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Neuronal control of microglia through the mitochondria

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ABSTRACT

The microbial toxin β-*N*-methylamino-L-alanine (BMAA), which is derived from cyanobacteria, targets neuronal mitochondria, leading to the activation of neuronal innate immunity and, consequently, neurodegeneration. Although known to modulate brain inflammation, the precise role of aberrant microglial function in the neurodegenerative process remains elusive. To determine if neurons signal microglial cells, we treated primary cortical neurons with BMAA and then co-cultured them with the N9 microglial cell line. Our observations indicate that microglial cell activation requires initial neuronal priming. Contrary to what was observed in cortical neurons, BMAA was not able to activate inflammatory pathways in N9 cells. We observed that microglial activation is dependent on mitochondrial dysfunction signaled by BMAA-treated neurons. In this scenario, the NLRP3 pro-inflammatory pathway is activated due to mitochondrial impairment in N9 cells. These results demonstrate that microglia activation in the presence of BMAA is dependent on neuronal signaling. This study provides evidence that neurons may trigger microglia activation and subsequent neuroinflammation. In addition, we demonstrate that microglial activation may have a protective role in ameliorating neuronal innate immune activation, at least in the initial phase. This work challenges the current understanding of neuroinflammation by assigning the primary role to neurons.

1. Introduction

Microglia are immune resident cells of the central nervous system (CNS) acting as the first line of defense in the brain [1–3]. During primitive hematopoiesis, myeloid precursors emerge from the yolk sac to the brain before differentiating to immature microglia [4,5]. This migration occurs before the generation of astrocytes and oligodendrocytes, concurrently with the birth of the neurons giving to microglia a unique opportunity to be part of several CNS developmental processes such as neurogenesis, synapses formation/elimination and neuronal circuits establishment and remodeling [6–10]. Due to its functions, microglial cells are thought to be responsible for the majority of the inflammatory processes in the CNS, although neurons are also able to mount immune responses. Indeed, neurons can express key players of the innate immunity, including Toll-like receptors (TLRs) [11], and can also produce cytokines used to interact with immune effector cells in response to pathogen infection or to damage-associated molecular patterns (DAMPs) [12–14].

β-*N*-methylamino-L-alanine (BMAA), a neurotoxin produced by cyanobacteria and by some other microbes, may be involved in the development of different neurodegenerative diseases [15] and recently our group showed that it can induce "gut-first" PD in mice [16]. According to the endosymbiotic hypothesis, mitochondria are believed to have originated from ancestral alphaproteobacteria [17]. As a result, they can be targeted by microbial toxins, which can cause neuronal mitochondrial damage, increased network fragmentation, and exposure of DAMPs [18,19]. This exposure activates innate immunity, leading to the production of cytokines and the recruitment and activation of immune cells such as microglia, ultimately triggering neuroinflammation [20].

BMAA can cross the blood-brain barrier (BBB) [21] and is involved in synaptic alterations related to acetylcholine, dopamine, and glutamate neurotransmission, including [22–24]. BMAA also leads to neurotoxicity by mimicking glutamate binding to NMDA and AMPA receptors [22]

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and is responsible for membrane depolarization by increasing intracellular Na^+, decreasing intracellular K^+ concentrations [25]. It has also been shown that BMAA administration leads to accumulation of intracellular Ca^{2+} in rat neurons and in human astrocytes, inducing mitochondrial fragmentation and dysfunction and consequently reactive oxygen species (ROS) overproduction and cytochrome *c* release, activating apoptosis [26–28]. Our group previously showed that BMAA targeted the mitochondria in pure neuronal cultures and due to this event, neurons activated innate immunity and produced pro-inflammatory cytokines [19]. The intracellular effects of BMAA include protein misfolding and aggregation [29–31], activation of inflammatory responses by the NOD-like receptor protein 3 (NLRP3) pathway, endoplasmic reticulum stress, and apoptosis [32]. These effects are associated with the inflammatory processes that occur during the progression of neurodegenerative diseases [19,33–36]. Our intention is to validate a new paradigm for neurodegenerative diseases in which neurons alone activate neuronal innate immunity and signal microglia to induce neuroinflammation. We provide evidence that in the presence of BMAA, microglial activation is dependent on neuronal inflammation signaling.

2. Materials and methods

2.1. BMAA preparation

S(+)-2-Amino-3-(methylamino) propionic acid hydrochloride (BMAA) (98 % purity) (iChemical CO, LTD, Shanghai; Cat. No. EBD28912) was dissolved in sterile deionized water to obtain a stock solution of 970 mM. A working solution with a concentration of 60 mM was prepared by diluting the stock solution of BMAA in sterile water. Both stock and working solution were aliquoted and stored at −20 °C to avoid thaw cycles.

2.2. Isolation and treatment of primary neuronal cultures

Primary neuronal cultures were obtained as described in [37] with minor modifications.

Frontal cortices were dissected from embryonic day 15–16 of C57BL/6 mice and transferred into Ca^{2+} and Mg^{2+} free Krebs buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO4, 13 mM glucose, 10 mM HEPES, pH 7.4). Then, the dissected tissue was incubated in Krebs solution supplemented with bovine serum albumin (0.3 g/L) (BSA, Sigma-Aldrich, St. Louis, MO, USA; Cat. No. 9048-46-8) containing trypsin (0.5 g/L) (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. T0303-1G) for 10 min at 37 °C. Tissue digestion was stopped by adding a trypsin inhibitor (type II-S; 0.75 g/L) (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. T9128-500MG) in Krebs buffer, followed by a centrifugation at 140 ×*g* for 5 min. The pellet was washed once with Krebs buffer and the cells were mechanically dissociated and resuspended in plating medium [MEM (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. M0268) supplemented with 10 % horse serum (Gibco, Carlsbad, CA, USA; Cat. No. 16050122), 0.6 % (*w/v*) glucose (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. G8270-1KG) and 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. S8636-100ML). The cells were seeded on poly-L lysine (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. P1399) (0.1 g/L)-coated 18 mm coverslips at a density of 0.75×10^6 cells/mL. After 4 h, the platting medium was replaced by fresh Neurobasal medium (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. 1.00289.0100), supplemented with 0.2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. G3126), 2 % B-27 supplement (Gibco, ThermoFisher Scientific, Waltham, MA, USA; Cat. No. 17504–044), penicillin (10,000 U/L) and streptomycin (100 mg/L) (Gibco, ThermoFisher Scientific, Waltham, MA, USA; Cat. No. 15140122-100 mL). The day after seeding, primary neuron cultures were treated with 5 μM 5-Fluoro-2′-Deoxyuridine (FDU) (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. F0503) to prevent glial cell proliferation. The cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO_2, 95 % air for 6 days before treatment, to allow neuronal differentiation. After 6 days in vitro, cultured primary neurons were treated with 3 mM BMAA or 1 μg/mL LPS (Lipopolysaccharides from *Escherichia coli* O26:B6, Sigma-Aldrich, St. Louis, MO, USA; Cat. No. L2654) for 48 h.

