supplemental Figures and Figure legends (S1-S7)

Whole representative WB membranes

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)

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- β 3-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-1 β	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	
NF κ B 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 IFN γ Quantikine ELISA	R&D Systems (Cat. No. MIF00)
Mouse IL-6 Quantikine ELISA	R&D Systems (Cat. No. M6000D)
Mouse IL-1 β 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 ₄ Cl)	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).

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

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

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 (mm²).

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 (mm²).

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 (mm²).

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 (mm²) 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 °C. 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 °C in a humidified chamber.

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 °C 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 °C for 10 min. The cell suspension was washed with PBS,

centrifuged at 250×g at 4 °C for 10 min. the pellet was fixed with 2% PFA solution for 10 min at 4 °C and washed with PBS. Finally, cells were centrifuged at 250×g at 4 °C 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 MgCl₂, 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 MgCl₂; 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 °C. 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-

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 MgCl₂, 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 °C. 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 cm² 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-p0 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% CO₂. 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.

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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 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 $140\times g$ 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 μ M 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 NH₄Cl and/or 20 μ M Leupeptin (Sigma, St. Louis, MO, USA) were added for 4 h to the culture medium. The combination of NH₄Cl 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 \times) and lysed in 1% Triton X-100 containing hypotonic lysis buffer (25 mM HEPES, 2 mM MgCl₂, 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 \times 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 \times) 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 \times g for 12 min at 4 °C and the resulting supernatant was further centrifuged at 11431 \times 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 MgCl₂; 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 °C 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 MgCl₂, 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.

Analysis of Oxygen Consumption (OCR)

Mesencephalic neurons

Neurons were seeded in 24-well XF culture plates and placed in a 37 °C, 5% CO₂ 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-CO₂ 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 °C. 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 KH₂PO₄, 5 mM MgCl₂, 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 μ M) caused an increase in OCR reflecting the maximal respiratory chain activity as well as the maximal substrate oxidation rate. Finally, antimycin A (4 μ M; 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% CO₂ 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-CO₂ 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 μ M 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 $\times 100$).

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 Ca²⁺ handling capacity

Mitochondrial Ca²⁺ uptake was measured fluorometrically in fresh mesencephalic or cortical mitochondria, in the presence of the Ca²⁺-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 μ g of protein per well) which were incubated with 0.5, 1 or 3 mM BMAA for 30 min at 30 °C. Calcium Green is a cell-impermeant visible light-excitable Ca²⁺ indicator that exhibits an increase in fluorescence emission intensity upon binding to Ca²⁺; thus, a decrease in the Calcium Green fluorescence is function of external Ca²⁺ concentration which indicates the capacity of mitochondria to handle Ca²⁺. Briefly, 5 μ g of isolated mitochondria were added to the standard incubation medium containing 125 mM KCl,

0.5 mM MgCl₂, 3 mM KH₂PO₄, 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 CaCl₂, were added every 4 min and Ca²⁺ 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 $\mu\text{g}/\mu\text{L}$ 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 \times 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_2PO_4 , 137 mM NaCl, 4.16 mM NaHCO_3 , 0.34 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 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 \times lens on an Axio Observer.Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software with a stage-based chamber (5% CO_2 , 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 \times magnification (Zeiss Plan-ApoChromat 63 \times , 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 ($\Delta\psi_m$) 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 $\Delta\psi_m$. 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 (λ_{ex} = 540 nm and λ_{em} = 590 nm). Subsequently, 1 μ M FCCP (protonophore) and 2 μ g/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 \pm 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.

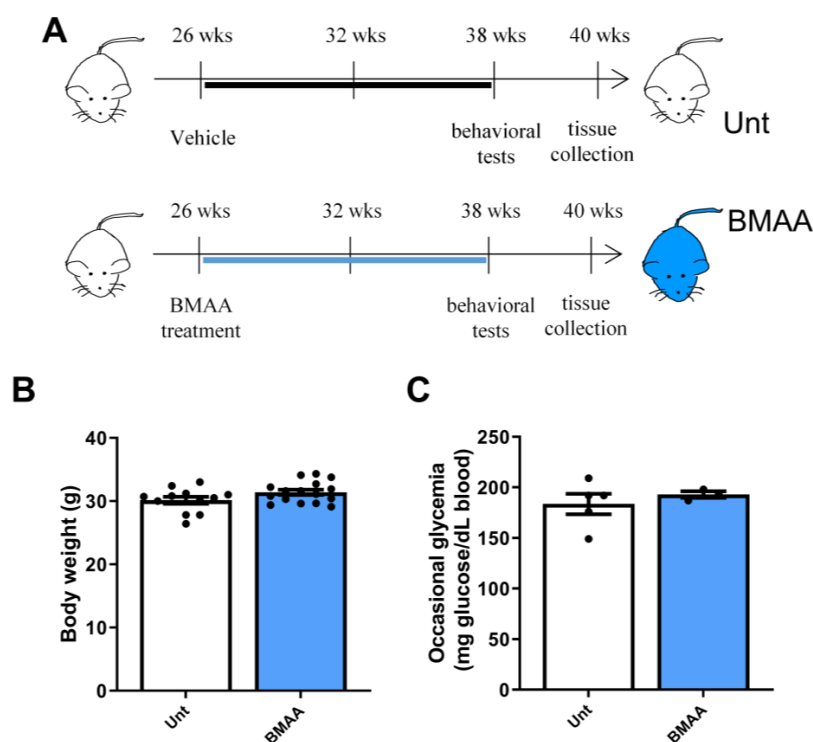
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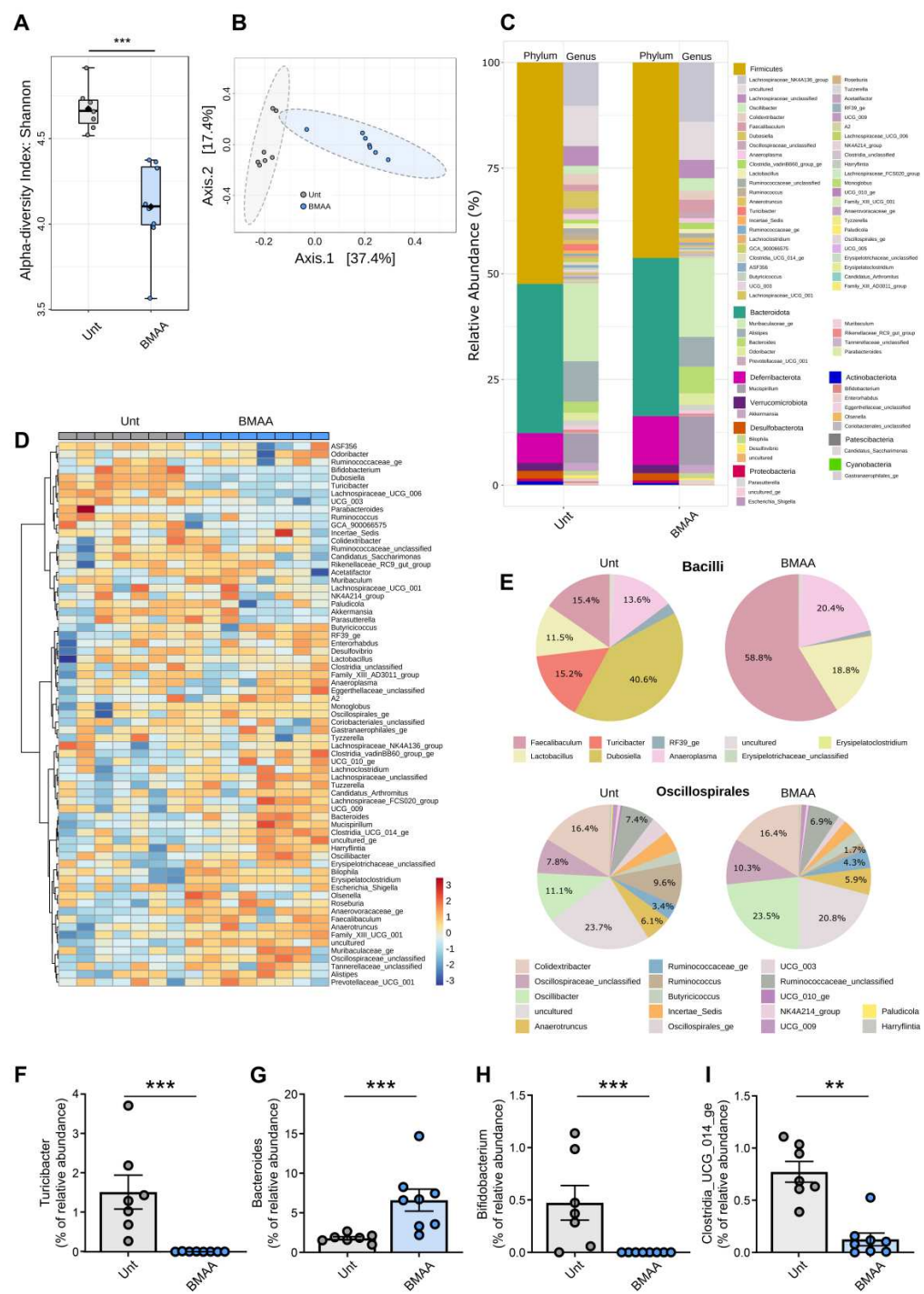
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Supplemental figures and respective legends

