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Microglial activation and the nitric oxide/cGMP/PKG pathway underlie enhanced neuronal vulnerability to mitochondrial dysfunction in experimental multiple sclerosis

Andrea Mancini[a,1], Michela Tantucci[a,1], Petra Mazzocchetti[a], Antonio de Iure[a], Valentina Durante[a], Lara Macchioni[b], Carmela Giampà[c], Alessandra Alvino[c], Lorenzo Gaetani[a], Cinzia Costa[a], Alessandro Tozzi[b,d], Paolo Calabresi[a,d], Massimiliano Di Filippo[a,*]

[a] Clinica Neurologica, Dipartimento di Medicina, Università degli Studi di Perugia, Ospedale Santa Maria della Misericordia, S. Andrea delle Fratte, 06132 Perugia, Italy
[b] Sezione di Fisiologia e Biochimica, Dipartimento di Medicina Sperimentale, Università degli Studi di Perugia, S. Andrea delle Fratte, 06132 Perugia, Italy
[c] Università Cattolica del Sacro Cuore, Istituto di Anatomia Umana e Biologia Cellulare, Largo Francesco Vito 1, 00168 Rome, Italy
[d] IRCCS, Fondazione Santa Lucia, via del Fosso di Fiorano 64, 00143 Rome, Italy

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ABSTRACT

During multiple sclerosis (MS), a close link has been demonstrated to occur between inflammation and neuro-axonal degeneration, leading to the hypothesis that immune mechanisms may promote neurodegeneration, leading to irreversible disease progression. Energy deficits and inflammation-driven mitochondrial dysfunction seem to be involved in this process. In this work we investigated, by the use of striatal electrophysiological field-potential recordings, if the inflammatory process associated with experimental autoimmune encephalomyelitis (EAE) is able to influence neuronal vulnerability to the blockade of mitochondrial complex IV, a crucial component for mitochondrial activity responsible of about 90% of total cellular oxygen consumption. We showed that during the acute relapsing phase of EAE, neuronal susceptibility to mitochondrial complex IV inhibition is markedly enhanced. This detrimental effect was counteracted by the pharmacological inhibition of microglia, of nitric oxide (NO) synthesis and its intracellular pathway (involving *soluble guanylyl cyclase*, sGC, and *protein kinase G*, PKG). The obtained results suggest that mitochondrial complex IV exerts an important role in maintaining neuronal energetic homeostasis during EAE. The pathological processes associated with experimental MS, and in particular the activation of microglia and of the NO pathway, lead to an increased neuronal vulnerability to mitochondrial complex IV inhibition, representing promising pharmacological targets.

1. Introduction

Multiple Sclerosis (MS) is a worldwide-diffused chronic central nervous system (CNS) disorder, affecting young adults and potentially leading to progressive accumulation of neurological disability, with a relevant impact on patient's quality of life and life expectancy (Ontaneda et al., 2017). Inflammatory processes characterize MS pathogenesis since its earliest phases, both in the form of recurrent immune attacks from the periphery to the CNS through the blood-brain barrier and *via* the activation of direct innate immune responses within the CNS itself, *e.g.* microglial cells. Together with inflammatory processes, neuro-axonal degeneration can be detected in the CNS of patients affected by MS since the earliest phases of the disease (Dutta and Trapp, 2007; Trapp and Nave, 2008) and it represents one of the major causes underlying the progressive accumulation of neurological disability (Hauser and Oksenberg, 2006; Trapp and Nave, 2008). In this *scenario*, the identification of the pathogenic link between inflammatory and neurodegenerative processes during MS (Geurts et al., 2009) could help in the development of effective therapies against disability accumulation. During last years, several studies suggested a potential pathogenic role for mitochondrial dysfunction in mediating neuro-axonal degeneration and irreversible disease progression during the course of MS (Calabrese et al., 2015; Campbell et al., 2014; Di Filippo et al., 2010; Witte et al., 2014). Mitochondria are intracellular organelles responsible for the majority of *adenosine triphosphate* (ATP) cellular production, but they also play a role in calcium metabolism, reactive oxygen species (ROS) production and cellular apoptosis (Andreyev et al., 2005; Di Mauro and Schon, 2003; Taylor and Turnbull, 2005).

* Corresponding author at: Clinica Neurologica, Dipartimento di Medicina, Università degli Studi di Perugia, Ospedale S. Maria della Misericordia, 06132 Perugia, Italy.
E-mail address: massimiliano.difilippo@unipg.it (M. Di Filippo).
[1] Equally contributed.

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ATP production mostly depends on the coordinated activity of *ATP-synthase* (complex V) and the mitochondrial *electron transport chain*, an enzymatic complex located within the inner mitochondrial membrane and constituted by four enzymes, *reduced nicotinamide adenine dinucleotide* (NADH) *dehydrogenase* (respiratory complex I), *succinate dehydrogenase* (respiratory complex II), *cytochrome c oxidoreductase* (respiratory complex III) and *cytochrome c oxidase* (COX, respiratory complex IV) (Andreyev et al., 2005; Di Mauro and Schon, 2003; Taylor and Turnbull, 2005). Complex IV is considered a crucial component for mitochondrial activity, since it represents the terminal of the *electron transport chain* and is responsible for approximately 90% of total cellular oxygen consumption (Di Mauro and Schon, 2003). Interestingly, pathological studies showed that complex IV defects during MS are significantly enhanced in brain areas characterized by a dense infiltration of activated microglial cells and macrophages, suggesting a potential key role for soluble inflammatory products in interfering with complex IV activity (Mahad et al., 2008, 2009). A better understanding of the intertwined relation among inflammatory mediators, neuronal energy failure and neuro-axonal degeneration during MS could be helpful in identifying new pharmacological targets for neuro-protective strategies. To address this purpose, we investigated with electrophysiological techniques if the acute CNS inflammatory process accompanying experimental autoimmune encephalomyelitis (EAE) (Amor et al., 2005; Al-Izki et al., 2012) was able to influence neuronal vulnerability to complex IV blockade. Specifically, to measure neuronal viability, we analyzed the amplitude of evoked excitatory field potentials before and after the *in vitro* application of a specific complex IV inhibitor, sodium-azide (NaN_3) (Bennett et al., 1996; Ziabreva et al., 2010), in control conditions and during experimental MS. Experiments were conducted in the nucleus striatum, a brain structure particularly prone to develop changes suggestive of neurodegeneration during MS (Hasan et al., 2009; Henry et al., 2008). We then investigated the potential strategy aimed at counteracting the deleterious synergistic effect between neuro-inflammation and mitochondrial dysfunction in experimental MS.

2. Materials and methods

2.1. Induction of chronic-relapsing EAE

Chronic-relapsing EAE was chosen as experimental model of MS, since it predictably follows a clinical course reminiscent of relapsing–remitting disease with subsequent disability accumulation (Amor et al., 2005). As previously described (Al-Izki et al., 2012; Di Filippo et al., 2013, 2016), six to eight-week-old Biozzi ABH mice (Envigo, Italy) were injected with 1 mg of syngeneic spinal cord homogenate (SCH) in Freund's adjuvant, supplemented with 100 μg of mycobacteria (*Mycobacterium (M.) tuberculosis* H37Ra and *M. butyricum* [8:1]) on day 0 and again on day 7 (Al-Izki et al., 2012; Di Filippo et al., 2013). Animals were monitored and weighed daily, from day 10 post-inoculation (p.i.) onwards, to assess the development of relapsing-remitting paralysis. Clinical signs were scored as follows: 0 = normal; 1 = fully flaccid tail; 2 = impaired righting reflex; 3 = hind limb paresis; 4 = complete hindlimb paresis; 5 = moribund/death (Al-Izki et al., 2012). Clinical signs that were less severe than the indicated grade were scored 0.5 less than the typical score. The initial (acute) phase of EAE usually occurred around 15–18 days p.i. (Al-Izki et al., 2012). During the first episode of neurological deficit, suggestive of CNS inflammation, animals were selected for the experiments. In order to select a homogeneous population of animals for the study, only mice with a significant clinical disability score (at least 3) were selected for the experiments in the EAE group. In a subgroup of mice, minocycline hydrochloride was dissolved in PBS and administered daily by intraperitoneal (i.p.) injection. This treatment was initiated on day 10 post immunization. EAE mice treated with minocycline received 50 mg/kg twice a day for the first 2 days, 50 mg/kg once a day for the next 5 days and 25 mg/kg once a day for the subsequent days until sacrifice (Di Filippo et al., 2016). For all the experimental plan 22 EAE mice and 25 control animals were utilized. All the procedures were conducted in conformity with the European Communities Council Directive of November 1986 (86/609/ECC), in accordance with a protocol approved by the Animal Care and Use Committee at the University of Perugia. All efforts were made to minimize the number of animals used and their suffering.

2.2. Preparation and maintenance of slices for electrophysiological recordings

For striatal recordings, Biozzi ABH and C57Bl/6 mice (Envigo, Italy) were sacrificed and the brain was dissected and immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 2.4 CaCl2, 10 glucose, and 25 NaHCO3, continuously bubbled with 95% O2 and 5% CO2, pH 7.4. Corticostriatal coronal slices (thickness, 240–270 μm) were cut using a vibratome (Leica VT 1200S) and every single slice was then transferred to a recording chamber and submerged in a continuously flowing ACSF (34 °C; 2.5–3 ml/min) bubbled with a 95% O2–5% CO2 gas mixture.