2.3. N9 microglial cell line culture and treatment

The murine N9 microglial cell line was kindly provided by Doctor Ana Luísa Cardoso from the Center for Neuroscience and Cell Biology, University of Coimbra, Portugal. N9 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. R1383), supplemented with 30 mM of glucose, 23.8 mM of sodium bicarbonate, 5 % of heat-inactivated fetal bovine serum (FBS) (Biowest, Bradenton, FL, USA; Cat. No. S1810-500 mL) and penicillin (10,000 U/L) and streptomycin (100 mg/L) (Gibco, ThermoFisher Scientific, Waltham, MA, USA; Cat. No. 15140122-100 mL), at pH 7.3.

The N9 cells were treated with 3 mM BMAA or 1 μg/mL LPS for 6 or 24 h. For the western blot, ELISA and caspase-1 assays, N9 cells were seeded at a density of 0.1×10^6 cells/mL in 6 well plates; for Seahorse XF24 apparatus measurements, N9 were seeded at a density of 0.25×10^5 cells/mL.

2.4. Co-culture strategy

For the co-culture strategy, primary neurons were seeded in poly-L lysine-coated coverslips with wax spheres and treated for 24 h. Then, the coverslips with neurons were added to the top of the N9 culture and the medium of the treated neurons was also added. The wax spheres allowed neurons to be in contact with N9 cells by keeping a minimum distance to avoid culture damage. The co-cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO_2, 95 % air for 24 h after the co-culture was set. The cell density used for N9 and primary neurons was 0.5×10^5 cells/mL and 0.75×10^6 cells/mL, respectively, in 12 well plate.

2.5. Mitochondrial function with Seahorse XF24 Extracellular Flux analyzer measurements in N9 cell line

N9 mitochondrial oxygen consumption rate (OCR) was evaluated by seeding approximately 10,000 cells per well in the 24 well cell culture microplates provided by the manufacturer (Agilent, Santa Clara, CA, USA; Cat. No. 100777-004). The N9 cells were treated with 3 mM BMAA or 1 μg/mL LPS for 6 or 24 h.

For the OCR measurement of the N9 co-cultured with treated neurons, the cortical neurons were seeded on poly-L lysine (0.1 g/L)-coated 10 mm coverslips at a density of 0.75×10^6 cells/mL and treated with 3 mM BMAA or 1 μg/mL LPS for 24 h. After this, the coverslips were placed on top of the 24 well cell culture microplates, where N9 were seeded, and the medium of the treated neurons was also added mimetizing the co-culture strategy. After 24 h, the coverslips with treated neurons were removed.

For the N9 culture alone or in co-culture, the protocol was the same. 1 h before placing the culture microplates in the Seahorse Analyzer, the cells were washed in assay medium, an unbuffered RPMI (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. R8755) containing 30 mM of glucose and 1 % of penicillin (10,000 U/L) and streptomycin (100 mg/L), at pH 7.3. The protocol for OCR respiration measurements consisted of 30 s mix, 30 s wait, and 3 min read during 3 cycles. The first three readings determined the basal OCRs. In the fourth reading, 1 μM of oligomycin (Alfa Aesar, Karlsruhe, Germany; Cat. No. J60211) was injected in each well and the resulting OCR was measured over three reading cycles. Next, the addiction of uncoupler FCCP (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. C2920) (2 μM) allowed the detection of the maximum respiration rate over 3 reading cycles. After this, a mixture of rotenone (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. R8875) (2 μM)