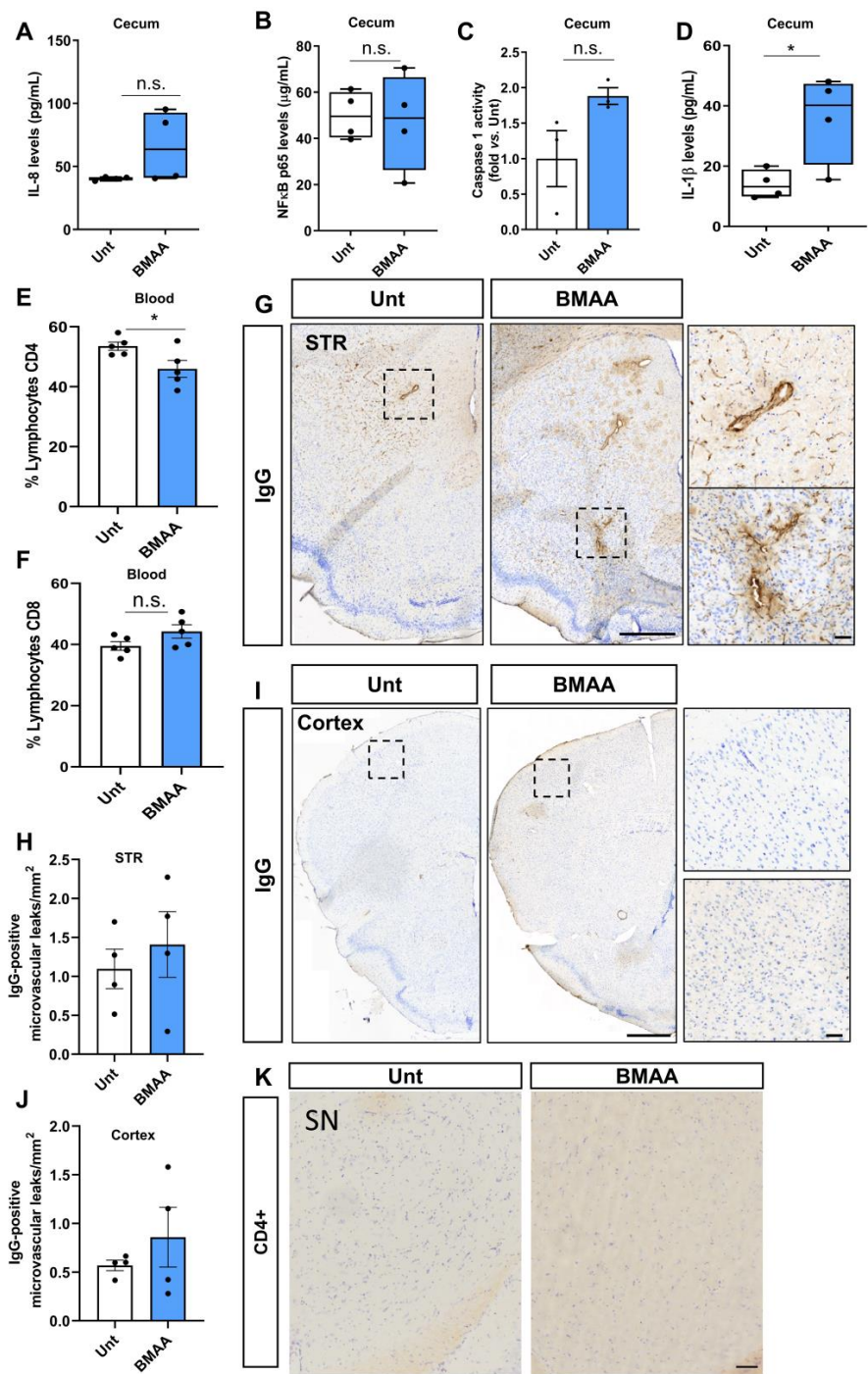


Supplemental Fig. S1. Mice body weight and glycemia were unaltered during the treatment. (A) Schematic representation of experimental design. (B) The body weights of mice were measured twice a week during the treatment. (C) Blood glucose was measured at the end of treatment and occasional glycemia were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



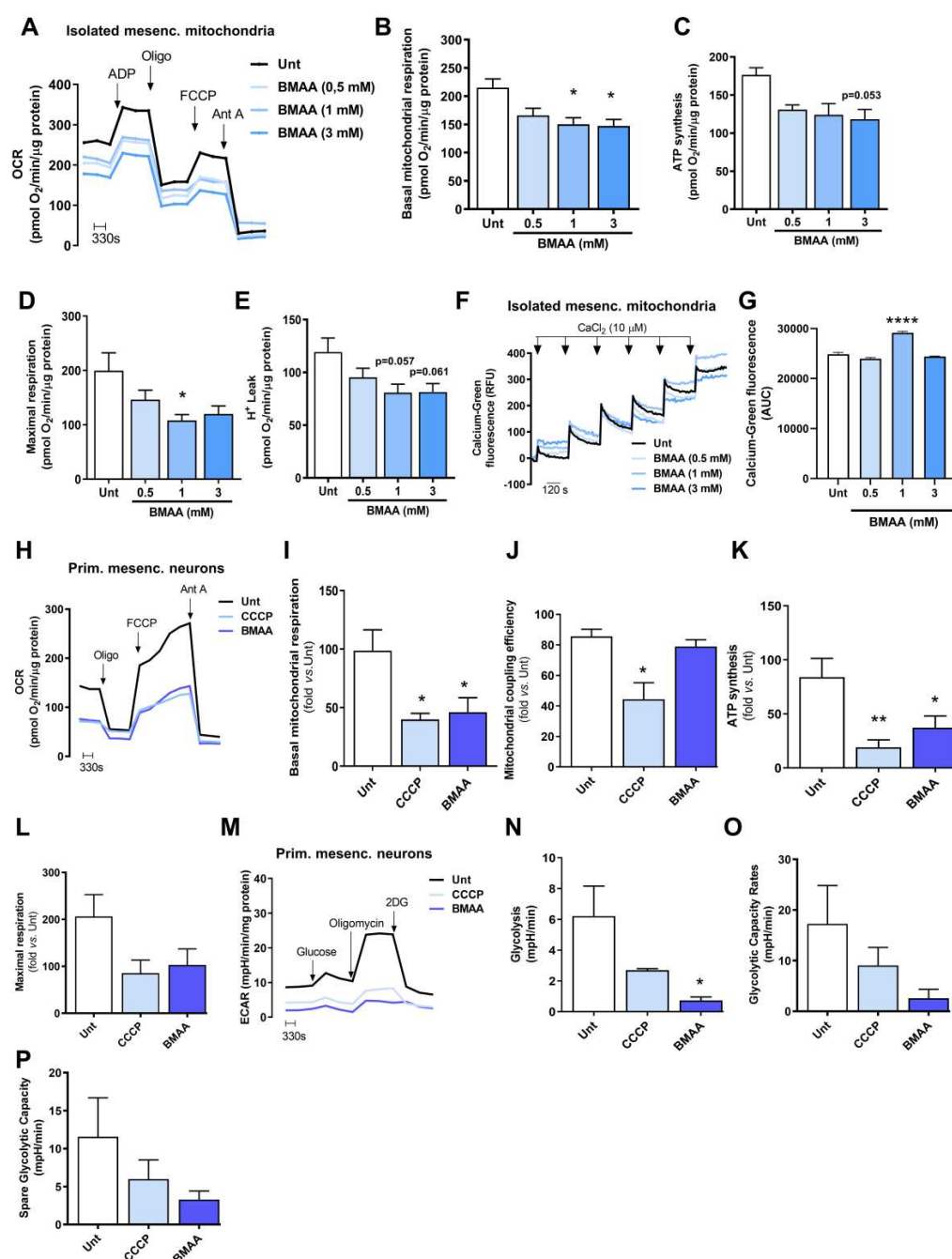
Supplemental Fig. S2. Cecum mucosa-associated microbiota diversity in BMAA-treated mice.

(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 Oscillospirales. (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).



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.

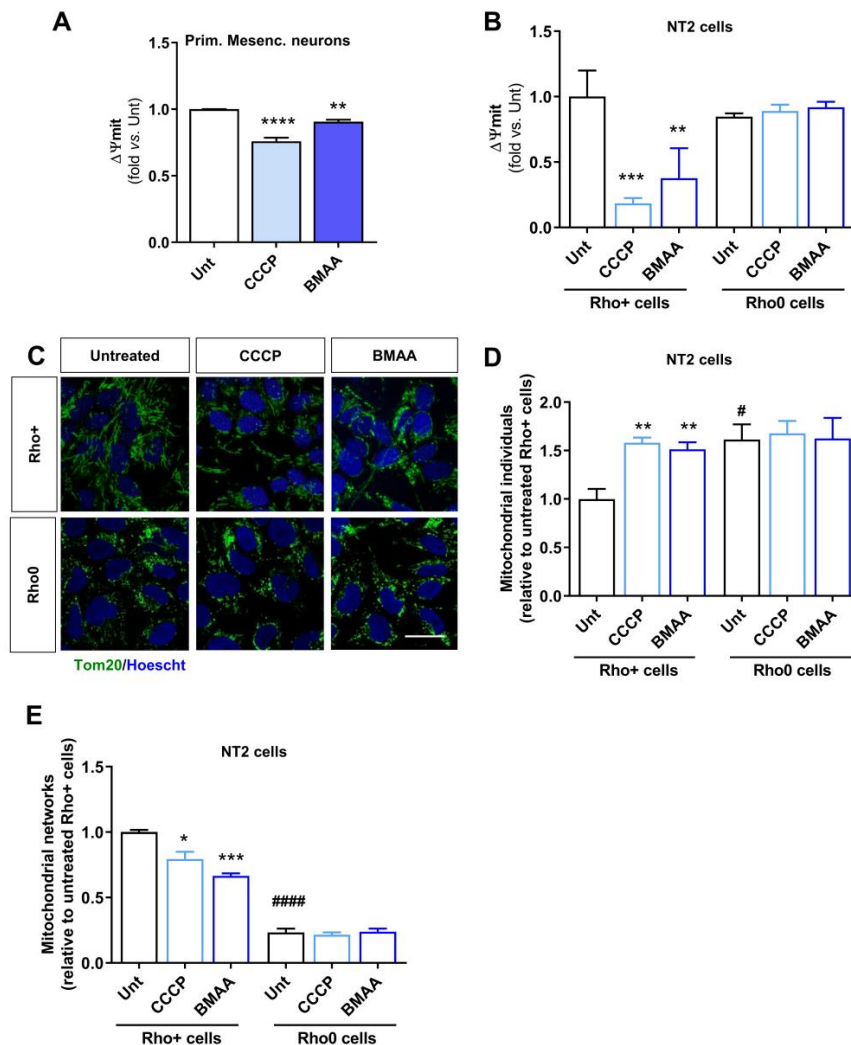
(A-D) Measurement of specific mediators involved in the inflammatory response in cecum intestinal samples from untreated (Unt) or BMAA-treated mice measured by ELISA. **(A)** IL-8 (n values for all conditions = 4, Unt vs BMAA, $p = 0.123$), **(B)** NF κ B (n values for all conditions = 4, Unt vs BMAA, $p = 0.8162$), **(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-1 β (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 mm² 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 μ m (enlarged inner boxes) and 500 μ m. 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);

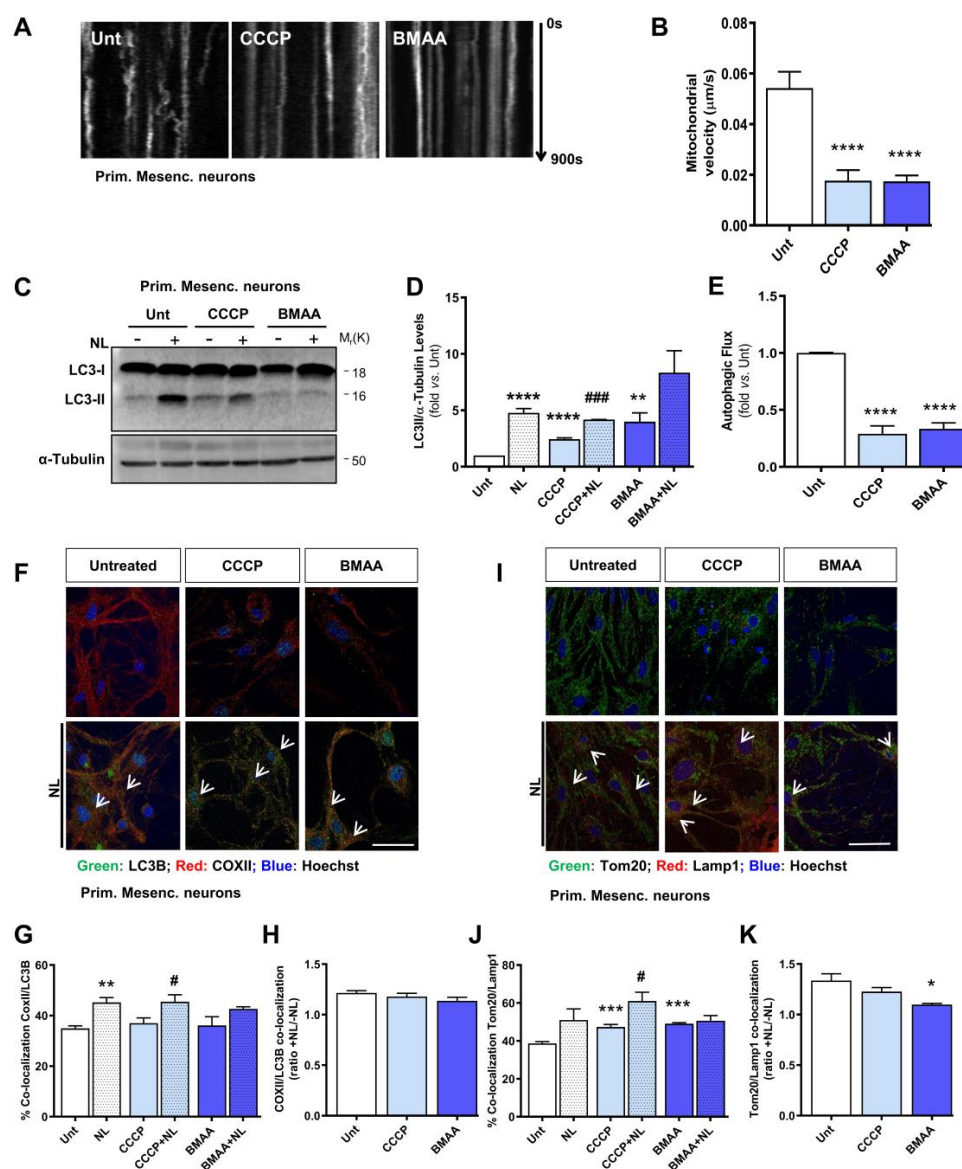
(C) ATP synthesis; (D) maximal respiration (Unt vs 1 mM BMAA, * $p = 0.0343$); (E) proton Leak. Values are pmol O₂/min/μg protein. n values for all conditions = 3, (F-G) Isolated mesencephalic mitochondria's ability to uptake calcium was evaluated with the fluorescent probe Calcium-green (n values for 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.