2.3. Electrophysiology

Extracellular recordings were obtained by using microelectrodes pulled from borosilicate glass pipettes backfilled with 2 M NaCl (15–20 MΩ), connected to an Axoclamp 2B amplifier (Molecular Devices, USA). A stimulating electrode was located in the white matter between the cortex and the striatum to activate cortico-striatal fibers. The recording electrodes were invariably placed within the striatum. An excitatory post-synaptic field potential (fEPSP) was evoked every 10 s by means of a bipolar electrode connected to a stimulator unit (Grass Telefactor, USA). The fEPSP amplitude was defined as the average of the amplitude from the peak of the early positivity to the peak negativity, and the amplitude from the peak negativity to peak of the late positivity (Centonze et al., 2006; Costa et al., 2008). Quantitative data were expressed as a percentage of the field potential amplitudes in respect to the relative baseline control amplitude values, the latter representing the mean of responses recorded during a stable period (15 to 20 min).

2.4. Immunohistochemistry

Tissue processing. Animals of the three experimental groups (control Biozzi mice, EAE mice, and EAE mice treated with minocycline) were transcardially perfused under deep anesthesia with chloral hydrate with 60 ml of saline solution containing 0.05 ml heparin, followed by 200 ml of 4% paraformaldehyde in saline solution. Brains were removed and post-fixed overnight at +4 °C, cryoprotected in 20% sucrose and 10% glycerol in 0.1 M phosphate buffer (PB) with sodium azide 0.02% for 48 h at 4 °C. Subsequently, they were sectioned frozen on a sliding microtome at 40 μm thickness. For the immune-histochemical detection of microglia activation, a single label immunofluorescence with rat anti-mouse-CD68 antibody was employed. Briefly, sections were incubated with rat anti-mouse CD68 (Immunological Science, Rome Italy) at a 1:500 concentration in a 0.1 M phosphate buffered (PB) solution containing Triton X 0.3% and 0.02 sodium azide for 72 h at +4 °C. Sections were then rinsed three times for 15 min at room temperature and subsequently incubated with donkey anti-rat cy3-conjugated secondary antibody (Jackson

Immunoresearch, West Grove, PA, USA) for 2 h at room temperature. Sections were then rinsed two times and then TOPRO counterstained. Images were acquired by using a CLSM (Zeiss LSM 510) microscope, under non-saturating exposure conditions and using the same acquisition settings for all samples. The conditions, in term of gain and laser power, were selected at levels that allowed optimal visualization of the fluorophore used as secondary antibody and standardized using sections from control mice. These settings were then applied as standards for all subsequent images. By a 40× objective, *Z*-stacks images of striatum from coronal sections were collected using computer-controlled microstepper stage of the confocal microscope. Stacks of images were, then, combined into a single two-dimensional (2D) projection image, exported in TIF file format using NIH ImageJ software.

2.5. Mitochondrial crude fraction preparation

Striatal tissue obtained from control mice and EAE mice were excised and homogenized in 0.32 M sucrose and 2 mM Hepes, pH 7.4 (S/H buffer). The omogenate was centrifuged at 1500*g* for 10 min to remove cell nuclei, unbroken cells, and debris. The supernatant was centrifuged at 8000*g* for 20 min; the crude mitochondrial fraction was resuspended in an appropriate amount of S/H buffer and treated with NP-40 for at least 30 min at 4 °C. The mitochondrial proteins concentration was determined by using the Bradford assay with bovine serum albumin as standard.

2.6. Complex IV activity evaluation

Complex IV activity was measured in 20 µg of crude mitochondria fraction by following the absorbance decrease (550 nm) of reduced cyt c (25 µM, reduced by ascorbic acid), in the reaction buffer (30 mM KH2PO4 and 1 mM EDTA, pH 7.2) with or without NaN_3, which was added after 30 s at final concentration of 1 mM. The enzymatic activity was expressed as nmol of oxidized cyt c/min/mg protein. Data are presented as mean ± standard deviation (SD).

2.7. Drugs

Drugs were bath applied by switching the flowing solution to ACSF containing known concentrations of each compound. Total replacement of the medium in the chamber occurred within 1 min. As specific mitochondrial complex IV inhibitor, we utilized sodium azide (NaN_3) (Bennett et al., 1996; Ziabreva et al., 2010). This compound was demonstrated to induce a mitochondrial complex IV inhibition similar to other compounds, such as potassium cyanide (Ziabreva et al., 2010), without inhibiting the activities of complex I and III of the mitochondrial respiratory chain (Bennett et al., 1996). The chosen dose (1 mM) and the time of exposure (30–35 min) had been already used for mitochondrial complex IV inhibition (Zambonin et al., 2010). Experiments with pro-inflammatory cytokines were performed with a pre-incubation of brain slices of approximately 1 h before the beginning of the electrophysiological recordings. Freund's incomplete adjuvant and sodium azide were purchased from Sigma-Aldrich (Milan, Italy); desiccated mycobacteria, *M. tuberculosis H37Ra* and *M. butyricum*, were obtained from Difco laboratories (Detroit, U.S.A.); minocycline hydrochloride was obtained by Tocris (Bristol, UK); IL-1β, IL-17, IFN-γ, TNF-α were obtained from R&D Systems (Minneapolis, U.S.A.); 7-NINA, ODQ, Rp-8Br-PET-cGMP, Snap, YC-1, 8BrcGMP were obtained from Tocris Biosciences (Bristol UK).

2.8. Statistical analysis

Values given in the figure and text are mean ± standard error (SE) with the exception of the data on mitochondrial complex IV activity in which standard deviation (SD) was provided. Analysis of variance (ANOVA) and Bonferroni's *post hoc* test were used for statistical analysis. The significance level was established at $p \leq .05$.

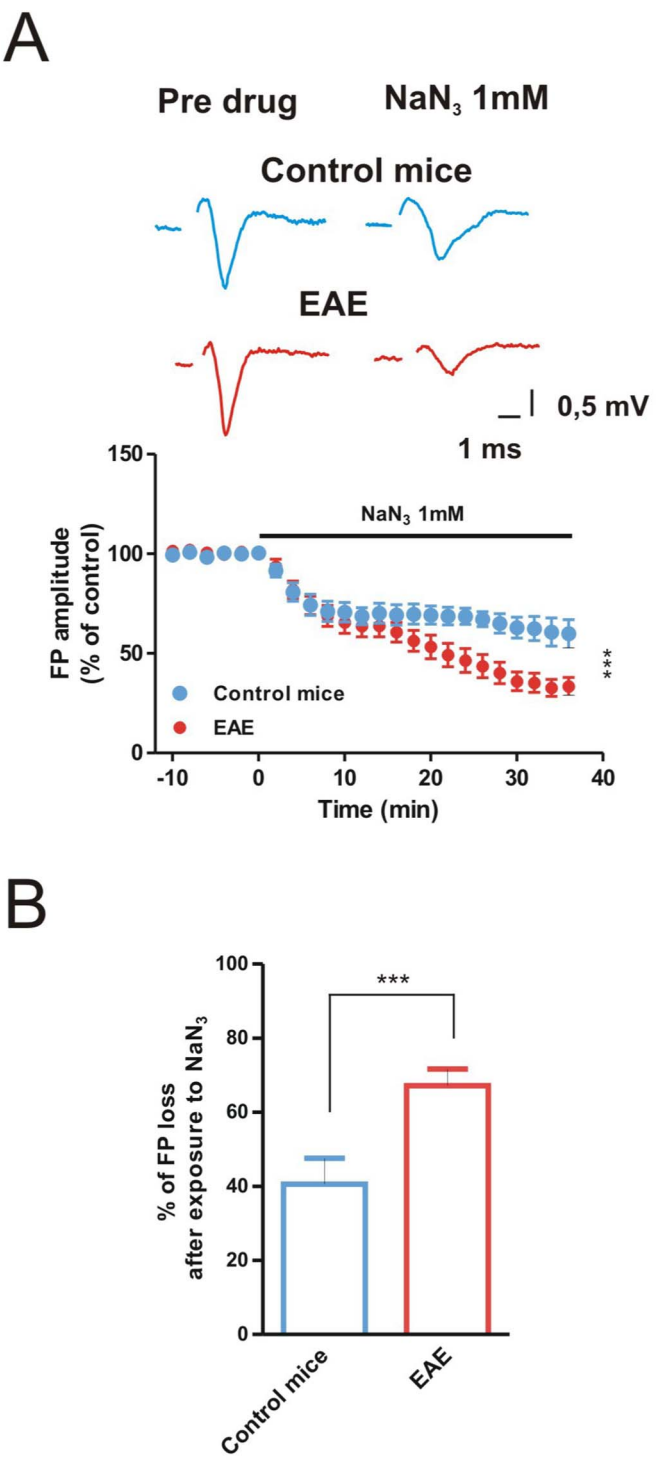

Fig. 1. Neuronal toxicity induced by mitochondrial respiratory chain complex IV inhibition is markedly enhanced during experimental MS. (A) Time-courses of striatal field potential (FP) amplitude in the presence of 1 mM sodium azide (NaN_3) in control Biozzi ABH mice (blue filled circles) and in mice affected by the acute clinical phase of EAE (red filled circles); in the upper panel, representative FP traces recorded before and 35 min after the application of 1 mM NaN_3 in control conditions and in EAE mice. (B) The histograms show the percentage of reduction of the striatal FP amplitude, 35 min after 1 mM NaN_3 application, in slices from control Biozzi ABH mice (blue bar) and EAE mice (red bar). ***$p < .001$.