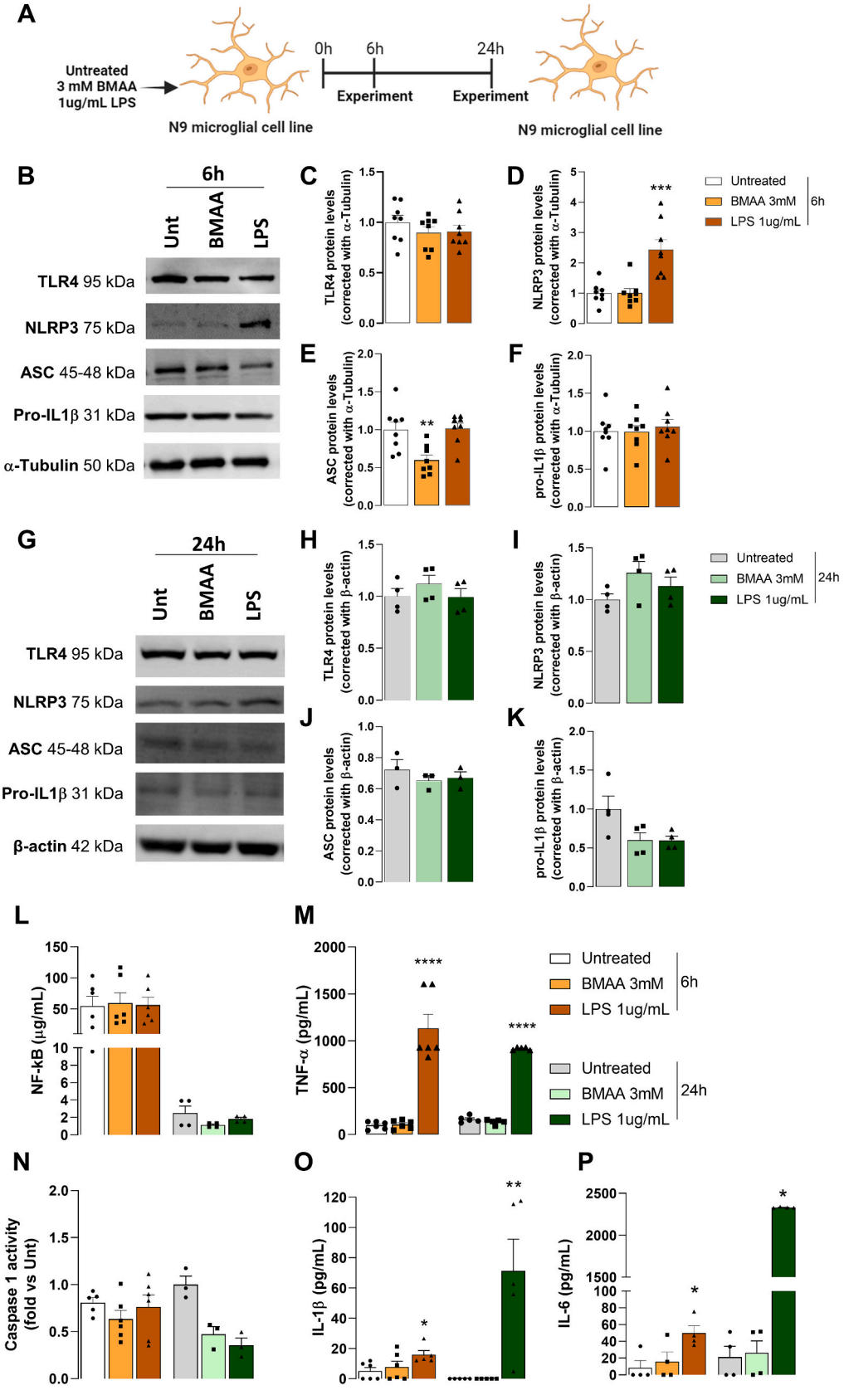

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Fig. 1. BMAA fails to activate inflammation pathways in N9 microglial cell line treated for 6 h or 24 h. (A) Representative scheme of the experiment. (B) Representative immunoblot and densitometric analysis of N9 microglial cell line treated for 6 h was performed by the loading of equal amounts of protein corrected with α-tubulin. (C-F) Evaluation of NLRP3-inflammatory pathway proteins by Western Blot. (G) Representative immunoblot and densitometric analysis of N9 microglial cell line treated for 24 h was performed by the loading of equal amounts of protein corrected with β-actin. (H-K) Evaluation of NLRP3-inflammatory pathway proteins by Western Blot. (L) Evaluation of NF-kB by ELISA kit. (N) Caspase-1 activation. (O) Assessment of IL-1β, (M) TNF-α and (P) IL-6 levels in the medium of N9-treated cells by ELISA kit. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ using One-way ANOVA with Dunnett's multiple comparison test. Results are expressed with the mean ± SEM derived from at least three independent experiments.

and antimycin A (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. A8674) (2 μM) was injected and the resultant OCR was detected over 3 reading cycles. Finally, the assay medium was the last injection, and the final OCR was measured for over three cycles.

2.6. Immunoblotting

To prepare cytosolic samples to detect pro-IL-1β, ASC, TLR4 and NLRP3 levels by western blot, after the incubations, N9 and cortical neurons were washed in ice-cold PBS and lysed in the hypotonic lysis buffer (25 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA) supplemented with 2 mM DTT, 0.1 mM PMSF, 1:1000 dilution of a protease inhibitor cocktail and 1 % Triton X-100. The cells were scraped on ice, transferred to the respective tube and, after 3 cycles of freezing (liquid nitrogen) and thawing (37 °C water bath), samples were centrifuged at 17000 ×*g* for 10 min and at 4 °C. The supernatant was collected and stored at −80 °C. Protein quantification was determined using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA USA; Cat. No. 23227). The samples containing 25 μg of protein were loaded onto a 10 % Tris-Tricine-SDS gel and separated by electrophoresis. After transfer to PVDF membranes (Millipore, Billerica, MA, USA; Cat. No. IPVH00010), non-specific binding was blocked by incubating the membranes in 3 % BSA with 0.1 % Tween-20 in Tris-Buffered Solution (TBS - 20 mM Tris, 150 mM NaCl and 0.1 % (*w/v*) Tween-20 ®, pH 7.6) for 1 h at room temperature. Then, the membranes were subsequently incubated with the respective primary antibodies overnight at 4 °C with gentle agitation: 1:750 anti-NLRP3 (ThermoFisher Scientific, Waltham, MA, USA; Cat. No. MA5–23919); 1:250 anti-TLR4 (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-293,072), 1:250 anti-pro-IL-1β (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-52,012); 1:250 anti-ASC (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-271,054). Finally, 1:5000 anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. A5441) or 1:5000 anti-α-tubulin (Cell Signaling Technology, Danvers, MA, USA; Cat. No. #2125) were used as loading control. After the overnight incubation with the primary antibodies, membranes were washed with TBS containing 0.1 % Tween for 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 the secondary incubation, the membranes were washed again and specific bands of each protein of interest were detected by developing with an alkaline phosphatase enhanced chemical fluorescence reagent (ECF, Sigma-Aldrich St. Louis, MO, USA; Cat. No. GERPN3685). Fluorescence signals were detected using a ChemiDoc Imaging System (BioRad, Hercules, CA, USA) and band densities were determined using ImageJ Software. Protein densities were corrected with the densities of β-actin or α-tubulin to account for possible differences in protein loading.

For the detection of α-synuclein, β-Amyloid and TOM20 protein levels in cell culture medium, supernatant was collected, centrifuged at 10000 ×*g* for 10 min at 4 °C to eliminate possible media contaminants. Then, 15 μL of supernatant were loaded onto a 7 % Tris-Tricine-SDS gel and separated by electrophoresis. The transfer to PVDF membranes, blocking, incubations, and detection were performed as described above. The membranes were incubated with the following primary antibodies: 1:750 anti-TOM20 (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-11,415), 1:1000 anti-α-synuclein (Cell Signaling Technology, Danvers, MA, USA; Cat. No. #2642) and 1:1000 anti-β-Amyloid (Cell Signaling Technology, Danvers, MA, USA; Cat. No. #8243).

2.7. Cytokines assessment by ELISA

For cytokines evaluation levels, before and after treatments, extracellular media was collected and centrifuged at 10000 ×*g* for 10 min at 4 °C. Then, N9 cells and neurons were washed in ice-cold PBS and proteins were extracted in hypotonic lysis as described above. After scrapping and cycles of freezing and thawing, the samples were centrifuged at 17000 ×*g* for 10 min at 4 °C and supernatant was collected. NF-kB p65 (Abcam, Cambridge, UK; Cat. No. ab133112), IL-1β (R&D Systems, Minneapolis, MN, USA; Cat. No. MLB00C), IL-6 (R&D Systems, Minneapolis, MN, USA; Cat. No. M6000B) and TNF-α (R&D Systems, Minneapolis, MN, USA; Cat. No. MTA00B) levels were determined using mouse ELISA kits according to manufacturer's instructions.

Specifically, extracellular levels were evaluated using 50 μL of each sample, whereas intracellular abundance of each cytokine was measured using 25 μg of protein for each condition.