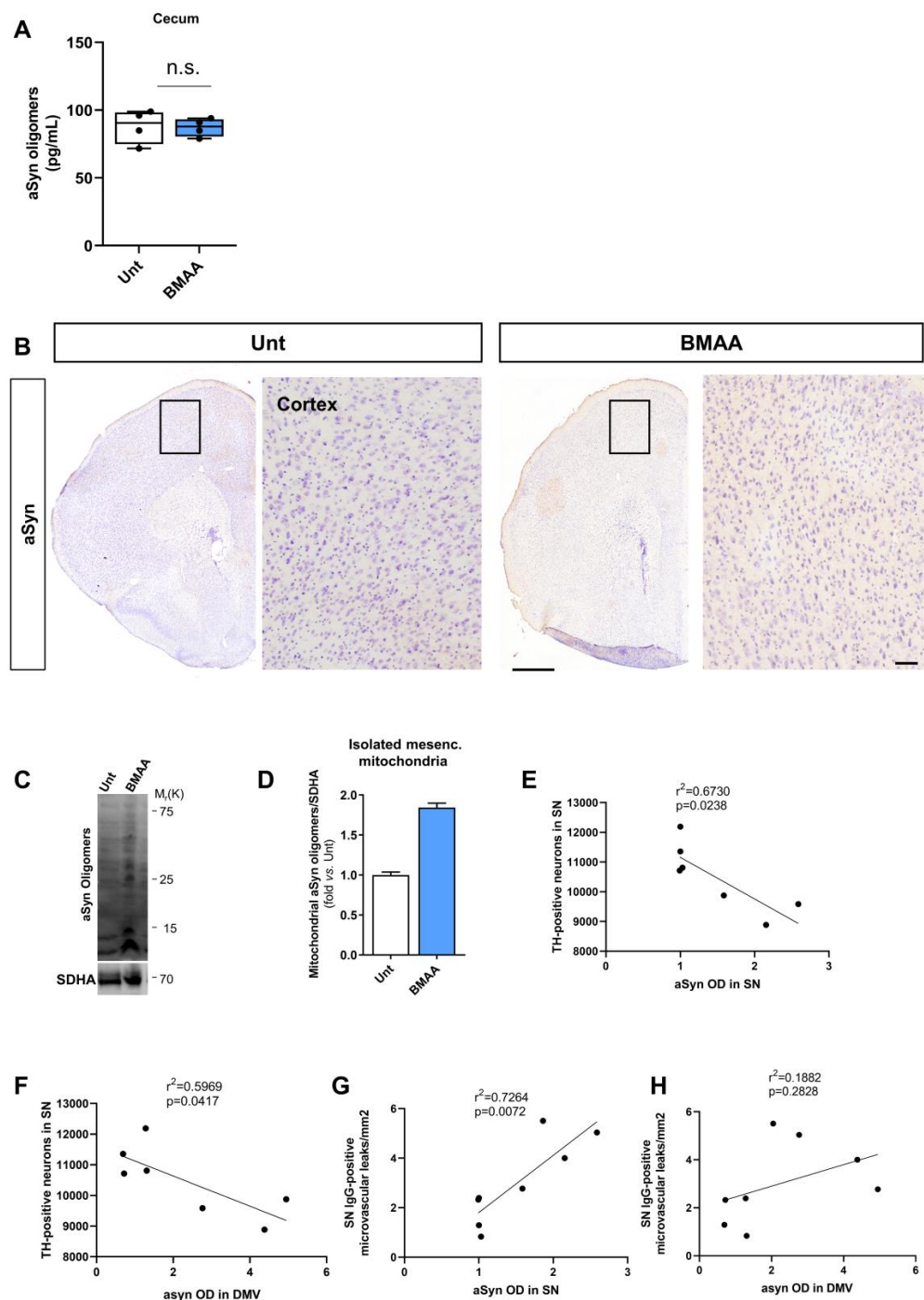
(A) Primary mice mesencephalic neurons treated with 1 μ M CCCP for 2 h and 3 mM BMAA for 48 h. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were assessed using the fluorescent cationic dye TMRM. Values are mean \pm S.E.M. (n values for Unt = 5, CCCP = 4 and BMAA = 5, Unt vs CCCP, ****p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

assessed using the fluorescent cationic dye TMRM. Values are mean \pm 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 \pm 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 \pm 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. aSyn 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

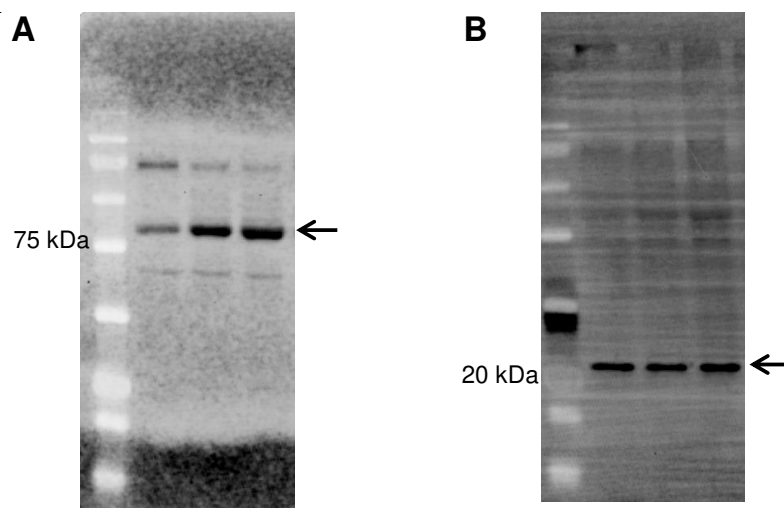


Figure 1. Whole representative western blots of Figure 4M showing phospho-Drp1 at 78 kDa (A) and Tom20 at 20 kDa (B).

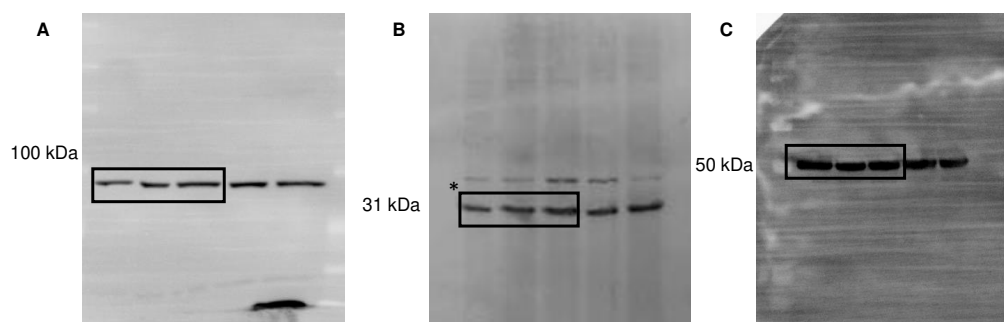


Figure 2. Whole representative western blots of Figure 5A showing TLR4 at 95 kDa (A), ProIL1 β at 31 kDa (B) and β III-Tubulin at 50 kDa (C). * This representative blot of ProIL1 β those not match the inset in figure 5B, which was lost.

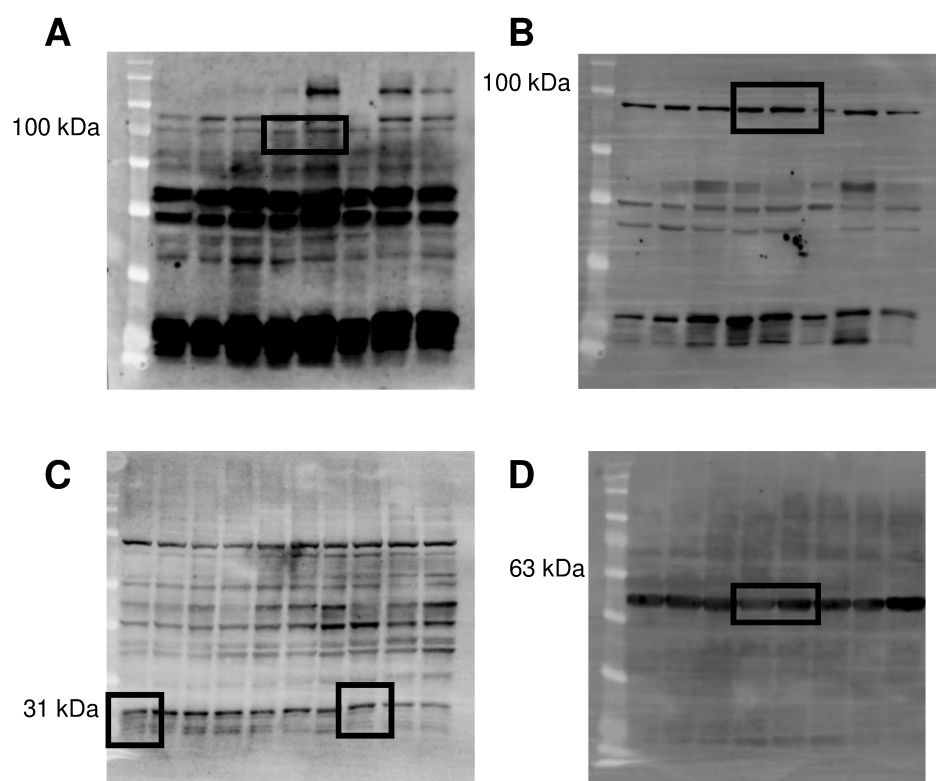


Figure 3. Whole representative western blots of Figure 5G showing TLR7 at 116 kDa (A), TLR4 at 95 kDa (B), ProIL1 β at 31 kDa (C) and respective β III-Tubulin at 50 kDa (D).