3. Results

3.1. Neuronal toxicity induced by mitochondrial respiratory chain complex IV inhibition is markedly enhanced during experimental MS

In order to test the effect of mitochondrial complex IV inhibition on neuronal viability in the nucleus striatum, striatal excitatory post-synaptic field potentials (fEPSPs) were recorded after the activation of glutamatergic cortico-striatal inputs, for 10 min to obtain a stable baseline and then for further 40 min in the presence of the mitochondrial complex IV inhibitor sodium azide (NaN_3) (Bennett et al., 1996; Ziabreva et al., 2010). Interestingly, the *in vitro* application of 1 mM NaN_3 for 30–35 min was able to induce a slow, progressive and irreversible reduction of the fEPSPs amplitude in control Biozzi ABH mice (Fig. 1A), reflecting irreversible neuronal dysfunction, since a prolonged *wash-out* from the mitochondrial toxin was not able to rescue physiological neuronal activity (Costa et al., 2008). In particular, 1 mM NaN_3 was able to reduce fEPSPs amplitude by 40.55% (± 7.05) in slices obtained from control Biozzi ABH mices ($n = 10$, $p < .001$) (Fig. 1A). A higher dose of NaN_3 was also tested (3 mM) but not considered for further experiments because it caused a rapid and almost complete loss of the fEPSPs amplitude (reduction of 90.5% ± 2.1 in EAE mice after 15 min of exposure, data not shown). The striatal toxic effect induced by 1 mM NaN_3 was then tested in slices from mice affected by the acute clinical phase of EAE. In this group, after 30–35 min of exposure to 1 mM NaN_3, we observed a reduction of fEPSPs amplitude of 67.19% (± 4.43) with respect to the baseline ($n = 14$, $p < .001$) (Fig. 1A). The analysis of these results showed that in mice evaluated during acute clinical phase of EAE the detrimental effect of the mitochondrial complex IV inhibitor was significantly enhanced (Fig. 1B), with a proportional 65.7% increase in the final absolute reduction of fEPSPs amplitude with respect to control conditions ($p < .001$, $F = 3.18$). These findings suggest that the pathologic process associated with EAE is able to make the striatum particularly vulnerable to the dysfunction of this mitochondrial complex.

3.2. Sodium azide (NaN_3) induces a significant mitochondrial complex IV inhibition both in control conditions and during EAE

In order to investigate the cause of the enhanced vulnerability of striatal neurons to NaN_3 during EAE, mitochondrial complex IV enzymatic activity was measured in striatal tissue obtained from controls ($n = 4$) and EAE mice ($n = 4$) with or without the application of 1 mM NaN_3 (Fig. 2). As expected, the application of 1 mM NaN_3 was able to induce a significant inhibition of COX activity both in controls and EAE mice (control mice 313.16 (± 27.68) nmol/min/mg protein, control mice plus NaN_3 117.98 (± 38.90) nmol/min/mg protein, $p \leq .001$; EAE 352.63 (± 32.73) nmol/min/mg protein, EAE plus NaN_3 114.91 (± 30.94) nmol/min/mg protein, $p \leq .001$). No significant differences were found in the basal mitochondrial complex IV activity (without exposure to NaN_3), suggesting the absence of an intrinsic complex IV dysfunction in EAE mice compared with controls (control mice 313.16 (± 27.68) nmol/min/mg protein, EAE 352.63 (± 32.73) nmol/min/mg protein, $p \geq .05$) (Fig. 2).

3.3. The increased neuronal vulnerability to mitochondrial complex IV inhibition during EAE depends on the nitric oxide/cGMP/PKG pathway

Nitric oxide (NO) seems to play a crucial role both in mitochondrial dysfunction and acute inflammatory processes (Giovannoni et al., 1998; Smith and Lassmann, 2002). Moreover, NO is a well-known direct complex IV inhibitor, since it has been demonstrated that low NO concentrations can compete with molecular oxygen (O2) in binding the functional site of COX, with a subsequent reversible and specific complex IV inhibition (Brown, 2001; Brown and Cooper, 1994; Brown et al., 1995; Cooper, 2003). Thus, we investigated the effect of specific

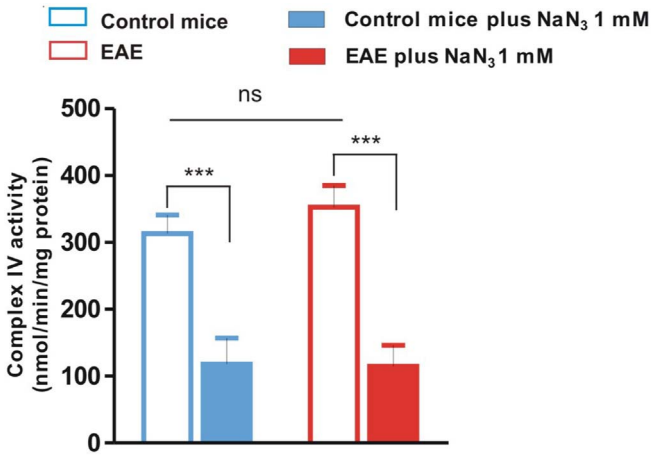

Fig. 2. Histograms show mitochondrial complex IV activity in control and EAE mice. Mitochondrial complex IV activity was determined by measuring the rate of oxidation of reduced cytochrome *c* at 550 nm. Mitochondrial fractions (20 µg of protein) were tested with or without exposure to 1 mM NaN_3. Data are expressed as nmol oxidized cytochrome *c*/min/mg protein and are presented as mean ± SD. ***$p \leq .001$, ns: not significant.

pharmacological inhibitors of the NO-activated pathway (Arcangeli et al., 2013; Costa et al., 2011) on the progressive loss of fEPSP amplitude induced by NaN_3 during the acute clinical phase of EAE. We applied the NO synthase (NOS) inhibitor *7-nitroindazole* (7-NINA) together with 1 mM NaN_3 on striatal brain slices and recorded the fEPSP amplitude for 30–35 min. Interestingly, in EAE mice, the co-exposure to 10 µM 7-NINA and 1 mM NaN_3 reduced the fEPSP amplitude of 32.66% (± 6.41) with respect to baseline fEPSP amplitude ($n = 7$, $p < .001$) (Fig. 3A). This detrimental effect on striatal synaptic transmission was significantly reduced with respect to the previously showed fEPSP amplitude loss of 67.19% (± 4.43) induced by the isolated application of 1 mM NaN_3 in EAE mice ($p < .001$) (Fig. 3A). The protective effect exerted by the inhibition of NOS on the neuronal susceptibility to complex IV dysfunction suggests a potential involvement of NO synthesis in mitochondrial dysfunction during EAE. To further characterize this potential role of NO, we investigated how the modulation of the other enzymes involved in the NO intracellular transduction pathway could influence NaN_3 toxicity. NO is known to activate *soluble guanylyl cyclase* (sGC), with a subsequent increase of intracellular levels of *cyclic guanosine monophosphate* (cGMP), that in turn activates *protein kinase G* (PKG), able to exerts several intra-cellular effects (Martínez-Ruiz et al., 2011). Thus, we tested the effect of ODQ (*1H-1,2,4 oxadiazolo-4,3-a quinoxalin-1-one*), a specific sGC inhibitor, and of *Rp-8Br-PET-cGMP*, an inhibitor of PKG, on the field potential loss induced by NaN_3 during EAE. Interestingly, we found that in EAE mice the application of 10 µM ODQ together with 1 mM NaN_3 reduced the fEPSP by 14.23% (± 4.54) with respect to the baseline ($n = 9$, $p < .001$) (Fig. 3B), while the co-exposure to 1 µM *Rp-8Br-PET-cGMP* and 1 mM NaN_3 reduced the fEPSP amplitude of 35.11% (± 3.23) with respect to the baseline fEPSP amplitude ($n = 5$, $p < .001$) (Fig. 3C). The reduction of the fEPSP amplitude produced combining the sGC or PKG inhibitors with NaN_3, was significantly lower compared to the effect of NaN_3 alone ($p < .001$), confirming the hypothesis of a possible role exerted by the NO/cGMP/PKG pathway in the worsening effect induced by the pathologic processes associated with EAE on mitochondrial complex IV dysfunction (Fig. 3D). To assess whether these pharmacological agents could cause, *per se*, changes in the fEPSP amplitude, we exposed slices of EAE mice to 7-NINA 10 µM ($n = 4$), ODQ 10 µM ($n = 5$) or *Rp-8Br-PET-cGMP* 1 µM ($n = 5$) without NaN_3, showing no significant alterations of the fEPSP amplitude ($p > .05$). Thus, the ameliorating role exerted by NO pathway modulators on mitochondrial complex IV dysfunction was not due to a generic effect of the compounds on the striatal neuronal synaptic transmission.