2.8. Caspase 1-like activity assay

Neuronal and N9 extracts were processed as described above. The resultant cell extracts were removed and stored at −80 °C. Protein content was determined using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA USA; Cat. No. 23227). Cortical neurons and N9 extracts containing 40 μg of protein were incubated in 25 mM HEPES, pH 7.5, containing 0.1 % (*w/v*) 3[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS), 10 % (w/v) sucrose, 2 mM DTT and 40 μM of Caspase 1 substrate (Ac-VAD-4-methoxy-2-naphtylamide) (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. SCP0066) at 37 °C for 2 h. Caspase 1 activation was determined by detecting the substrate cleavage at 405 nm using a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.9. Data analysis

Data are expressed as means ± standard error of the means (SEM) of at least 4 independent experiments. Statistical analyses were performed using GraphPad Prism 8.4.0 for Windows (GraphPad Software, San Diego, CA, USA). Normality distribution analysis (Shapiro-Wilk test) was applied to establish the appropriate parametric or non-parametric tests for subsequent analysis. Unpaired *t*-test and One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons or Kruskal-Wallis's multiple comparisons test (comparisons between untreated cells versus treatments) were used to compare means between experimental groups. *p*-values <0.05 were considered significant.

3. Results

3.1. BMAA failed to activate innate immunity in microglial cells

The mainstream theory states that microglial activation is responsible for neuroinflammatory events that will trigger neurodegeneration

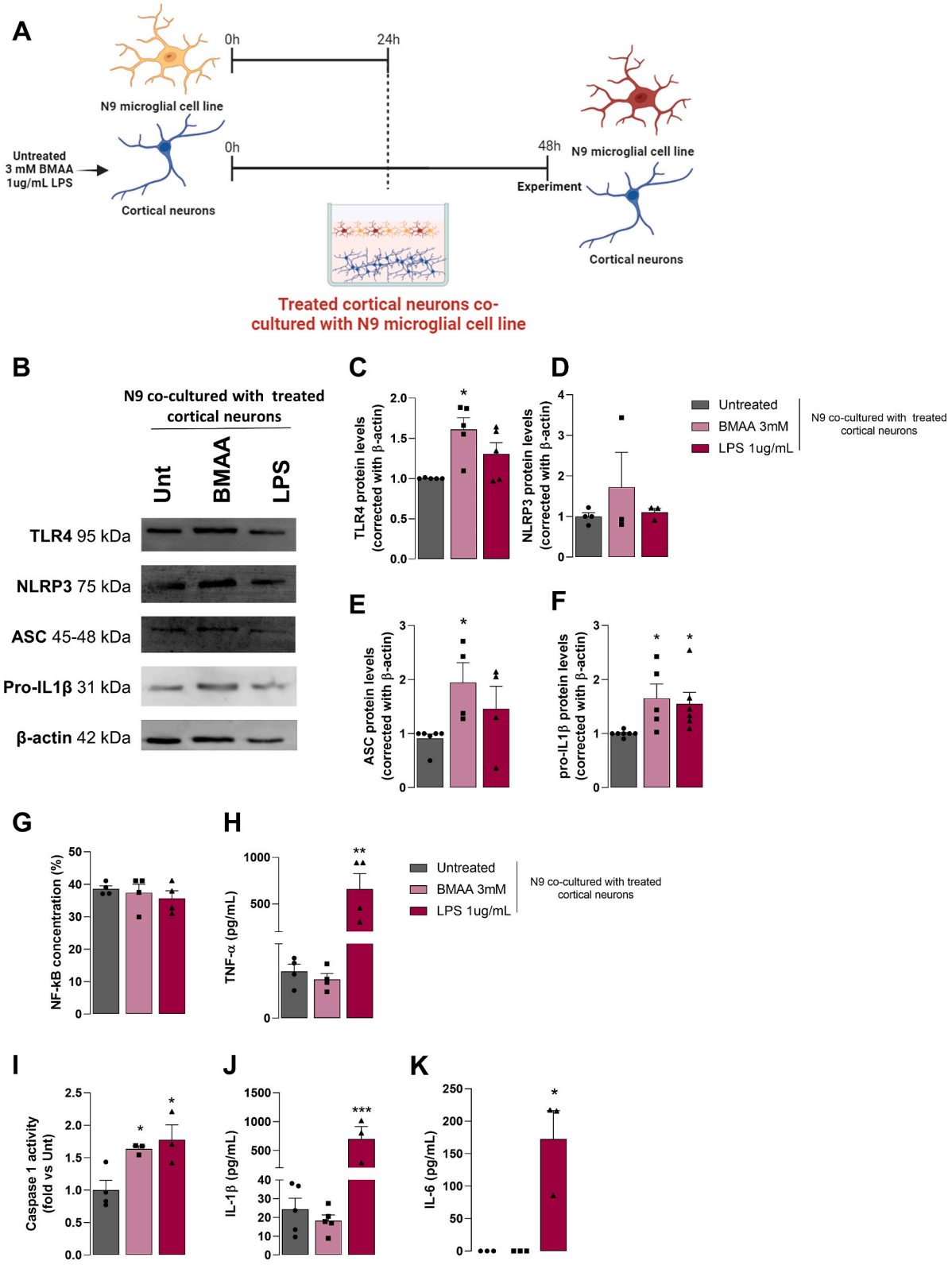

Fig. 2. Presence of neurons treated with BMAA induced N9 inflammatory pathways activation. (A) Representative scheme of the experiment. (B) Representative immunoblot and densitometric analysis of N9 microglial cell line co-cultured with treated cortical neurons was performed by the loading of equal amounts of protein corrected with β-actin. (C-F) Evaluation of NLRP3-inflammatory pathway proteins by Western Blot. *$p < 0.05$ using Kruskal-Wallis test. (G) Evaluation of NF-kB by ELISA kit. (I) Caspase-1 activation. *$p < 0.05$ using One-way ANOVA with Dunnett's multiple comparison test against Untreated condition. (J) Assessment of IL-1β and (H) TNF-α, (K) IL-6 levels by ELISA kit in the N9 extracts that were co-cultured with treated cortical neurons. **$p < 0.01$ ***$p < 0.001$ One-way ANOVA with Dunnett's multiple comparison test. Results are expressed with the mean ± SEM derived from at least three independent experiments.