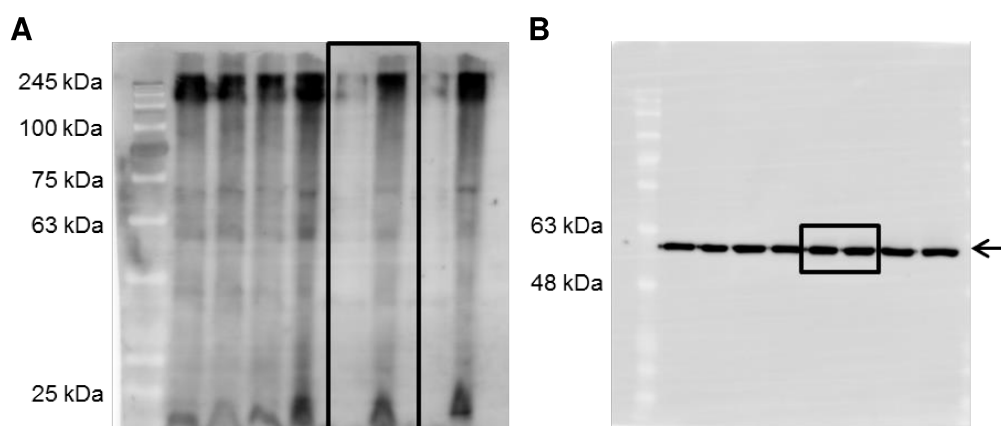


Figure 4. Whole representative western blots of Figure 6D showing aSyn Oligomers (A) and β III-Tubulin at 50 kDa (B).

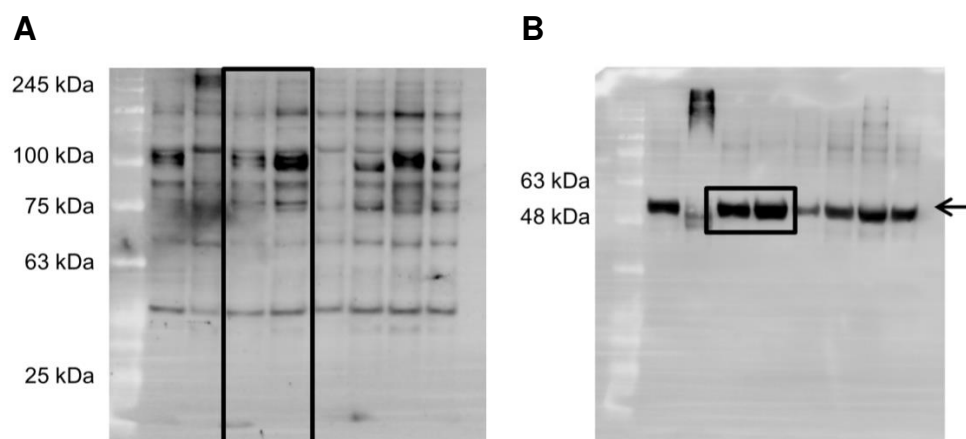


Figure 5. Whole representative western blots of Figure 6K showing aSyn Oligomers (A) and βIII-Tubulin at 50 kDa (B).

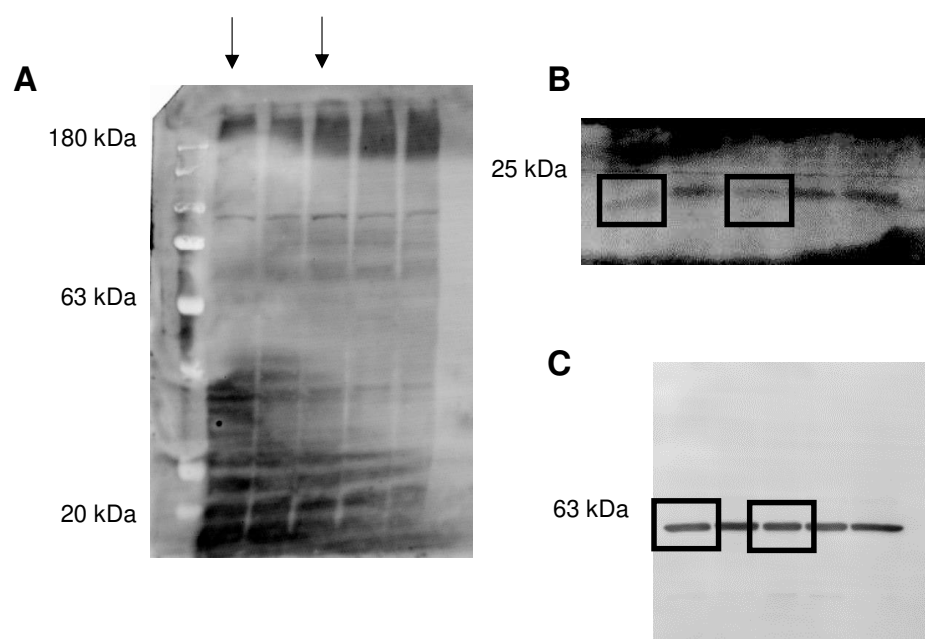


Figure 6. Whole representative western blots of Figure 6N showing aSyn Oligomers (A), aSyn monomer (B) and β-actin at 40 kDa (C).

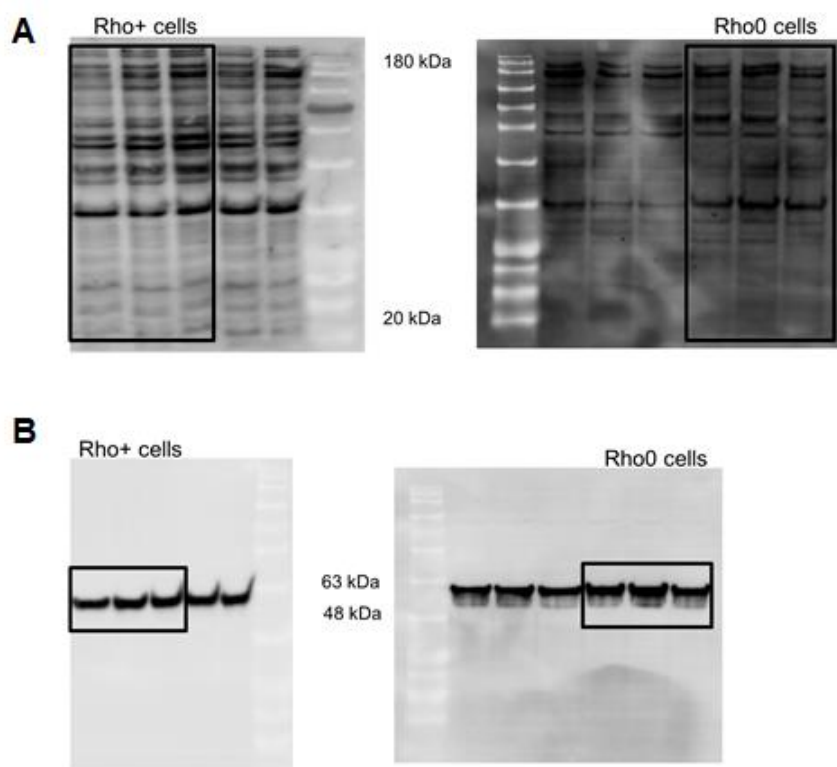


Figure 7. Whole representative western blots of Figure 6P showing aSyn Oligomers (**A**) and α -Tubulin at 50 kDa (**B**).

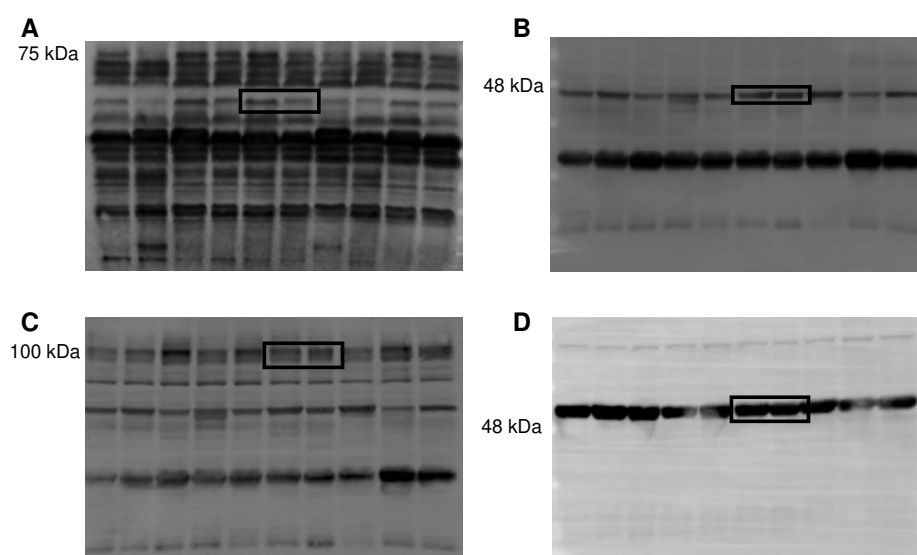


Figure 8. Whole representative western blots of Figure 7D showing TH at 62kDa (**A**), Synaptophysin at 38 kDa (**B**), PSD95 at 95 kDa (**C**) and β III-Tubulin at 50 kDa (**D**).

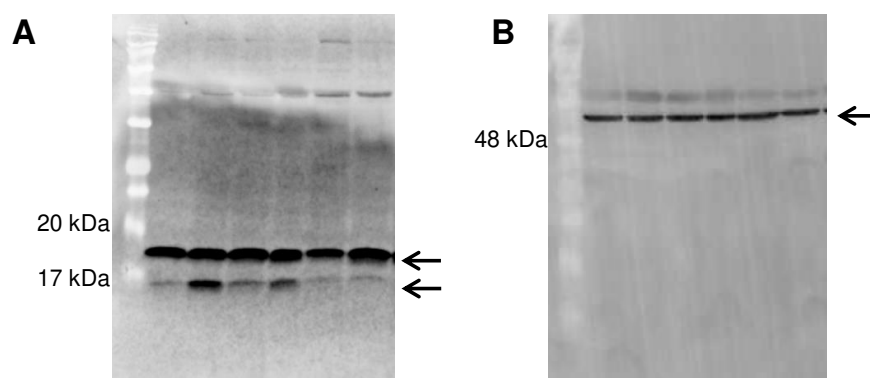


Figure 9. Whole representative western blots of Supplementary Figure S6C showing LC3B at 16 and 18 kDa (**A**) and α -Tubulin at 50 kDa (**B**).

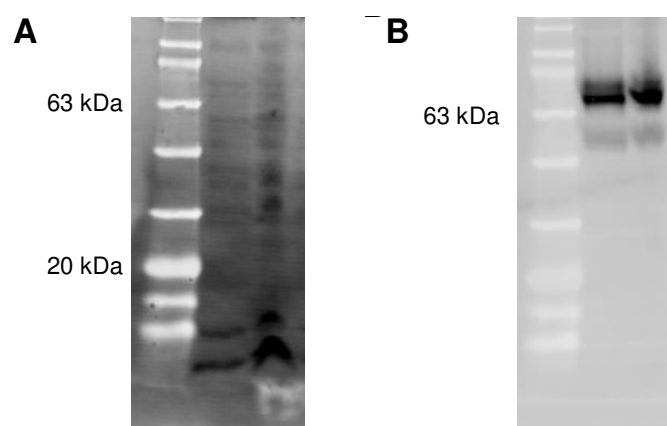


Figure 10. Whole representative western blots of Supplementary Figure S7C showing mitochondrial aSyn Oligomers (**A**) and SDHA at 70 kDa (**B**).