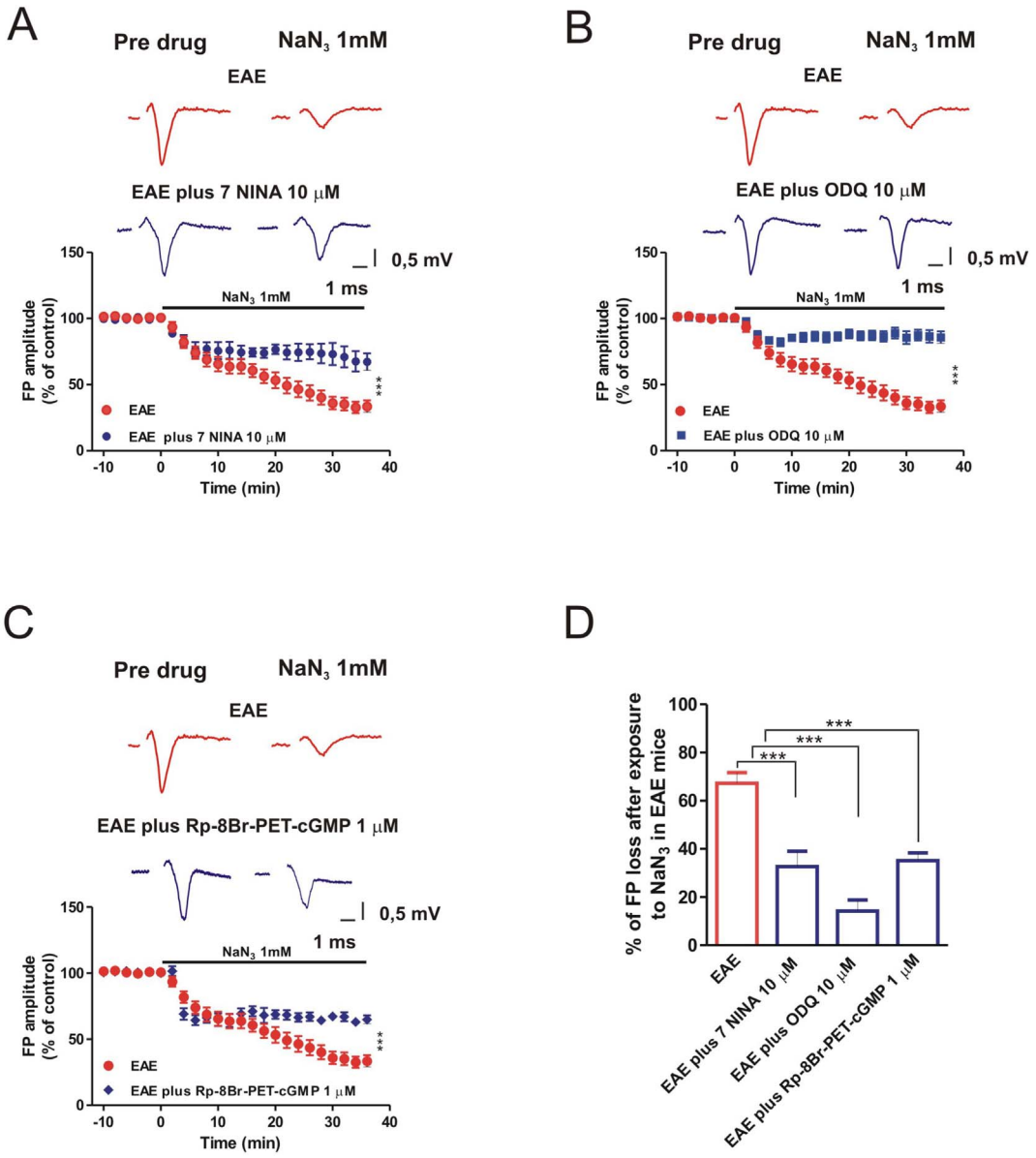

Fig. 3. The inhibition of NO synthesis and of NO-activated intracellular pathway reduces neuronal vulnerability to mitochondrial complex IV inhibition during EAE. Time-courses of striatal field potential (FP) amplitude in the presence of 1 mM NaN_3 and either 10 μM 7-NINA (A), 10 μM ODQ (B) or 1 μM Rp-8Br-PET-cGMP (C) in mice affected by the acute clinical phase of EAE. In the upper panels, representative FP traces recorded before and after the application of 1 mM NaN_3 in EAE mice with and without the co-application of 10 μM 7-NINA (A), 10 μM ODQ (B) or 1 μM Rp-8Br-PET-cGMP (C). (D) The histograms show the percentage of reduction of the striatal FP amplitude, 35 min after 1 mM NaN_3 application alone and with the co-application of 10 μM 7-NINA, 10 μM ODQ or 1 μM Rp-8Br-PET-cGMP in slices from EAE mice. ***p < .001.

3.4. Exposure to specific inhibitors of NO-activated pathway does not protect from neuronal toxicity induced by mitochondrial respiratory chain complex IV inhibition in control conditions

In order to investigate if modulators of the NO pathway counteracted the NaN_3-induced toxicity also in control conditions we analyzed the effects of the different pharmacological compounds previously described (NOS, sGC and PKG inhibitors) in control mice. The exposure of striatal brain slices obtained from control mice to 1 mM NaN_3 for 30–35 min induced a progressive and irreversible reduction of the field potential amplitude, of about 32.84% (± 4.98) with respect to baseline (n = 14) (Fig. 4A). The co-application of 10 μM of the NOS inhibitor, 7-NINA, together with 1 mM NaN_3 did not modify in control mice the progressive fEPSP amplitude reduction with respect to NaN_3 alone (n = 6, p > .05) (Fig. 4A). No significant differences in neuronal susceptibility to 1 mM NaN_3-toxicity were shown also when the toxin was applied together with 10 μM ODQ (n = 5, p > .05, (Fig. 4B)) or 1 μM *Rp-8Br-PET-cGMP* (n = 4, p > .05, (Fig. 4C)) in control mice. These results suggest that the neuro-protective effect exerted by the negative modulators of the NO-pathway on mitochondrial complex IV dysfunction could be detected only during the pathologic process associated with EAE, probably due to the inflammation-related enhanced production of NO and the subsequent sustained activation of the sGC/PKG pathway, without influencing neuronal susceptibility to complex IV inhibition in control conditions (Fig. 4D).

3.5. Specific activators of the NO-activated pathway do not enhance neuronal toxicity induced by mitochondrial respiratory chain complex IV inhibition in control conditions

The results described above suggest that neuro-inflammation is able to enhance neuronal toxicity induced by mitochondrial complex IV inhibition and that a potentially crucial role in this process could be attributable to NO and its pathway, involving sGC and PKG. To further

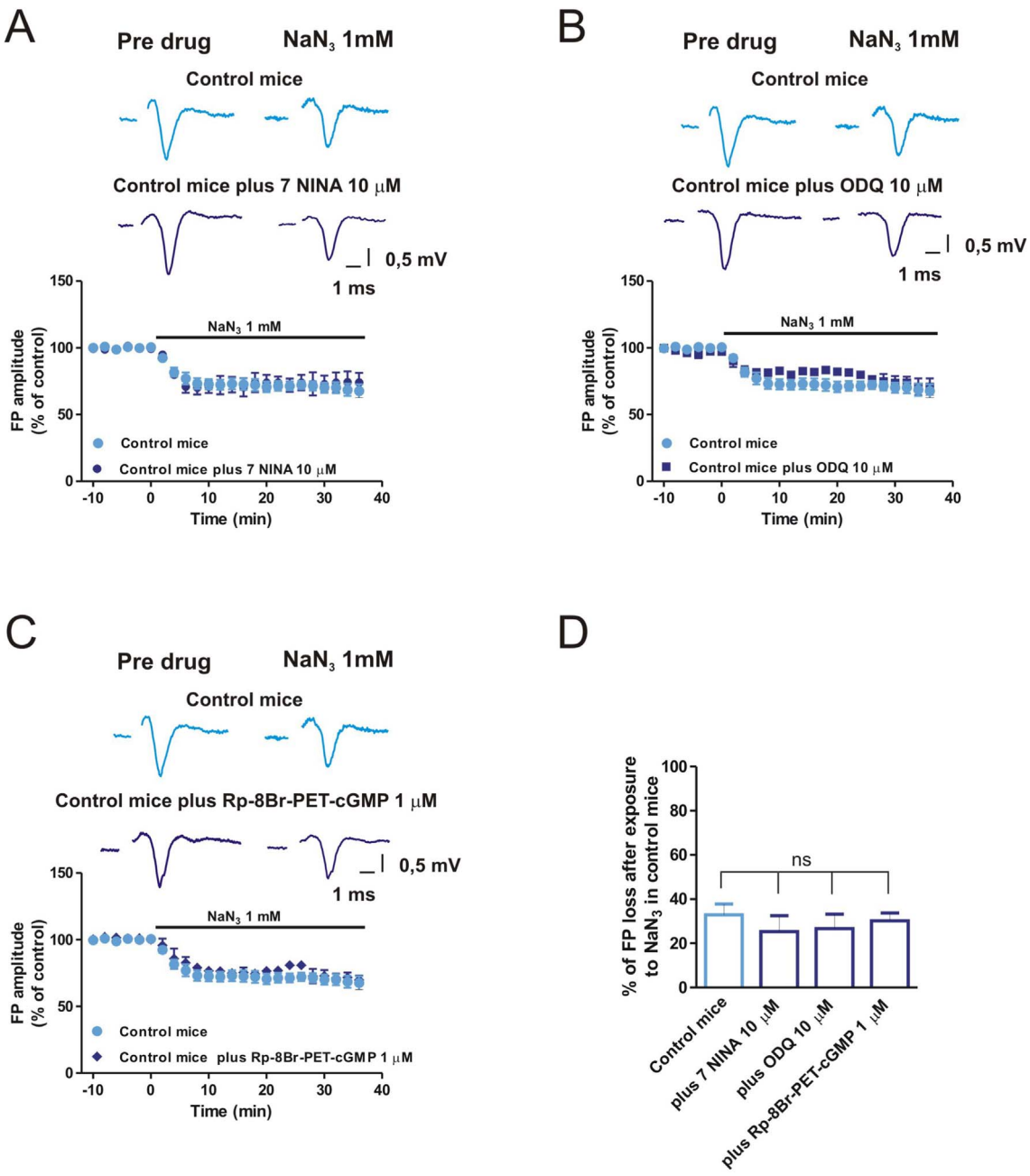

Fig. 4. The application of inhibitors of NO synthesis and of NO-activated intracellular pathway does not influence neuronal vulnerability to mitochondrial complex IV inhibition in control condition. Time-courses of striatal field potential (FP) amplitude in the presence of 1 mM NaN_3 plus either 10 μM 7-NINA (A), 10 μM ODQ (B) or 1 μM Rp-8Br-PET-cGMP (C) in control mice, with representative traces in the upper panels. (D) The histograms show the percentage of reduction of the striatal FP amplitude, 35 min after 1 mM NaN_3 application alone and with the co-application of 10 μM 7-NINA, 10 μM ODQ or 1 μM Rp-8Br-PET-cGMP in slices from control mice. ns: not significant.