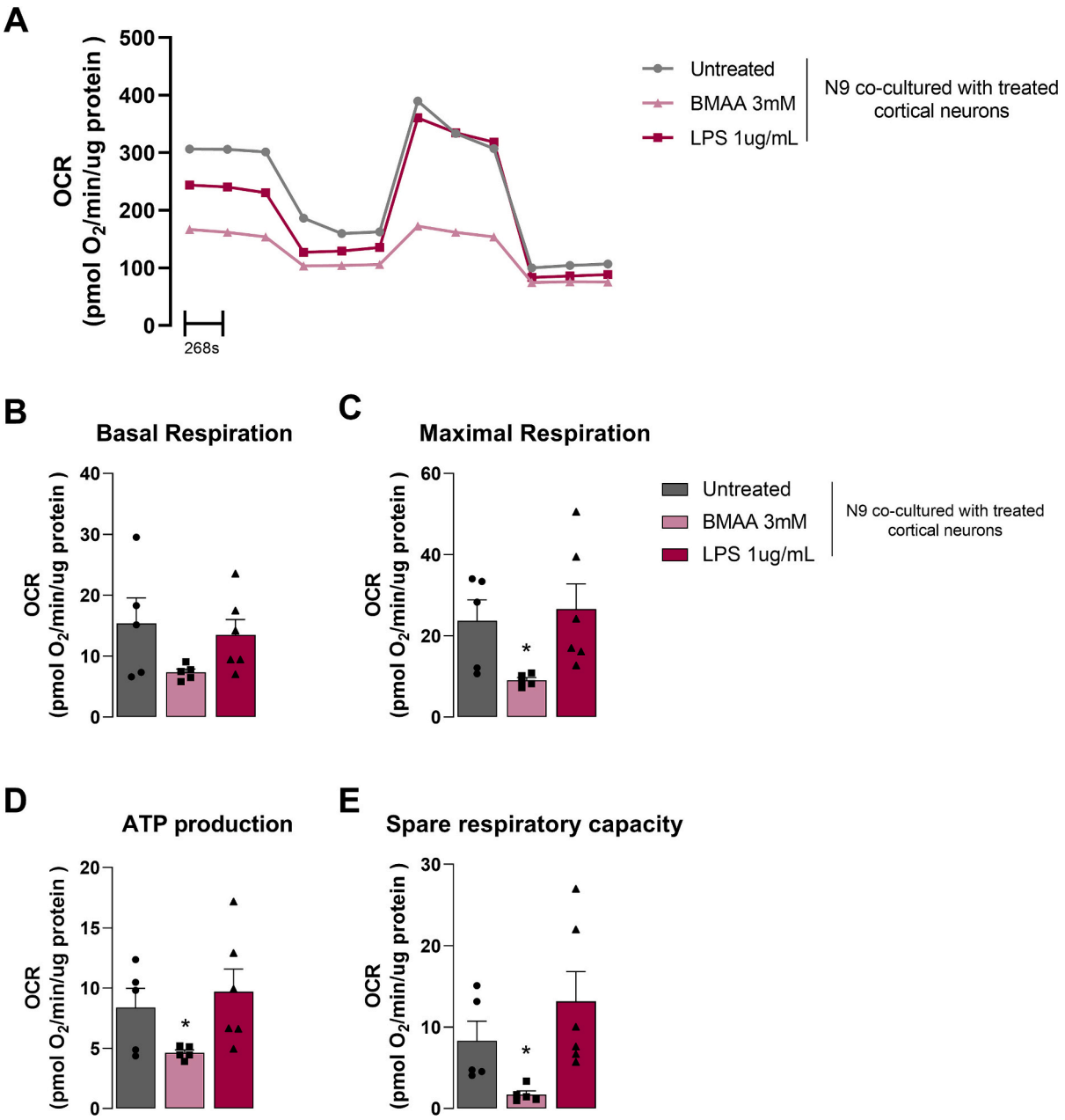

Fig. 3. BMAA-treated cortical neurons led to mitochondrial dysfunction in N9 microglial cell line when co-cultured for 24 h. (A) OCR representative curve using Seahorse Apparatus. (B) Histograms of Basal O_2 consumption; (C) Maximal O_2 consumption; (D) ATP production; (E) Spare respiratory capacity of N9 microglial cell line co-cultured with cortical neurons that were previously treated with 3 mM BMAA and 1 μg/mL LPS in the presence of 1 μM oligomycin, 2 μM FCCP and 2 μM antimycin 2 μM plus rotenone. *$p < 0.05$ Unpaired *t*-test comparison test between Untreated vs BMAA conditions. Results are expressed with the mean ± SEM derived from at least five independent experiments.

[3]. We hypothesize that microglial activation in neurodegenerative disorders and the correspondent microglial-dependent inflammation is dependent on neuronal signaling. Indeed, N9 microglial cells were not affected by BMAA (Fig. 1), but LPS was able to activate these cells and induce the release of proinflammatory cytokines (Fig. 1M, O-P). Interestingly, N9 activation by LPS was not due to NLRP3 inflammasome activation as was previously demonstrated in cortical neurons [38]. Previously we showed that BMAA targeted the mitochondria and induced mitochondrial network fragmentation with the exposure of cardiolipin, a well-known DAMP in pure neuronal cultures [19]. Under such scenario NLRP3 inflammasome is activated and pro-inflammatory cytokines are produced [19]. Herein, we observed that BMAA neither LPS, a recognized microglia activator, induce mitochondrial dysfunction (Fig. S1) in N9 cells. These results indicate that the activation of microglial cells by LPS is independent of both mitochondria and NF-kB (Fig. S1 and Fig. 1L).

3.2. BMAA activates microglia through neuronal innate immunity signaling

To prove our premise, we performed co-cultures with neurons pre-treated with BMAA for 24 h, co-cultured with N9 cell line for an additional 24 h. We observed that the presence of neurons treated with BMAA induced N9 mitochondrial dysfunction (Fig. 2), namely a decrease in ATP production (Fig. 3D). Microglial mitochondrial impairment induced innate immune activation through NF-kB and