Supplemental material for:

“Footprints of a microbial toxin from the gut microbiome to mesencephalic mitochondria”

Table of Contents

Table S1. List of Reagents

Detailed Methods

Supplemental Figures and Figure legends (S1-S7)

Whole representative WB membranes

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)

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- β 3-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-1 β	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	
NF κ B 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 IFN γ Quantikine ELISA	R&D Systems (Cat. No. MIF00)
Mouse IL-6 Quantikine ELISA	R&D Systems (Cat. No. M6000D)
Mouse IL-1 β 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 ₄ Cl)	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).

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

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

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 (mm²).

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 (mm²).

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 (mm²).

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 (mm²) 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 °C. 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 °C in a humidified chamber.

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 °C 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 °C for 10 min. The cell suspension was washed with PBS,

centrifuged at 250×g at 4 °C for 10 min. the pellet was fixed with 2% PFA solution for 10 min at 4 °C and washed with PBS. Finally, cells were centrifuged at 250×g at 4 °C 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 MgCl₂, 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 MgCl₂; 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 °C. 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-

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 MgCl₂, 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 °C. 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 cm² 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-p0 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% CO₂. 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.

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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 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 $140\times g$ 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 μ M 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 NH₄Cl and/or 20 μ M Leupeptin (Sigma, St. Louis, MO, USA) were added for 4 h to the culture medium. The combination of NH₄Cl 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 \times) and lysed in 1% Triton X-100 containing hypotonic lysis buffer (25 mM HEPES, 2 mM MgCl₂, 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 \times 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 \times) 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 \times g for 12 min at 4 °C and the resulting supernatant was further centrifuged at 11431 \times 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 MgCl₂; 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 °C 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 MgCl₂, 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.

Analysis of Oxygen Consumption (OCR)

Mesencephalic neurons

Neurons were seeded in 24-well XF culture plates and placed in a 37 °C, 5% CO₂ 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-CO₂ 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 °C. 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 KH₂PO₄, 5 mM MgCl₂, 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 μ M) caused an increase in OCR reflecting the maximal respiratory chain activity as well as the maximal substrate oxidation rate. Finally, antimycin A (4 μ M; 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% CO₂ 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-CO₂ 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 μ M 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 $\times 100$).

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 Ca²⁺ handling capacity

Mitochondrial Ca²⁺ uptake was measured fluorometrically in fresh mesencephalic or cortical mitochondria, in the presence of the Ca²⁺-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 μ g of protein per well) which were incubated with 0.5, 1 or 3 mM BMAA for 30 min at 30 °C. Calcium Green is a cell-impermeant visible light-excitable Ca²⁺ indicator that exhibits an increase in fluorescence emission intensity upon binding to Ca²⁺; thus, a decrease in the Calcium Green fluorescence is function of external Ca²⁺ concentration which indicates the capacity of mitochondria to handle Ca²⁺. Briefly, 5 μ g of isolated mitochondria were added to the standard incubation medium containing 125 mM KCl,

0.5 mM MgCl₂, 3 mM KH₂PO₄, 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 CaCl₂, were added every 4 min and Ca²⁺ 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 $\mu\text{g}/\mu\text{L}$ 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 \times 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_2PO_4 , 137 mM NaCl, 4.16 mM NaHCO_3 , 0.34 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 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 \times lens on an Axio Observer.Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software with a stage-based chamber (5% CO_2 , 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 \times magnification (Zeiss Plan-ApoChromat 63 \times , 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 ($\Delta\psi_m$) 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 $\Delta\psi_m$. 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 MgCl₂, 6 mM glucose, 10 mM HEPES, 10 mM NaHCO₃, and 1 mM CaCl₂. Basal fluorescence was recorded for 5 min at 37 °C (λ_{ex} = 540 nm and λ_{em} = 590 nm). Subsequently, 1 μ M FCCP (protonophore) and 2 μ g/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 \pm 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.

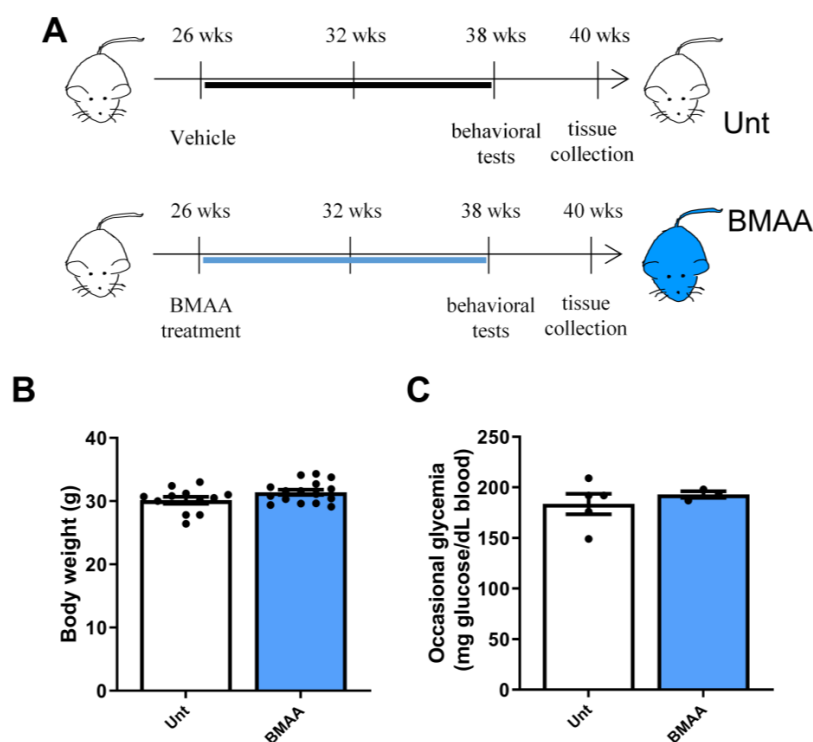
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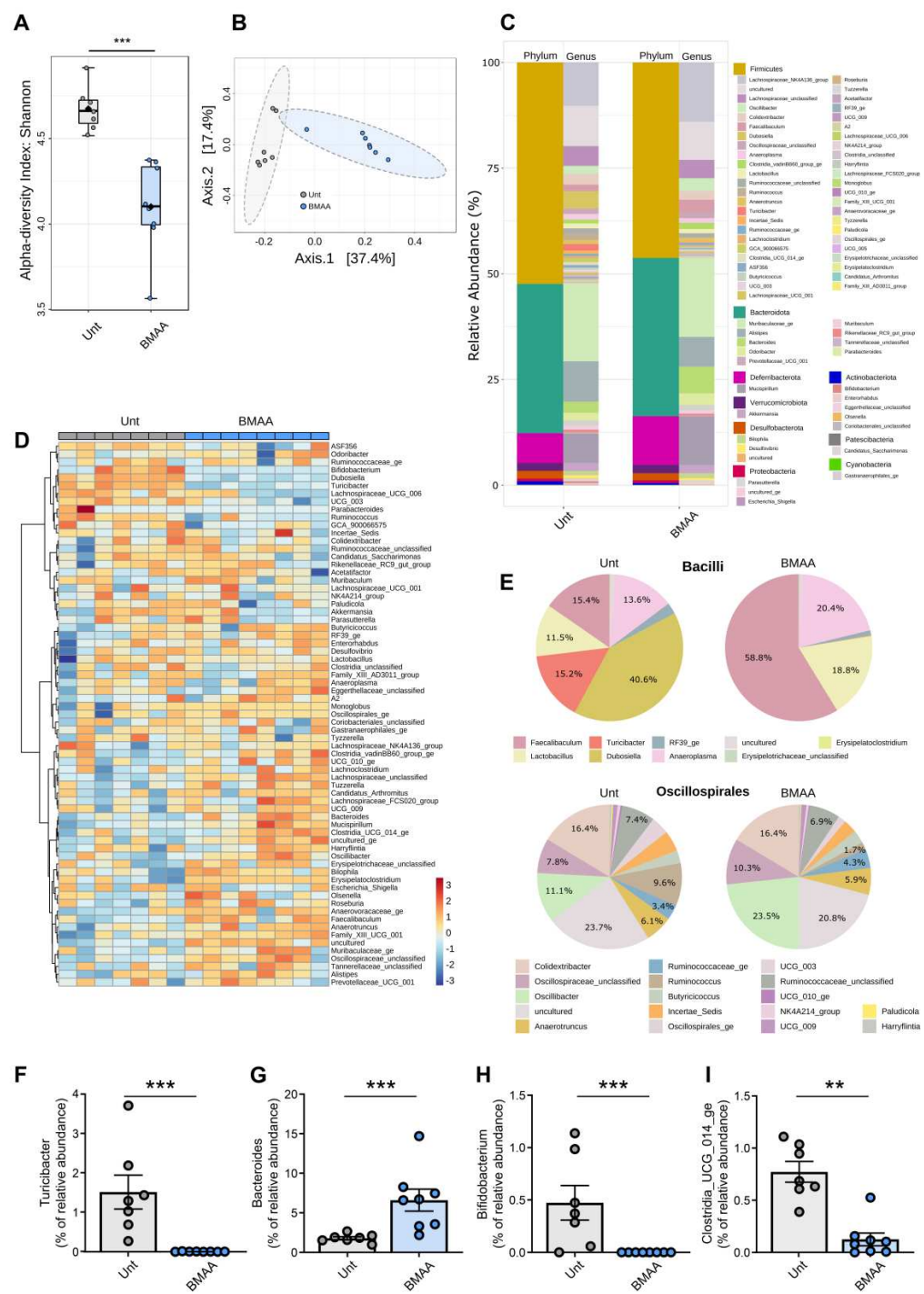
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Supplemental figures and respective legends