investigate this hypothesis, we tested if the isolated activation of the NO pathway, in control conditions without inflammatory processes of the CNS, is able to mimic the worsening effect of EAE on mitochondrial complex IV dysfunction. We then utilized specific pharmacological activators of the NO pathway, in order to demonstrate if the exposure to these agents is able, *per se*, to enhance NaN_3-induced neuronal toxicity in the absence of neuroinflammation. In particular, we utilized SNAP (*S-nitroso-N-acetylpenicilla*mine*)*, a NO donor, YC-1 (*3-5-hydroxymethyl-2-furyl-1-benzyl-indazole*), an activator of sGC, and *8Br-cGMP*, a PKG activator (Arcangeli et al., 2013; Costa et al., 2011). The co-exposure of brain slices obtained from control C57Bl/6 mice to 100 μM SNAP and 1 mM NaN_3 induced a similar fEPSP amplitude loss, after 30–35 min of exposure, with respect to that induced by 1 mM NaN_3 alone, with a non-statistically significant difference ($n = 5$, $p > .05$) (Fig. 5A). Similarly, the co-application of 1 μM YC-1, an activator of sGC, or 1 μM *8Br-cGMP*, a PKG activator, together with 1 mM NaN_3 was not able to significantly enhance the absolute fEPSP amplitude loss, previously described with the isolated exposure to 1 mM NaN_3 (respectively $n = 6$, $p > .05$, Fig. 5B; $n = 7$, $p > .05$, Fig. 5C). These findings suggest that the isolated and transient *in vitro* pharmacologic release of NO and the transient activation of its pathway are not sufficient to mimic the worsening effect of EAE on mitochondrial complex IV activity (Fig. 5D), suggesting that the molecular pathways leading to this detrimental effect probably need either the concomitant presence of other inflammatory-related mediators or a sustained and prolonged exposure to high NO concentrations and sGC/PKG activation.

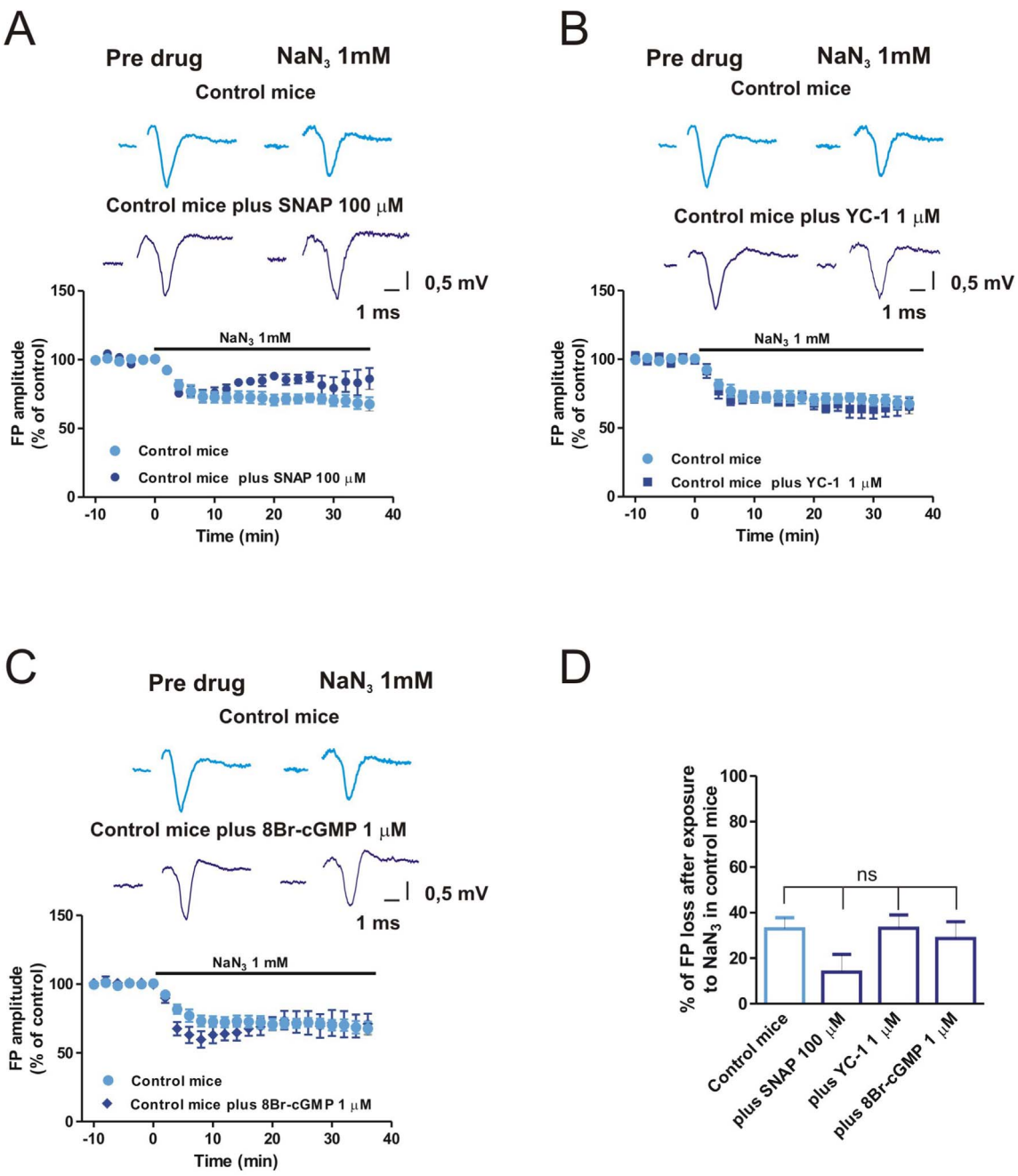

Fig. 5. Pharmacological activation of the NO intracellular pathway does not enhance sodium-azide-induced neuronal toxicity in control conditions. Time-courses of striatal field potential (FP) amplitude in the presence of 1 mM NaN_3 plus either 100 μM of an NO-donor (SNAP) (A), 1 μM YC-1, an activator of sGC (B) or 1 μM of the PKG activator 8Br-cGMP (C) in control mice. In the upper panels, representative FP traces recorded before and after the application of 1 mM NaN_3 in control mice with and without the co-application of SNAP (A), YC-1 (B) or 8Br-cGM (C). (D) The histograms show the percentage of reduction of the striatal FP amplitude, 35 min after 1 mM NaN_3 application alone and with the co-application of 100 μM SNAP, 1 μM YC-1 or 1 μM 8Br-cGMP in slices from control mice. ns: not significant.

3.6. Exposure to pro-inflammatory cytokines does not influence neuronal toxicity induced by mitochondrial complex IV inhibition

An inverse correlation between complex IV activity and macrophage/microglial density has been demonstrated in brain MS tissue (Mahad et al., 2008, 2009). We thus investigated if the *in vitro* exposure to some of the pro-inflammatory molecules that are known to be released by immune cells during neuro-inflammation was able to mimic the worsening effect of EAE on NaN_3 neuronal toxicity. As soluble products of inflammation we utilized *interleukin-17* (IL-17, 20 ng/ml), *interleukin-1β* (IL-1β, 40 pg/ml and 100 pg/ml), *tumour necrosis factor-α* (TNF-α, 40 ng/ml and 80 ng/ml) and *interferon-γ* (IFN-γ, 10 ng/ml and 50 ng/ml). We incubated striatal brain slices obtained from control mice with each single concentration of the pro-inflammatory cytokines before the application of 1 mM NaN_3, and recorded fEPSP amplitude for 30–35 min. The exposure of brain slices to both 1 mM NaN_3 and 20 ng/ml IL-17 determined a total reduction of fEPSP amplitude that was not significantly different compared to that induced by 1 mM NaN_3 alone in control conditions ($n = 8$, $p > .05$) (Fig. 6A). Similar results were obtained applying increasing doses of IL-1β ($n = 4$ for the 40 pg/ml dose and $n = 8$ for the 100 pg/ml dose) together with 1 mM NaN_3 (Fig. 6B). We then analyzed the time course of fEPSP amplitude loss during the application of 40 ng/ml ($n = 4$) and 80 ng/ml ($n = 5$) TNF-α in association with 1 mM NaN_3, showing no significant differences