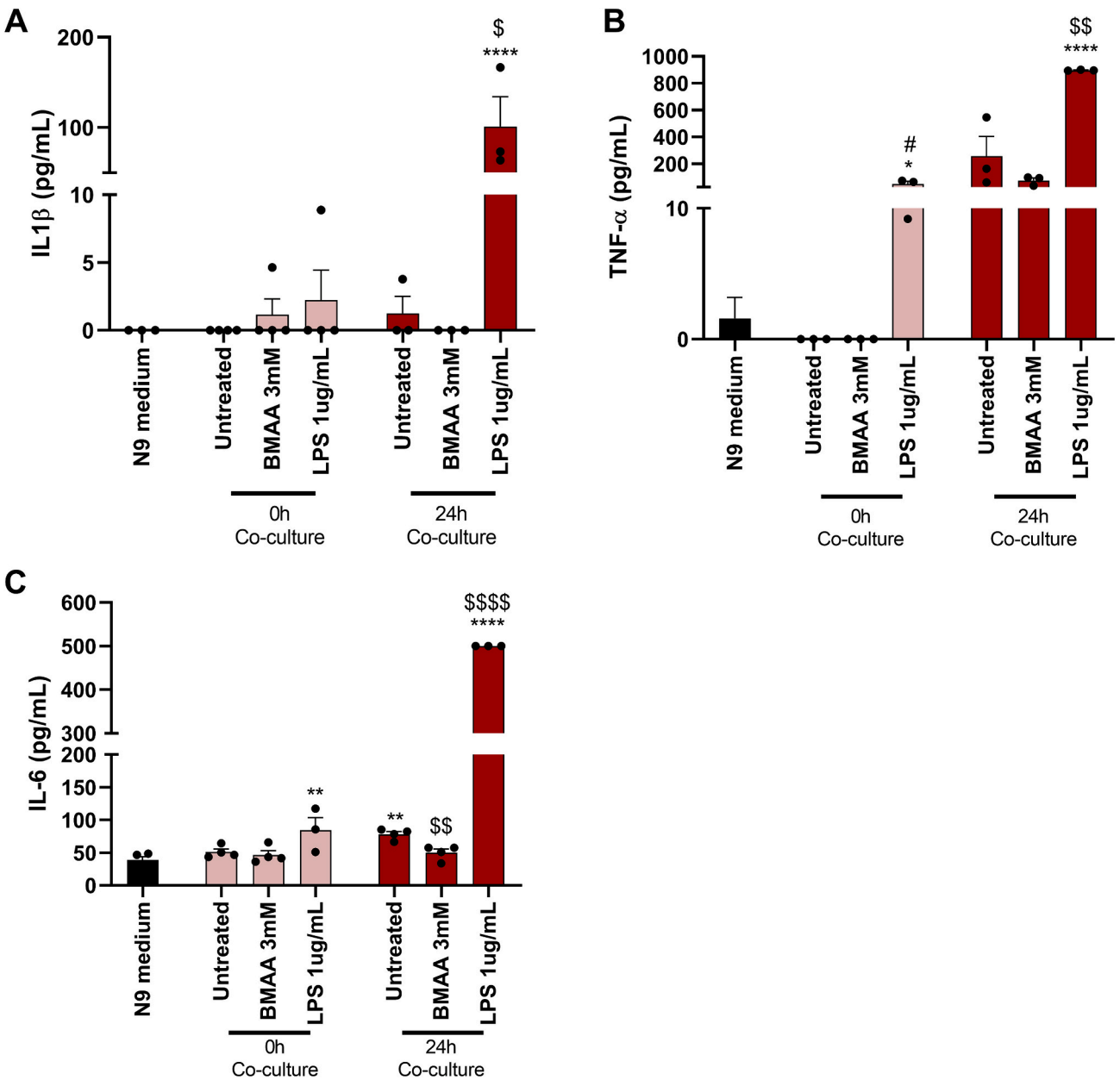

Fig. 4. Increased inflammatory profile in the co-culture medium after 24 h. (A) Assessment of IL-1β, (B) TNF-α and (C) IL-6 levels by ELISA kit in the N9 cell medium (0 h co-culture) and after 24 h of co-culture with treated cortical neurons (24 h of co-culture). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ One-way ANOVA with Dunnett's multiple comparison test (N9 medium condition vs all conditions), #$p < 0.05$ One-way ANOVA with Dunnett's multiple comparison test (Untreated 0 h of co-culture condition vs 0 h co-culture conditions), \$$p < 0.05$, \$\$$p < 0.01$ One-way ANOVA with Dunnett's multiple comparison test (Untreated 24 h of co-culture condition vs 24 h of co-culture conditions). Results are expressed with the mean ± SEM derived from at least three independent experiments.

NLRP3 pathway (Fig. 2B-G). Although we did not see NF-kB activation at 24 h, we do see an increase in pro-IL1β levels (Fig. 2F) as well as an increase in caspase1 activation in N9 cell (Fig. 2I). Neurons may signal microglia cells by releasing small amounts of pro-inflammatory cytokines. Nevertheless, we did not observe alterations in the levels of IL-1β (Fig. 4A), TNF-α (Fig. 4B) or IL-6 (Fig. 4C) released after 24 h of co-culture with BMAA. LPS differentially induced the release of TNF-α and IL-6 from neurons at 0 h of co-culture and at 24 h (Fig. 4B, C).

3.3. Transfer of neuronal mitochondria and protein aggregates activate microglial cells and rescue neurons

Our previous work [19] has shown that BMAA leads to neuronal mitochondrial dysfunction and innate immunity activation, demonstrating that neurons can mount inflammatory responses, a function that is usually associated with microglial cells. Here, we observed that microglial cells became activated after neuronal deficient mitochondria transfer to microglia (Fig. 5A). Given that neuron-microglia communication can occur through the transfer of protein aggregates, such as amyloid-beta (Aβ) peptides or α-synuclein (αSyn), we measured protein levels in the medium of co-cultures. After 24 and 48 h, we observed an increase in Aβ (Fig. 5B) and αSyn (Fig. 5C) in the medium, suggesting that neuron-microglia signaling may be due to the transfer of toxic protein aggregates. It has been demonstrated that BMAA induces mitochondrial dysfunction through the buildup of Aβ [19] or αSyn oligomers [16]. Neurons send dysfunctional mitochondria and protein aggregates to microglial cells, reducing their burden. In co-cultures, neurons do not fully activate NF-KB and NLRP3 dependent innate immunity (Fig. 6A-G), contrary to previous observations in pure neuronal cultures [19]. A reduction in neuronal innate immune activation was observed, as