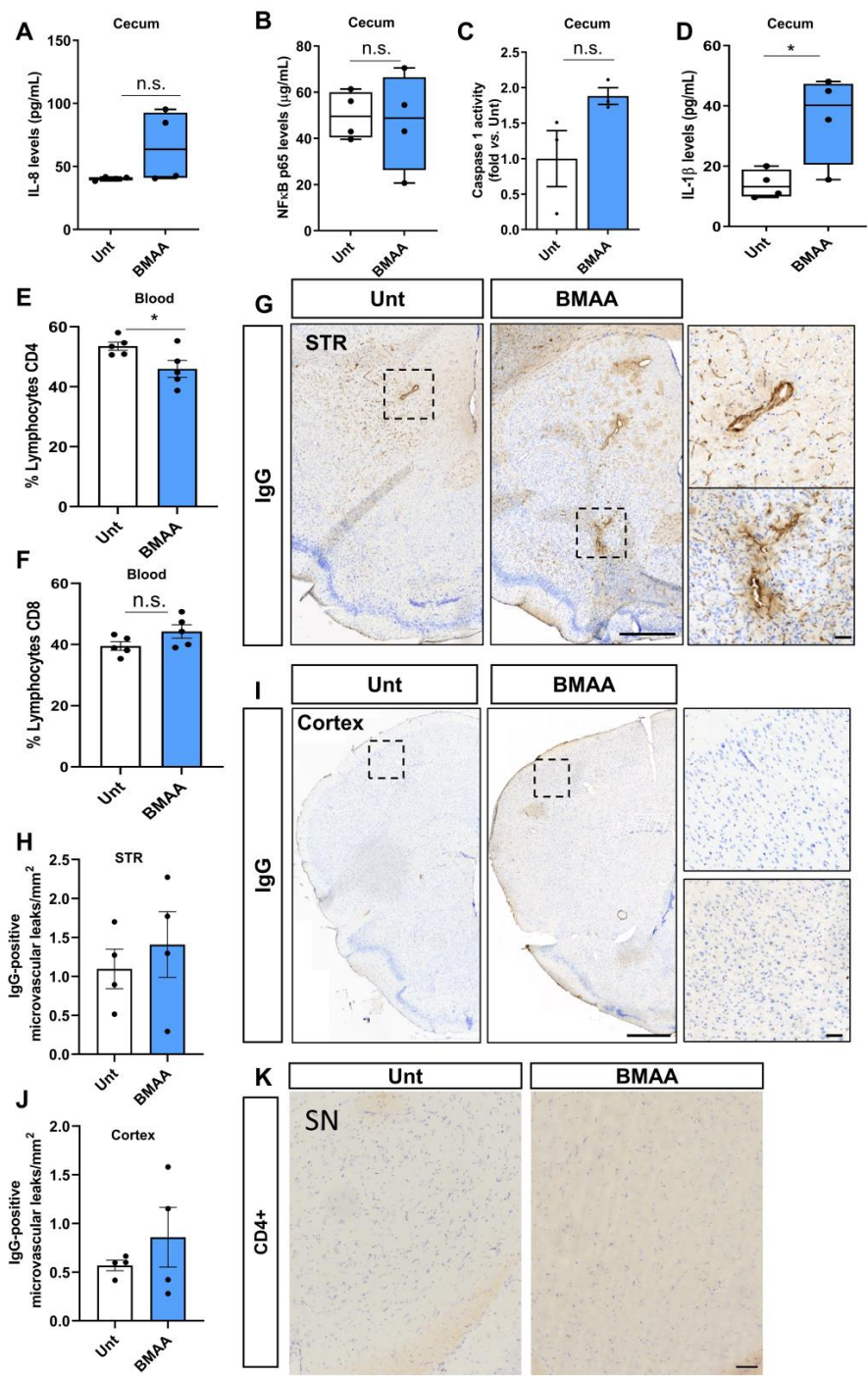


Supplemental Fig. S1. Mice body weight and glycemia were unaltered during the treatment. (A) Schematic representation of experimental design. (B) The body weights of mice were measured twice a week during the treatment. (C) Blood glucose was measured at the end of treatment and occasional glycemia were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



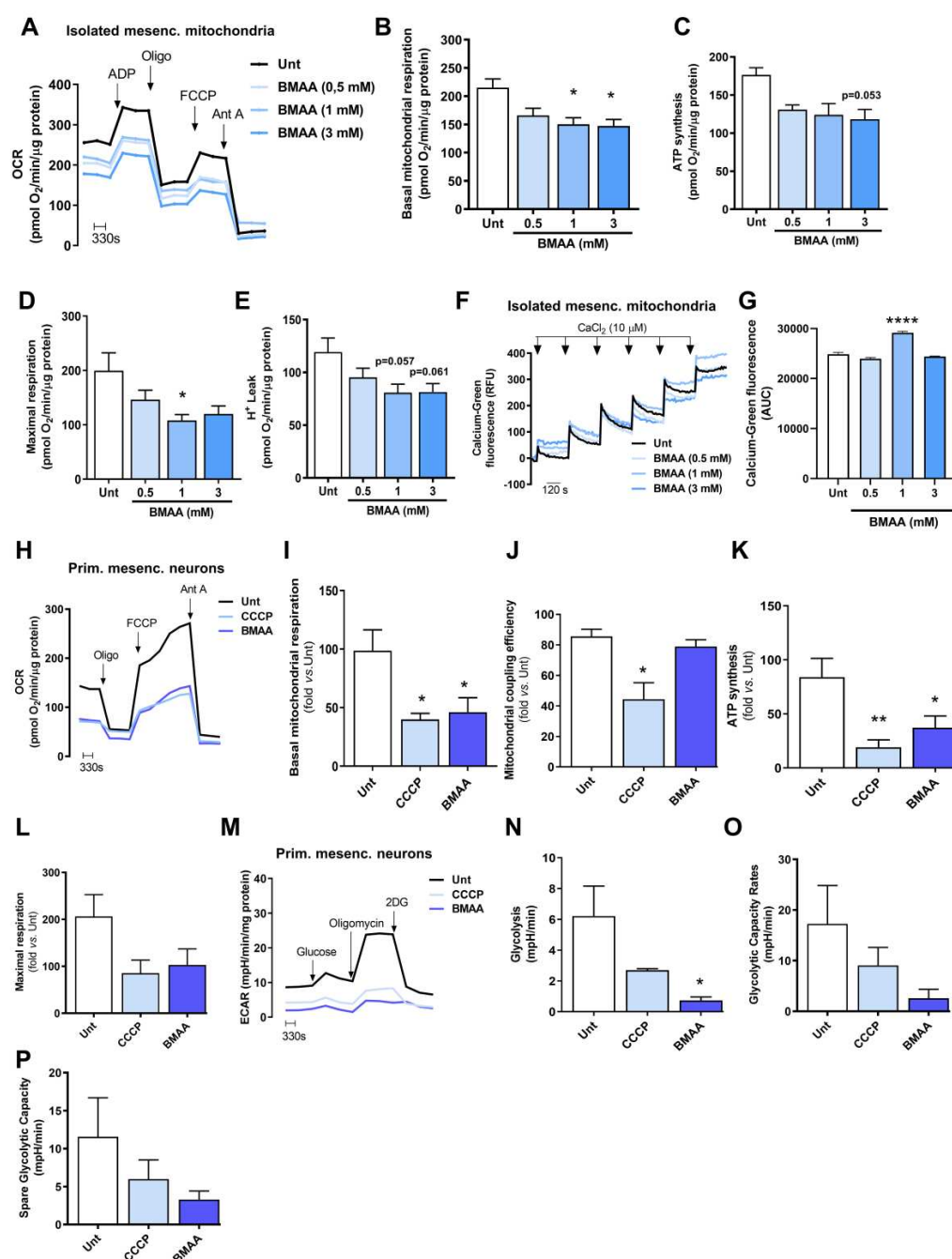
Supplemental Fig. S2. Cecum mucosa-associated microbiota diversity in BMAA-treated mice.

(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 Oscillospirales. (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).



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.

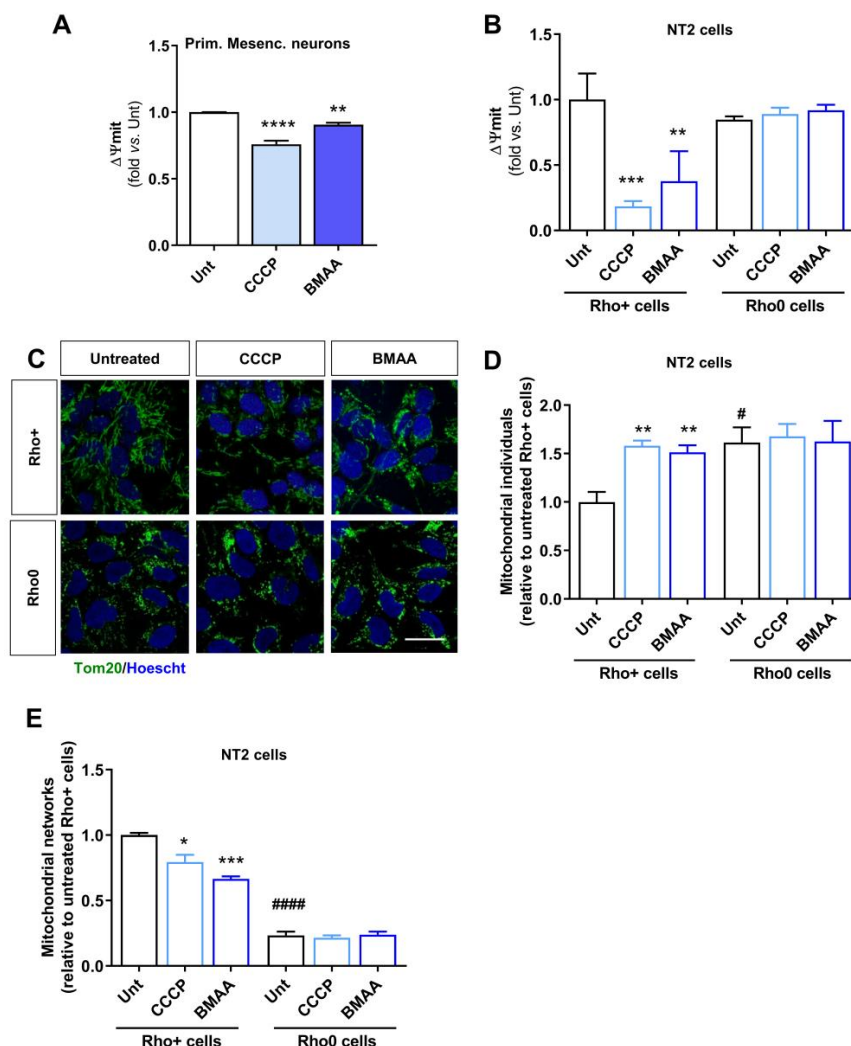
(A-D) Measurement of specific mediators involved in the inflammatory response in cecum intestinal samples from untreated (Unt) or BMAA-treated mice measured by ELISA. **(A)** IL-8 (n values for all conditions = 4, Unt vs BMAA, $p = 0.123$), **(B)** NF κ B (n values for all conditions = 4, Unt vs BMAA, $p = 0.8162$), **(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-1 β (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 mm² 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 μ m (enlarged inner boxes) and 500 μ m. 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);

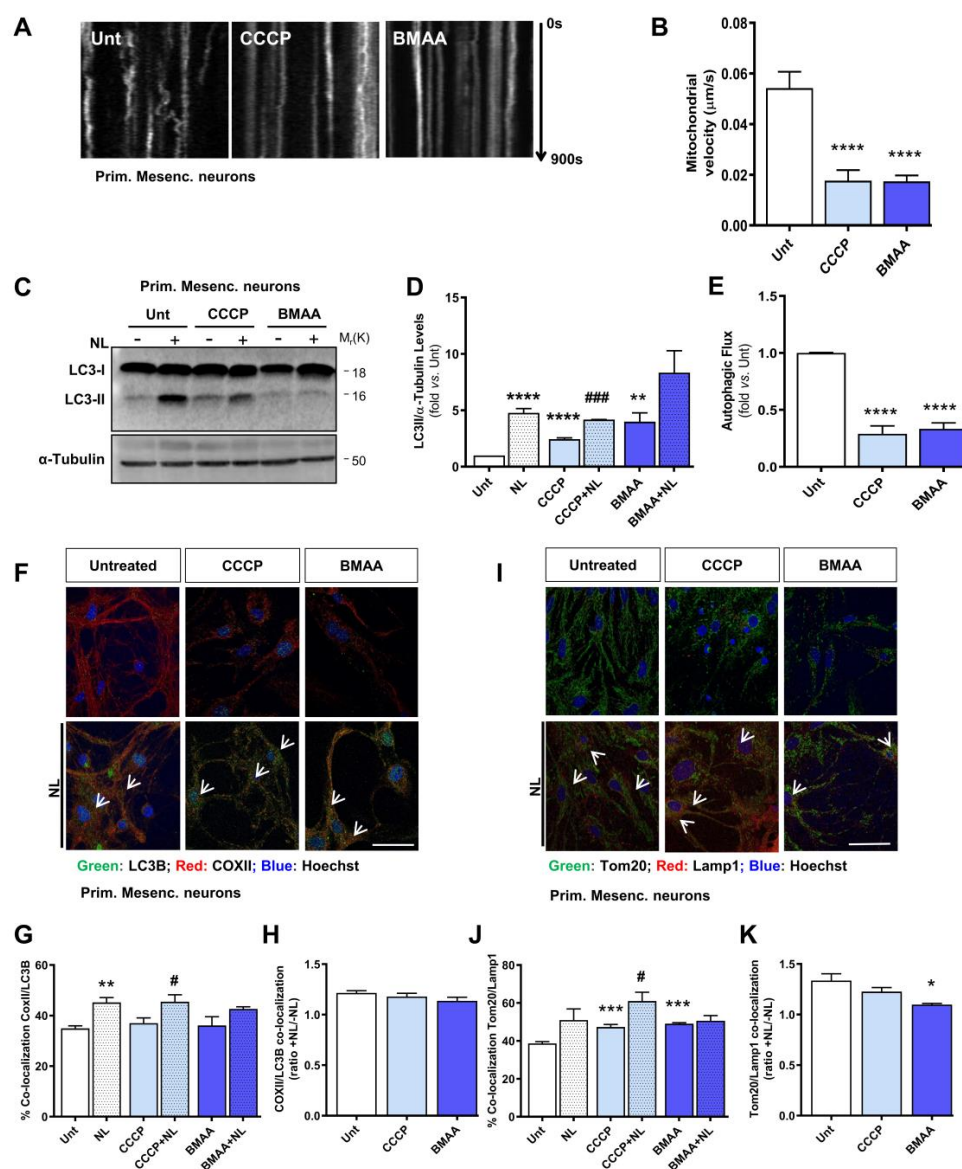
(C) ATP synthesis; (D) maximal respiration (Unt vs 1 mM BMAA, * $p = 0.0343$); (E) proton Leak. Values are pmol O_2 /min/ μ g protein. n values for all conditions = 3, (F-G) Isolated mesencephalic mitochondria's ability to uptake calcium was evaluated with the fluorescent probe Calcium-green (n values 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 μ M 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 \pm 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.