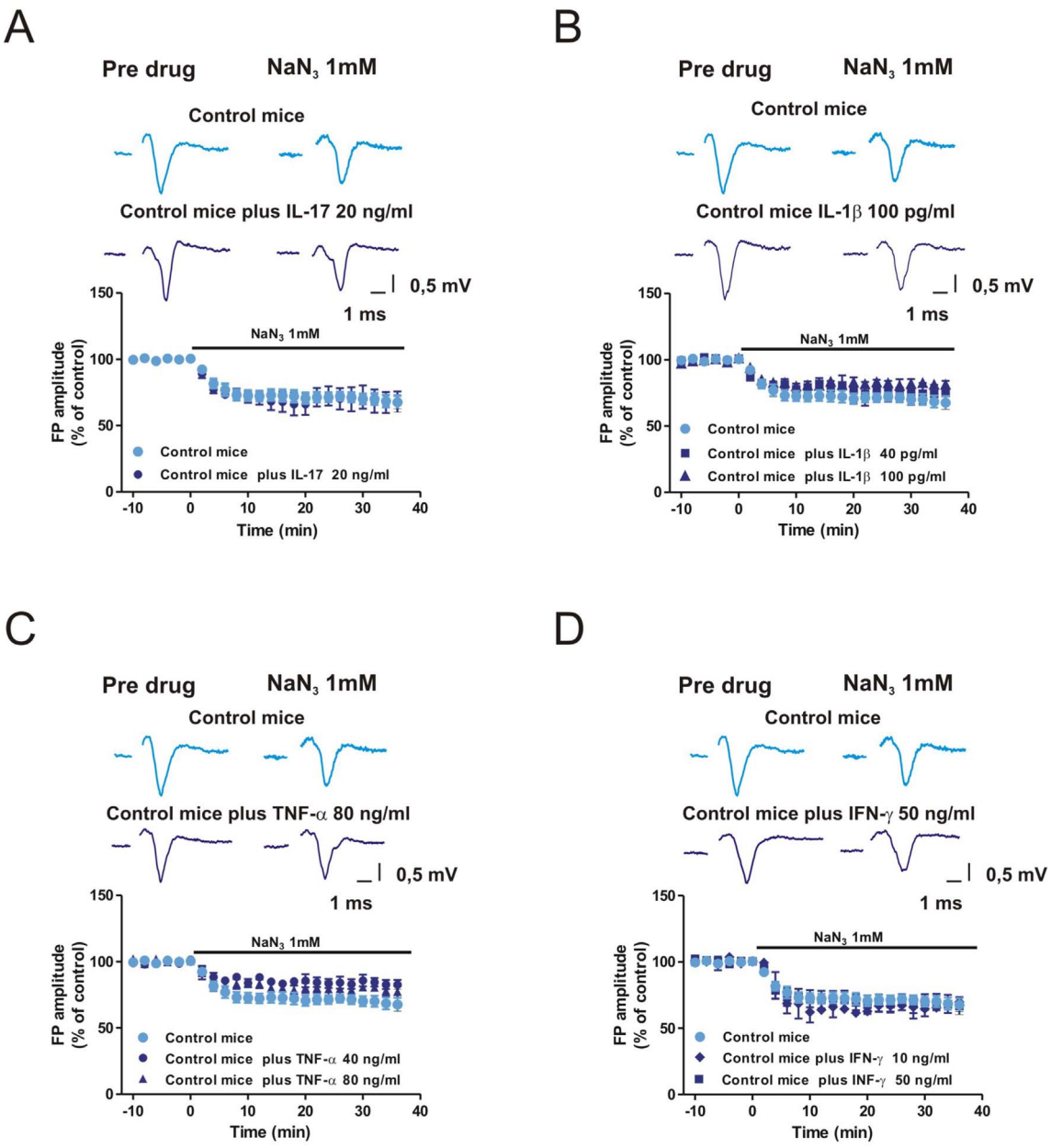

Fig. 6. The *in vitro* application of pro-inflammatory cytokines does not influence neuronal susceptibility to mitochondrial complex IV inhibition in control conditions. (A) Time-courses of striatal field potential (FP) amplitude in the presence of 1 mM NaN_3 and 20 ng/ml IL-17 in control mice. In the upper panel, representative FP traces recorded before and after the application of 1 mM NaN_3 in control mice with and without the co-application of 20 ng/ml IL-17. (B) FP amplitude loss induced by the application of 1 mM NaN_3 plus either 40 pg/ml (blue filled squares) or 100 pg/ml (blue filled triangles) IL-1β in control mice, with representative FP traces in the upper panel for the higher IL-1β concentration. (C) Time-courses of striatal FP amplitude in the presence of 1 mM NaN_3 plus either 40 ng/ml (blue filled circles) or 80 ng/ml (blue filled triangles) TNF-α in control mice, with representative FP traces in the upper panel for the higher TNF-α concentration. (D) Time-courses of striatal FP amplitude in the presence of 1 mM NaN_3 plus either 10 ng/ml (blue filled diamonds) or 50 ng/ml (blue filled squares) IFN-γ in control mice, with representative FP traces in the upper panel for the higher IFN-γ concentration.

compared to the fEPSP amplitude reduction induced by NaN_3 alone ($p > .05$) (Fig. 6C). Finally, IFN-γ at increasing doses (10 ng/ml, n = 2, and 50 ng/ml, n = 10) in association with 1 mM NaN_3, was able to significantly influence the fEPSP amplitude loss induced by NaN_3 ($p > .05$) (Fig. 6D). We then analyzed if the combined exposure to the soluble products of inflammation that were tested in isolation (TNF-α 80 ng/ml plus IFN-γ 50 ng/mln n = 4; TNF-α 80 ng/ml plus IFN-γ 50 ng/ml plus IL-1β 80 pg/ml, n = 3) together with 1 mM NaN_3 was able to influence neuronal toxicity induced by NaN_3 in control conditions. However, also this co-application of different pro-inflammatory cytokines and NaN_3 did not influence the absolute fEPSP amplitude loss induced by this mitochondrial toxin in control conditions ($p > .05$). Finally, in order to better reproduce the inflammatory microenvironment, we tested if the exposure to 100 μM of the NO donor SNAP, together with all the inflammatory cytokines previously tested (TNF-α 80 ng/ml plus IFN-γ 50 ng/ml plus IL-1β 80 pg/ml plus IL-17 20 ng/ml, n = 3) was able to influence the neuronal toxicity induced by 1 mM NaN_3, finding no significant differences with respect to 1 mM NaN_3 alone ($p > .05$). These results collectively show that IL-17, IL-1β, TNF-α and IFN-γ, when applied acutely, alone or together, are not able to mimic the worsening effect of EAE on mitochondrial complex IV toxicity.

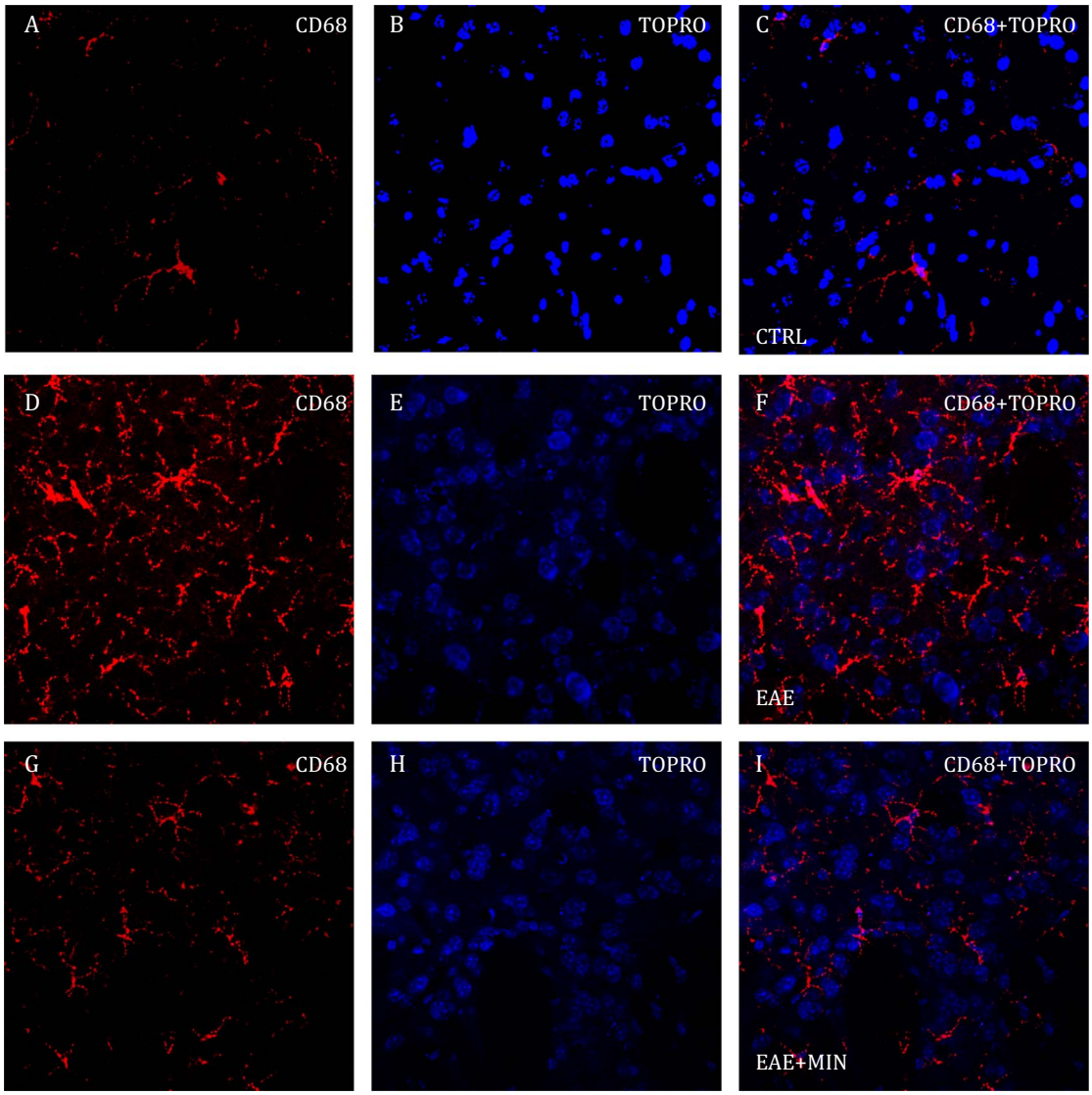

Fig. 7. Confocal laser scanning microscope (CLSM) images showing double-label immunofluorescence for activated microglia CD68 (visualized in red cy3 fluorescence) counterstained with TOPRO (visualized in blue). The immunofluorescence was performed in striatum of control mice (A–C); EAE mice (D–F) and minocycline-EAE treated mice (G–I). Note the intense activation of microglia in EAE mice and the inhibitory effect of minocycline.