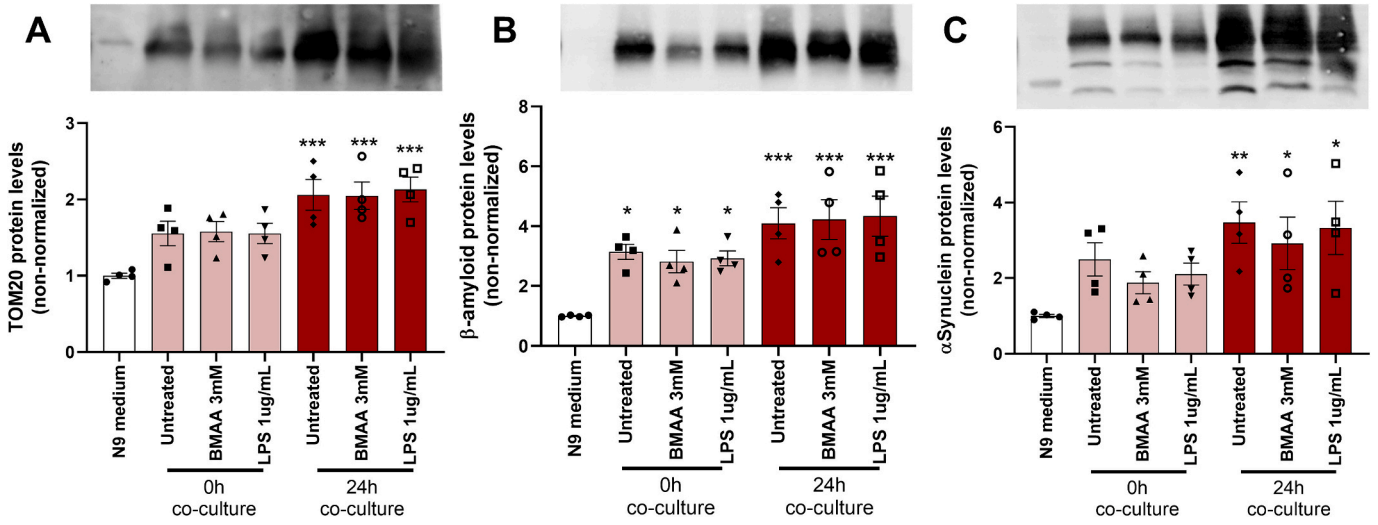

Fig. 5. Neuron-microglia communication is due to transfer of mitochondria and protein aggregates. (A) Representative immunoblot and densitometric analysis of TOM20, (B) Aβ and (C) αSyn protein levels in the medium of co-cultures. *p < 0.05, **p < 0.01, ***p < 0.001 One-way ANOVA with Kruskal-Wallis's multiple comparisons test (N9 medium vs all conditions). Results are expressed with the mean ± SEM derived from at least four independent experiments.

increased levels of TLR4 (Fig. 6C), increased levels of pro-IL1β (Fig. 6F), caspase 1 activation (Fig. 6I), and higher increases in IL-1β production (Fig. 6J) were not observed, as previously shown [19]. Our data clearly show that depending on the trigger, neurons may be the first sensing cells. By activating the microglial immune response, neurons can resist for longer periods and also signal microglia for defense purposes.

4. Discussion

It is well accepted that microgliosis is a typical histopathological hallmark of age-related neurodegenerative disorders, like AD and PD [39]. Growing evidence including the studies showing reactive microglia in the hippocampus and in the *Substantia Nigra* (SN) of AD and PD patients [40–42], indicates that chronic inflammation can contribute to the progressive loss of neurons in specific brain areas that are associated with the disease. Additionally, increased expression of inflammatory mediators in affected brain areas are also associated with disease progression [43,44]. Our work shows that microglia activation is mediated by neuronal signaling. Recent data suggests that there may be an association between neuroinflammation, mitochondrial dysfunction, and impaired proteostasis [38]. Mitochondrial dysfunction, associated with an increase in reactive oxygen species (ROS), a decrease in mitochondrial inner membrane potential, and reduced ATP production, is a well-accepted feature of the neurodegenerative processes of AD and PD [45–48]. To deal with the accumulation of defective mitochondria and their harmful consequences, cells activate fragmentation and elimination through mitophagy [49]. Additionally, mitochondrial and protein aggregates may transfer between cells [50,51]. However, prolonged mitochondrial fragmentation can expose DAMPs that will activate innate immune responses [49]. Previously we showed that a bacterial metabolite, BMAA, targeted the mitochondria and induced mitochondrial network fragmentation with the exposure of cardiolipin, a well-known DAMP in pure cortical neuronal cultures [19] and in midbrain cultures [16]. Under such scenario NLRP3 inflammasome is activated and pro-inflammatory cytokines are produced [16,19]. Herein, we demonstrate that neurons can activate microglia while simultaneously reducing the burden of dysfunctional mitochondria, resulting in a decrease in neuronal innate immunity.

Recent evidence shows that microbial dysbiosis and altered bacterial metabolite production are related to sporadic body-first AD and PD [52,53]. Alterations in the gut microbiome and bacterial metabolite production may be closely interconnected with neuronal mitochondrial function [16,54]. Neuroinflammation associated with mitochondrial dysfunction is currently one of the distinctive hallmarks of neurodegenerative diseases [45]. As mitochondria have a proteobacterial lineage, they are susceptible to microbial byproducts, such as BMAA, a microbial toxin that can be misincorporated into proteins, leading to endoplasmic reticulum stress, redox imbalance, and caspase-dependent apoptotic cell death [34,35]. Interestingly, we showed that extracellular alphaproteobacteria acts largely through the modulation of mitochondria activity, and often mitochondria are themselves immunogenic organelles that can trigger protective mechanisms through DAMPs mobilization [55]. Due to this common origin of bacteria and mitochondria, several microbial metabolites are known to target mitochondria and, consequently, neuronal death. Toxins such as pneumolysin [56,57], α-toxin [58] and the vacuolating cytotoxin (VacA) [59] from *Streptococcus pneumoniae*, *Staphylococcus aureus* or *Helicobacter pylori*, respectively, are pore-forming toxins that can alter the calcium influx into cells leading to mitochondrial impairment and apoptosis [60,61]. Regarding BMAA, in vitro studies have shown that BMAA decreased oxidative phosphorylation, altered calcium homeostasis and exacerbated ROS production in NSC-34 motor neurons cell line [62]. Moreover, BMAA was found to impact the viability of the N2a neuronal cell line by interfering with the mitochondrial enzyme succinate dehydrogenase (SD) activity [63]. Our group showed that *Listeria monocytogenes* infection in mice led to gut and systemic inflammation that ultimately resulting in neuronal mitochondria dysfunction [64].

Microglia, which are immunocompetent brain cells, play a crucial role in regulating brain development and function. They shape neuronal circuits, maintain homeostasis, and respond to central nervous system injuries. The interaction between microglia and neurons is essential for a healthy brain, but can be harmful during injury stages due to continuous microglial activation, which can lead to chronic neuroinflammation [65]. While microglia function is associated with the neuroinflammatory response, it has been proven that neurons are also capable of mounting neuroimmune responses against external triggers [11–14].

Our hypothesis is that microglial activation in neurodegenerative diseases and the associated microglial-mediated inflammation are dependent on neuronal signaling.