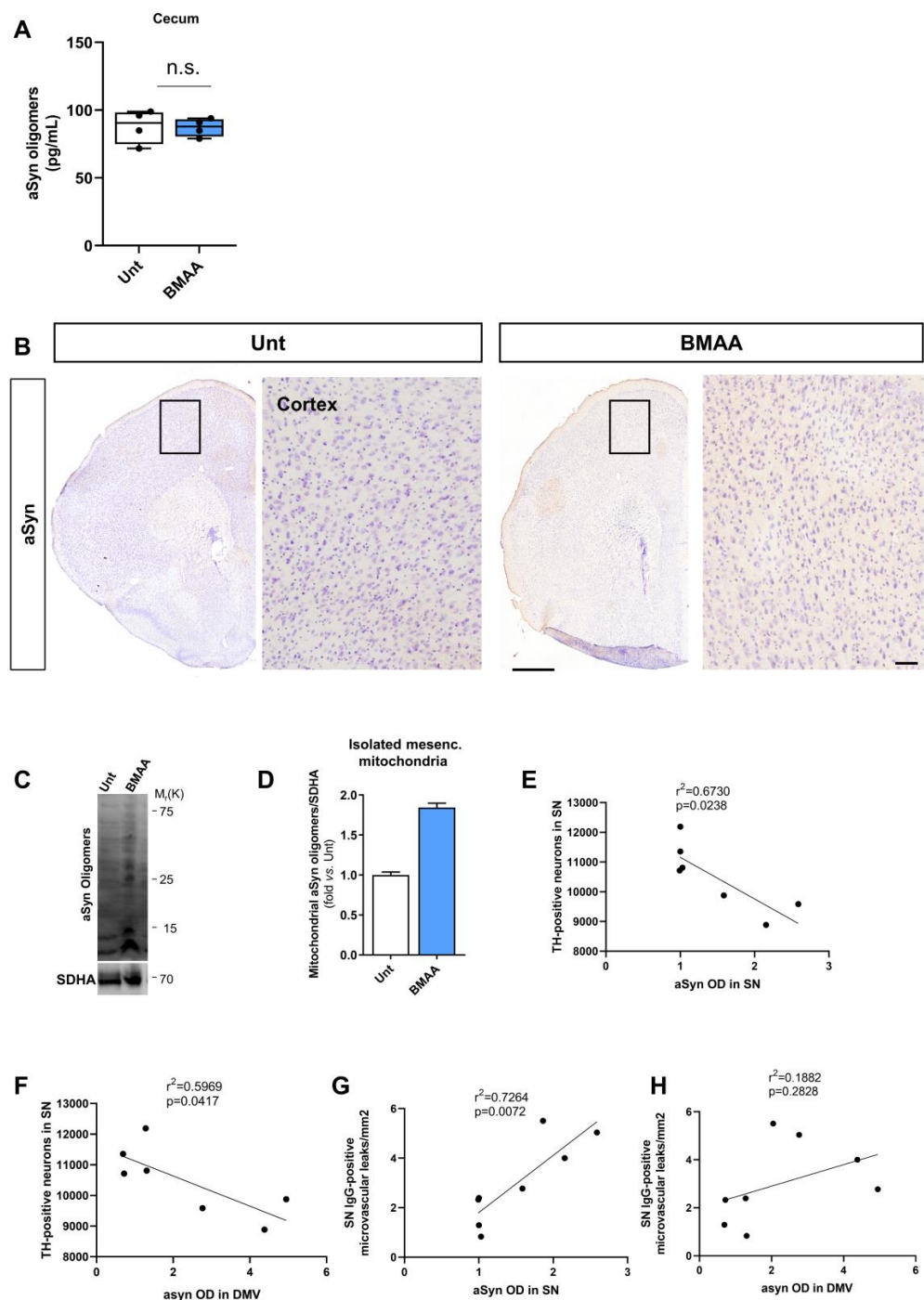
(A) Primary mice mesencephalic neurons treated with 1 μ M CCCP for 2 h and 3 mM BMAA for 48 h. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were assessed using the fluorescent cationic dye TMRM. Values are mean \pm S.E.M. (n values for Unt = 5, CCCP = 4 and BMAA = 5, Unt vs CCCP, ****p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

assessed using the fluorescent cationic dye TMRM. Values are mean \pm 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 \pm 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 \pm 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. aSyn 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

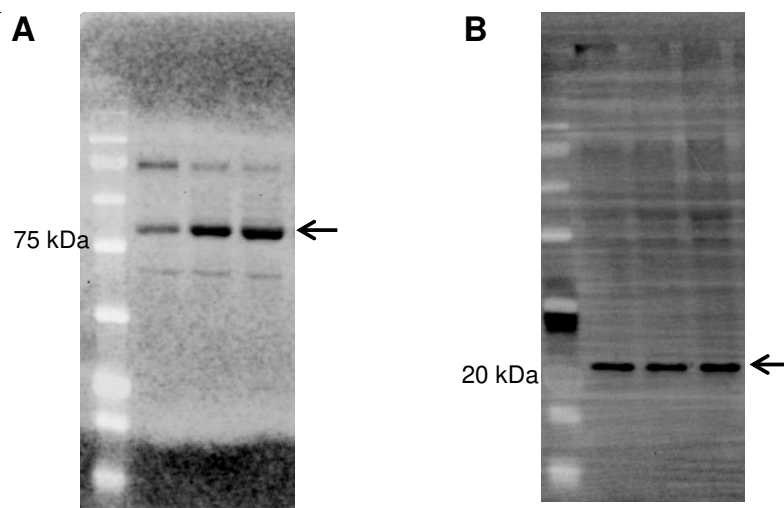


Figure 1. Whole representative western blots of Figure 4M showing phospho-Drp1 at 78 kDa (A) and Tom20 at 20 kDa (B).

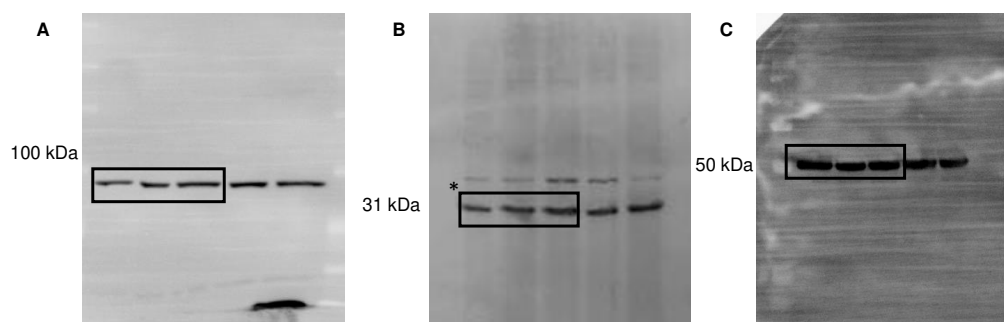


Figure 2. Whole representative western blots of Figure 5A showing TLR4 at 95 kDa (A), ProIL1 β at 31 kDa (B) and β III-Tubulin at 50 kDa (C). * This representative blot of ProIL1 β those not match the inset in figure 5B, which was lost.

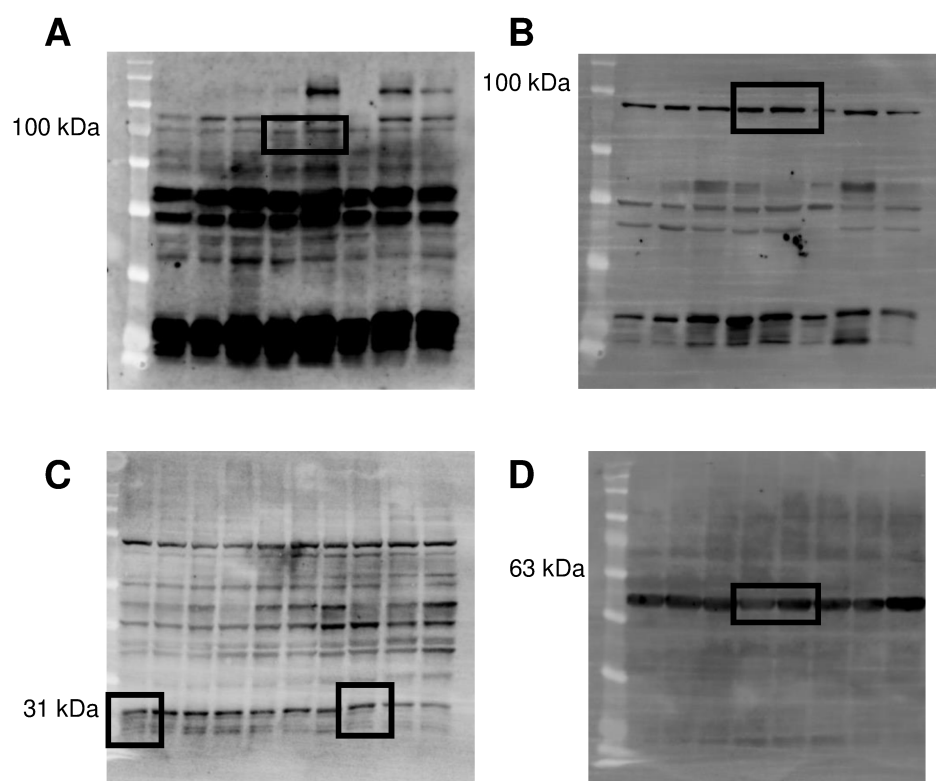


Figure 3. Whole representative western blots of Figure 5G showing TLR7 at 116 kDa (A), TLR4 at 95 kDa (B), ProIL1 β at 31 kDa (C) and respective β III-Tubulin at 50 kDa (D).