3.7. Microglial activation drives the increased neuronal susceptibility to mitochondrial complex IV inhibition during EAE

The EAE pathogenic process is associated with an intense activation of microglial cells in the CNS (Centonze et al., 2009; Di Filippo et al., 2016; Nisticò et al., 2013; Ziehn et al., 2012) coupled with the over-expression of ROS-producing enzymes like NADPH oxidase (Di Filippo et al., 2016; Gao et al., 2012) whose activation has been related to mitochondrial injury (Fischer et al., 2012). Thus, we decided to investigate if an activation of microglial cell could be responsible of the observed increase of neuronal vulnerability in EAE mice. First, we confirmed the presence of microglial activation in the striatum by immunolabeling striatal tissue with an antibody for activated microglia (rat-anti mouse-CD68). Immunostaining for CD68 in the EAE mice group revealed an intense microglial reaction (Fig. 7 A–C), where microglial cells appeared numerous and the intensity of the reaction was higher compared to that observed in control mice (Fig. 7 D–F). Then, in order to test if the activated microglia could influence the observed neuronal susceptibility to mitochondrial complex IV inhibition, we treated EAE mice with intra-peritoneal injections of minocycline, an antibiotic able to prevent microglial activation and to modulate T lymphocytes (Chen et al., 2011). As expected, microglial reaction appeared markedly attenuated in EAE mice treated with minocycline (Fig. 7G–I). Extracellular striatal fEPSPs recordings showed that in the minocycline-treated group, exposure to 1 mM NaN_3 caused a reduction of the fEPSPs amplitude (21.54% ± 8.91, n = 8) that was significantly lower with respect to what observed in the EAE group (67.00% ± 11.07, n = 5, $p \leq .001$) and was similar to that observed in control mice (37.42%, ± 4.64, n = 4, $p \geq .05$) (Fig. 8).

4. Discussion

The obtained results indicate that the neuroinflammatory process associated with EAE is able to markedly enhance the neuronal susceptibility to mitochondrial complex IV inhibition. Interestingly, the exposure to specific pharmacological inhibitors of NO-synthesis and its intracellular pathway was able to reverse this detrimental effect, suggesting a potential key role for this soluble inflammatory mediator in linking inflammation and neuronal damage and a detrimental interaction between the inflammatory-related activation of sGC/PKG and

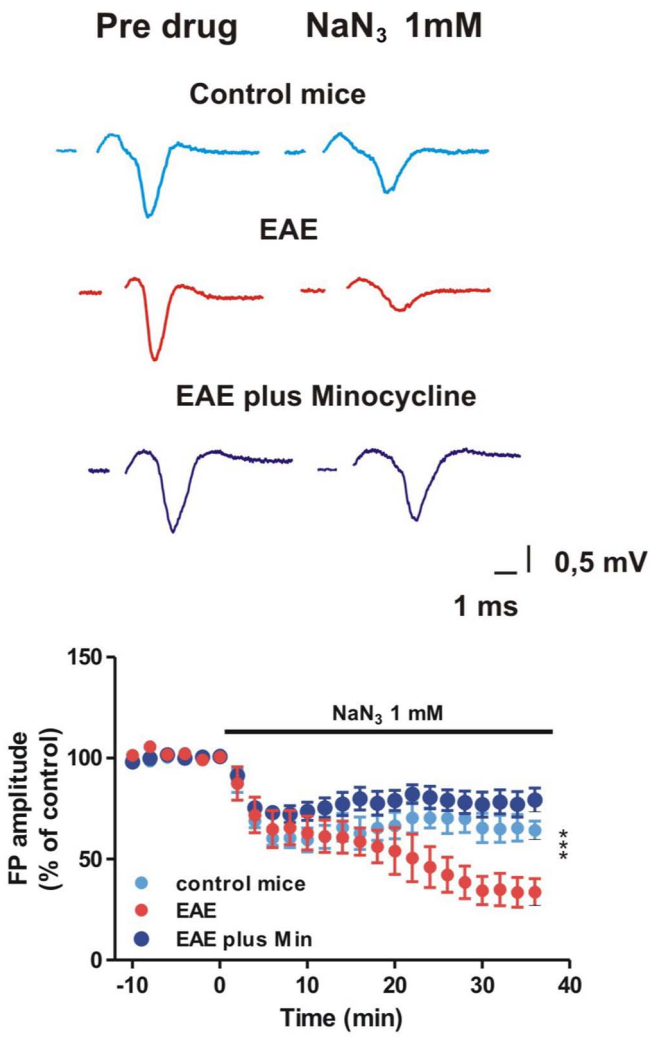

Fig. 8. Neuronal toxicity induced by mitochondrial respiratory chain complex IV inhibition is reduced in EAE mice treated with the microglial inhibitor minocycline. Time-courses of striatal field potential (FP) amplitude in the presence of 1 mM sodium azide (NaN_3) in control Biozzi ABH mice (blue filled circles), in mice affected by the acute clinical phase of EAE (red filled circles) and in minocycline treated EAE mice (dark blue filled circles). In the upper panel, representative FP traces recorded before and 35 min after the application of 1 mM NaN_3 for all the three groups. ***$p < .001$.

mitochondrial complex IV activity (Fig. 9).

Mitochondrial dysfunction seems to play a potential pathogenic role in the neuro-axonal degeneration during the course of MS since the earliest phases of the disease (Campbell et al., 2014; van Horssen et al., 2012; Witte et al., 2010, 2014). Several studies showed that neuronal damage and axonal transection can be detected in the context of acute demyelinating lesions (Bitsch et al., 2000; Ferguson et al., 1997; Trapp et al., 1998), suggesting that an inflammation-related neuronal injury occurs early during the course of the disease. It has been recently showed that, during both experimental and human MS, a reversible form of focal axonal swelling, potentially evolving into axonal disruption, can be detected during the earlier phase of neuro-inflammation, when the myelin sheet has not been affected yet (Nikić et al., 2011). The observed axonal damage, which has been named *focal axonal degeneration* (FAD), was found to be more pronounced in CNS areas with an intense immune cells infiltration, reinforcing the hypothesis of a causative correlation with the production of soluble and diffusible inflammatory products, such as reactive oxygen and nitrogen species (ROS and RNS), by activated macrophages/microglia (Nikić et al., 2011). Interestingly, the first ultrastructural alteration observed during FAD was an intra-axonal mitochondrial swelling, suggesting that an early mitochondrial dysfunction could start the pathogenic process leading to FAD (Nikić et al., 2011). NO-donor and RNS application were able to induce similar mitochondrial abnormalities, suggesting a key role of these compounds in inducing axonal degeneration not related to myelin sheet loss (Nikić et al., 2011).

Our results support the hypothesis that, during EAE, CNS neurons might be particularly susceptible to the failure of the mitochondrial respiratory function. It is worth to note that, although the vulnerability of striatal neurons to mitochondrial complex IV inhibition was markedly enhanced during EAE, complex IV basal activity was not reduced in this experimental group. The presence of a preserved complex IV function could thus be a key factor in maintaining neuronal homeostasis and preventing cellular energetic failure during MS. Interestingly, several reports demonstrated an enhanced intra-axonal mitochondrial density and increased axonal complex IV activity in chronic MS lesions, that has been suggested to represent a compensatory mechanism (Lu et al., 2000; Smith et al., 2013; van Horssen et al., 2012; Zambonin et al., 2011).

Since the application of pharmacological inhibitors of NO synthesis was able to rescue neurons from mitochondrial complex IV dysfunction during EAE but not in control conditions, this mediator could represent one of the major responsible in linking inflammation and mitochondrial disruption in our experimental setting. This hypothesis is further supported by the evidence that electrically active axons, with an increased energetic demand, were more prone to degeneration when exposed to low NO concentrations, similar to those present in active demyelinating lesions (Smith et al., 2001). This could be related to the pathological consequences of the NO-induced mitochondrial dysfunction with a reduced ATP production on the ionic axonal homeostasis. In particular, a sustained electrical activity could increase sodium (Na^+) influx through voltage-gated sodium channel, requiring an increased activity of energy dependent sodium-extruding mechanism (Smith et al., 2001; Trapp and Stys, 2009; Waxman, 2006). In an inflammatory environment, mediators such as NO could thus induce an energetic failure through mitochondrial inhibition, contributing to a chronic imbalance between energetic axonal demand and mitochondrial ATP production in chronically demyelinated axons.