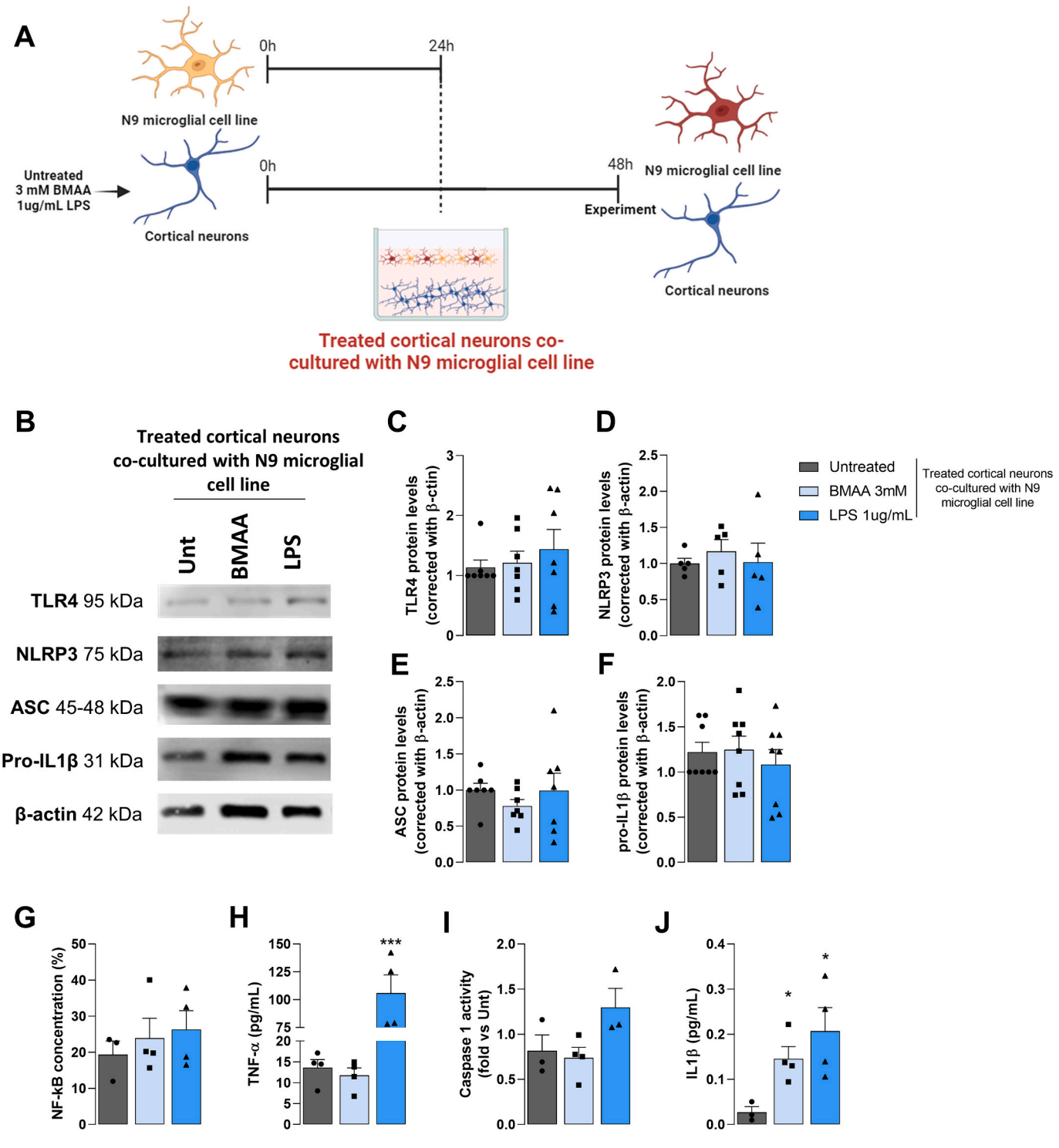

Fig. 6. Cortical neurons treated with BMAA and LPS for 24 h and co-cultured with microglia with additional 24 h do not fully activate NF-KB and NLRP3 dependent innate immunity. (A) Representative scheme of the experiment. (B) Representative immunoblot and densitometric analysis of cortical neurons treated for 24 h and co-cultured with N9 microglial cell line for another 24 h, was performed by the loading of equal amounts of protein corrected with β-actin. (C—F) Evaluation of NLRP3-inflammatory pathway proteins by Western Blot. (G) Evaluation of NF-kB by ELISA kit. (I) Caspase-1 activation. (J) Assessment of IL-1β and (H) TNF-α levels in treated cortical neurons extracts that were co-cultured with N9 cells. # < 0.05 Unpaired t-test Untreated vs BMAA condition. *$p < 0.05$ ***$p < 0.001$ One-way ANOVA with Dunnett's multiple comparison test. Results are expressed with the mean ± SEM derived from at least three independent experiments.

5. Conclusions

The recognition that neurons may be the initial cells to react to harmful stimuli in the brain provides new avenues for investigating the causes of neurodegenerative diseases. By examining the effects of bacterial toxins on the central nervous system and the communication between neurons and microglia, we can consider a potential body-first etiology and explore new possibilities for developing effective therapeutic strategies to slow the progression of neurodegenerative disorders.

Abbreviations

AD	Alzheimer's Disease
ALS-PDC	Amyotrophic lateral sclerosis-parkinsonism dementia complex
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BMAA	β-*N*-methylamino-L-alanine
CHAPS	dimethylammonio]-propanesulfonic acid
CNS	Central nervous system
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
DAMPs	Damage-associated molecular patterns
LPS	Lipopolysaccharides
NLR	Nod-like receptor
OCR	Oxygen consumption rate
PD	Parkinson's Disease
ROS	reactive oxygen species
SN	*Substantia nigra*
TLR	Toll-like receptor

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2024.167167.

Ethics approval and consent to participate

The use of C57BL/6 mice was approved by Direção Geral de Alimentação e Veterinária the Portuguese Entity that regulates animal use in research (reference number: 0421/000/000/2017).

Consent for publication

Not applicable.

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CRediT authorship contribution statement

A.R. Pereira-Santos: Formal analysis, Investigation, Methodology, Writing – original draft. **Emanuel Candeias:** Formal analysis, Investigation, Methodology. **J.D. Magalhães:** Formal analysis, Investigation, Methodology. **Nuno Empadinhas:** Validation, Writing – review & editing. **A. Raquel Esteves:** Investigation, Methodology. **Sandra M. Cardoso:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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