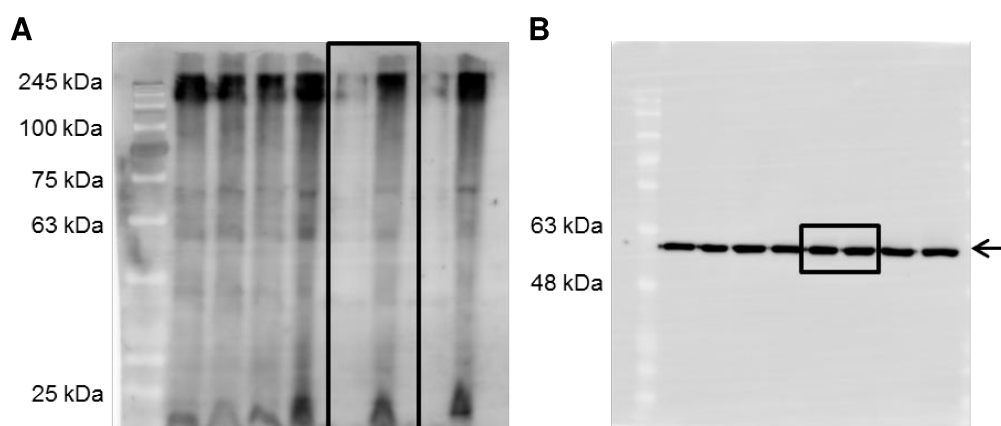


Figure 4. Whole representative western blots of Figure 6D showing aSyn Oligomers (A) and β III-Tubulin at 50 kDa (B).

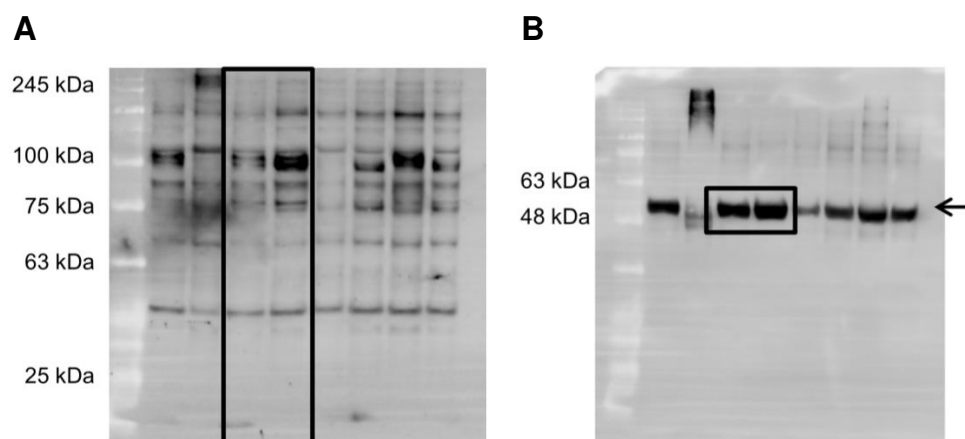


Figure 5. Whole representative western blots of Figure 6K showing aSyn Oligomers (A) and βIII-Tubulin at 50 kDa (B).

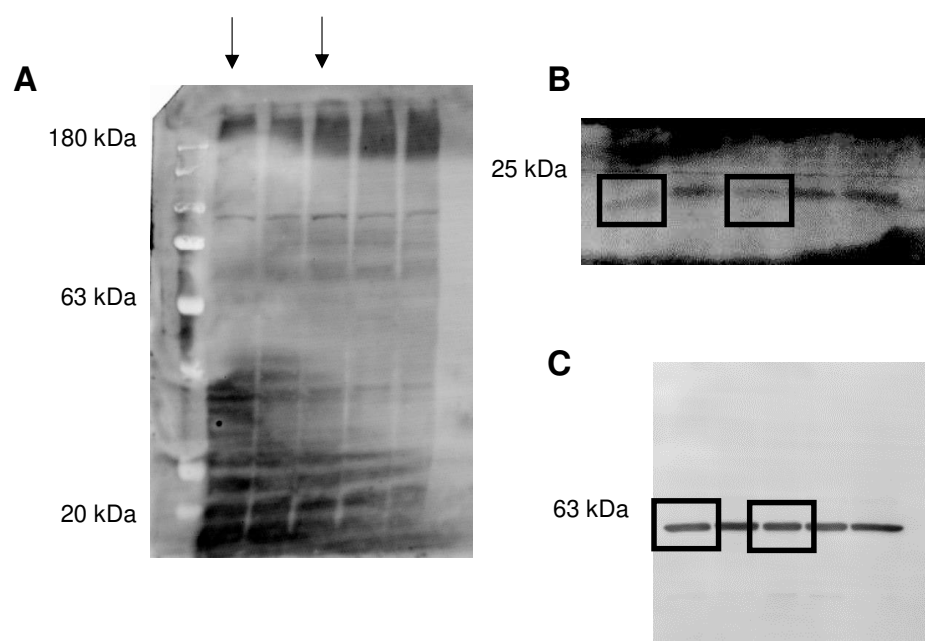


Figure 6. Whole representative western blots of Figure 6N showing aSyn Oligomers (A), aSyn monomer (B) and β-actin at 40 kDa (C).

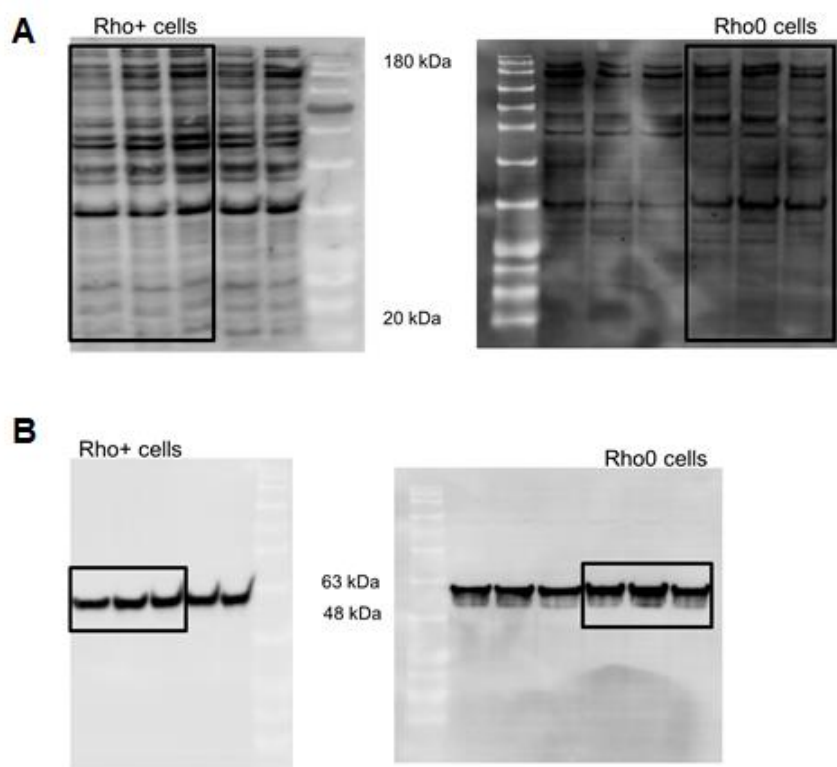


Figure 7. Whole representative western blots of Figure 6P showing aSyn Oligomers (**A**) and α -Tubulin at 50 kDa (**B**).

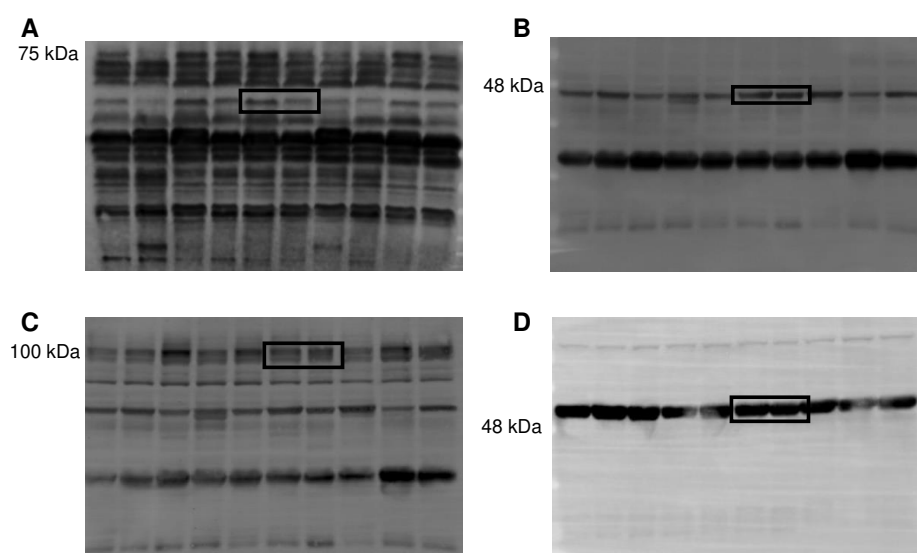


Figure 8. Whole representative western blots of Figure 7D showing TH at 62kDa (**A**), Synaptophysin at 38 kDa (**B**), PSD95 at 95 kDa (**C**) and β III-Tubulin at 50 kDa (**D**).

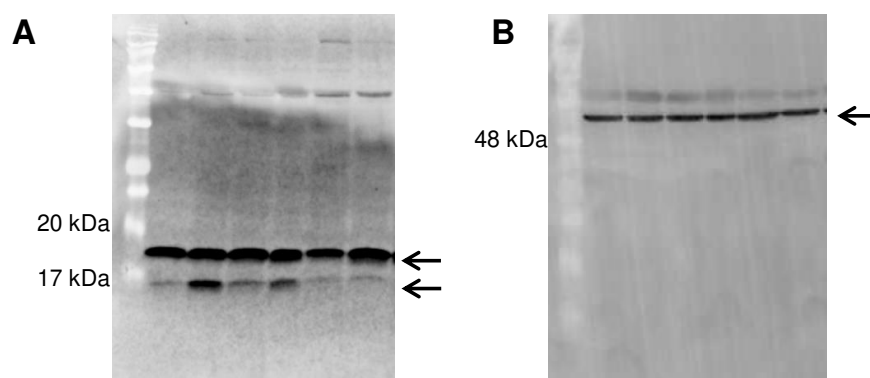


Figure 9. Whole representative western blots of Supplementary Figure S6C showing LC3B at 16 and 18 kDa (**A**) and α -Tubulin at 50 kDa (**B**).

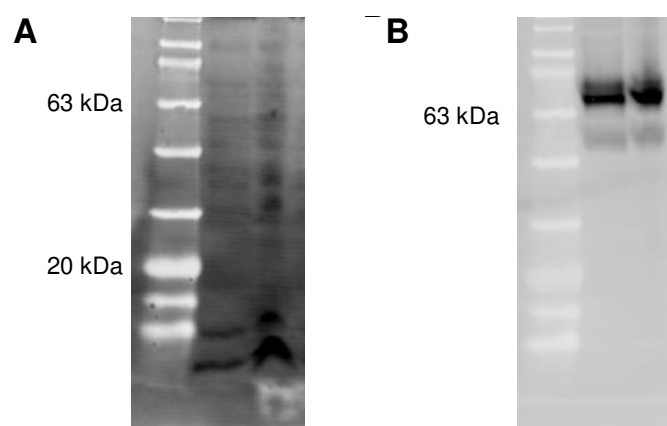


Figure 10. Whole representative western blots of Supplementary Figure S7C showing mitochondrial aSyn Oligomers (**A**) and SDHA at 70 kDa (**B**).