There is still debate about the potential harmful or protective effect on cellular viability of the sGC/PKG pathway activation. Indeed, some authors showed that, both in nervous and myocardic tissues, the transient *in vitro* exposure to NO was able to exert an anti-apoptotic role after ischemia through a sGC/PKG mediated inhibition of mitochondrial permeabilisation, *cytochrome c* release, and caspase activation, all processes that are involved in the cellular apoptosis induced by mitochondrial dysfunction (Arandarcikaite et al., 2015; Borutaite et al., 2009). On the other side, others showed that the sustained activation of sGC/PKG can induce diffuse cellular apoptosis and necrosis in pancreatic tissues, which could be reversed by the inhibition of PKG (Tejedo et al., 1999). It is possible that the activation of NO intracellular pathway exerts either anti- or pro-apoptotic cellular effects through the interaction with different mitochondrial targets, depending on the type of stimulation (transient *vs* sustained), cellular type and NO concentrations. In our study, we showed a significant protective role exerted by sGC/PKG inhibition against neuronal death induced by mitochondrial complex IV dysfunction during EAE. Interestingly, the acute and transient *in vitro* pharmacological activation of sGC/PKG was not able to mimic the enhancing effect on mitochondrial dysfunction exerted by the neuroinflammatory process associated with EAE. This result seems to suggest that, during EAE, the enhanced release of NO and the activation of sGC/PKG could alter mitochondrial dysfunction because of the concomitant presence of other inflammatory mediators and/or activation of brain innate immunity. In our setting, the *in vitro* exposure to soluble inflammatory mediators, such as IL-17, IL-1β, TNF-α, IFN-γ did not alter neuronal susceptibility to mitochondrial complex IV inhibition. Conversely, the inhibition of the EAE-associated striatal microglial activation was able to clearly rescue the enhanced neuronal susceptibility to mitochondrial complex IV inhibition.

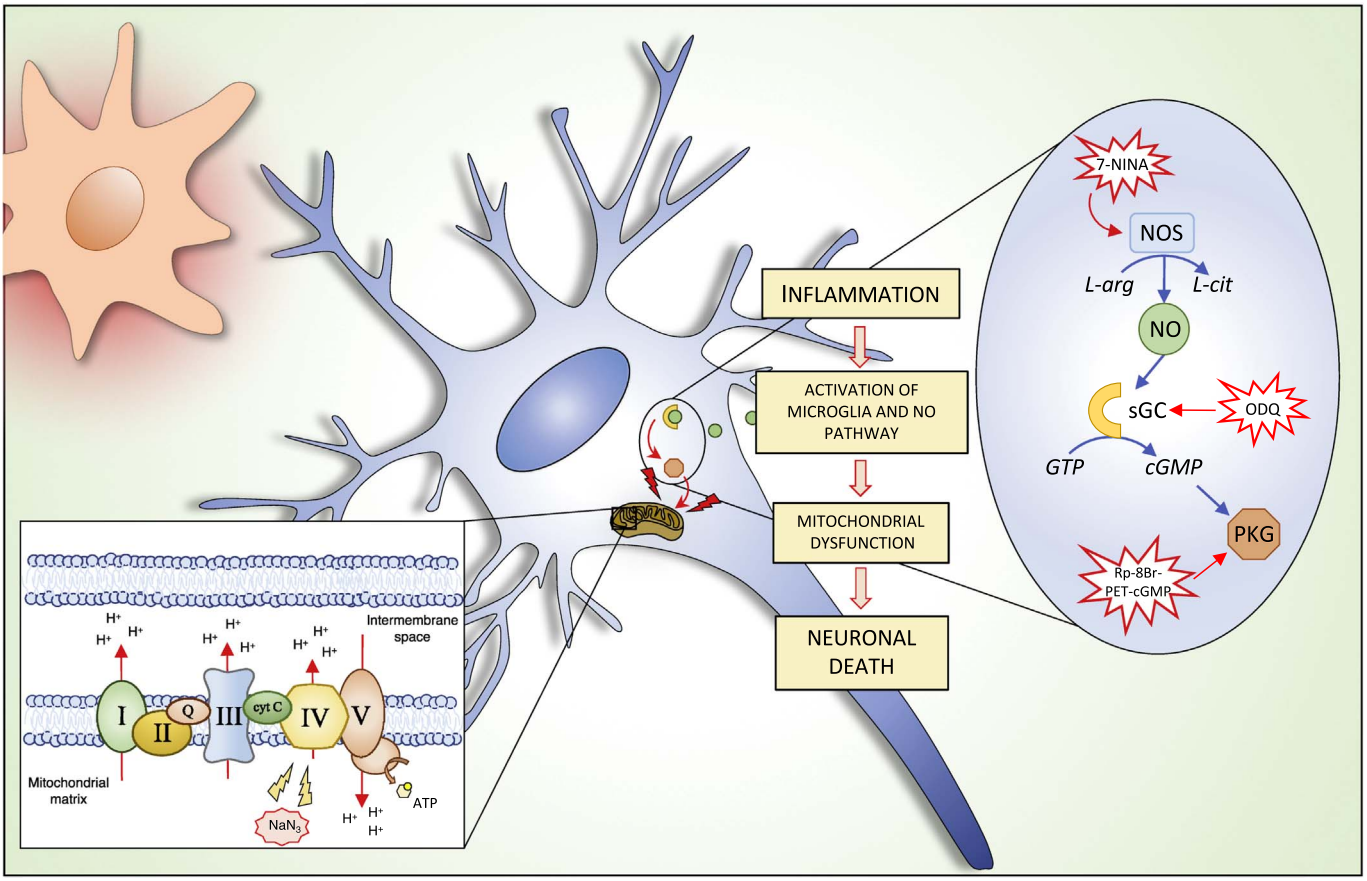

Fig. 9. NO-activated pathway as a possible link between neuroinflammation, mitochondrial dysfunction and neuro-axonal degeneration. During CNS inflammation, the activation of microglia, together with the release of NO and the subsequent sustained activation of its intracellular pathway (showed in the oval box on the right), may result in mitochondrial dysfunction and in an enhanced vulnerability to inhibition of mitochondrial complex IV (showed in the squared box on the left), causing neuronal dysfunction/death. The figure shows the pathway activated by NO, and in particular *soluble guanylyl cyclase*, sGC, and *protein kinase G*, PKG and the mitochondrial *respiratory chain complexes*. In the present work sodium-azide (NaN_3) has been utilized as complex IV inhibitor and 7-NINA, ODQ and Rp-8Br-PET-cGMP as inhibitors, respectively, of NOS, sGC and PKG. Abbreviations: cGMP: *cyclic guanosine monophosphate*; cyt c: *cytochrome c*; GTP: *guanosine triphosphate* L-arg: *L-arginine*; L-cit: *L-citrulline*; 7-NINA: *7-nitroindazole*; NOS, *nitric oxide synthase*; ODQ: *1H-1,2,4 oxadiazolo-4,3-a quinoxalin-1-one*; Q: *coenzyme Q*.

Activated microglia deeply influence the CNS microenvironment, releasing pro-inflammatory cytokines and ROS thanks to the over-expression of ROS-producing enzymes like NADPH oxidase (Gao et al., 2012). Interestingly, the concomitant activation of NADPH oxidase and NO-synthase during neuroinflammation could lead to the production of RNS able to irreversible inhibit mitochondrial chain complexes (Brown, 2001).

5. Conclusions

Inflammation and mitochondrial dysfunction seem to be intertwined in a vicious cycle, ultimately converging into a common synergistic process responsible for neuro-axonal degeneration during the course of MS. The obtained data support the evidence that microglia could play a key role, together with the activation of the NO pathway, in linking the inflammatory process associated with experimental MS to the observed neuronal susceptibility to mitochondrial dysfunction.

Inflammation-related NO synthesis and the sustained activation of its intracellular pathway, involving sGC and PKG may represent a promising pharmacological target to counteract the synergic detrimental effect of inflammation and mitochondrial dysfunction (Colombo et al., 2012, 2014) and to design effective neuro-protective strategies.

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Competing interests

AM received travel grants from Teva and Sanofi Genzyme to attend national conferences. LG received travel grants from Biogen-Idec, Biogen, Novartis, Teva, Genzyme and Almirall to attend national and international conferences. MDF participated to advisory boards of Biogen Idec, Novartis, Teva and Bayer, received travel grants from Bayer Schering, Biogen-Dompé, Biogen-Idec, Merck-Serono, Novartis and Sanofi-Aventis to attend national and international conferences and speaker and writing honoraria from Biogen Idec, Genzyme, Merck, Novartis, Sanofi-Genzyme and Teva. PC received/receives research support from Bayer Schering, Biogen:Dompé, Boehringer Ingelheim, Eisai, Lundbeck, Merck:Serono, Novartis, Sanofi:Aventis, Sigma:Tau and UCB Pharma. PC also receives/received support from Ricerca Corrente IRCCS, Ricerca Finalizzata IRCCS, European Community Grant REPLACES (restorative plasticity at corticostriatal excitatory synapses), the Italian Minister of Health, and AIFA (Agenzia Italiana del Farmaco). MT, PM, AdI, VD, LM, CG, AA, CC and AT report no competing interests.

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