Neuroprotective Role of Metformin in a Model of Cerebelar Ataxia

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NEUROPROTECTIVE ROLE OF METFORMIN IN A MODEL OF CEREBELAR ATAXIA

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#*In memoriam* of Dr. Mario Alberto Cardoso Silva Neto

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Abstract

Cerebellar ataxia is a heterogeneous group of neural disorders clinically characterized by cerebellar dysfunction. The diagnosis of patients with progressive cerebellar ataxia is complex due to the direct correlation with other neuron diseases. Although there is still no cure for this

pathological condition, some metabolic, hereditary, inflammatory, and immunological factors affecting cerebellar ataxia are being studied and which may become therapeutic targets. Advances in studying the neuroanatomy, pathophysiology, and molecular biology of the cerebellum contributes to better understand the mechanisms behind the development of this disorder. In this study, we analyzed the neuromodulatory role of the AMP-activated protein kinase (AMPK) on cerebellar ataxia induced by the neurotoxin 3-acetylpyridine (3-AP) in brain stem and cerebellum, after pre-treatment with metformin, a pharmacological indirect activator of AMPK. The results shown here suggest that AMPK activation in the brain stem and cerebellum leads to a significant reduction in neuroinflammation in these regions. AMPK was able to restore the changes in fatty acid composition and pro-inflammatory cytokines caused by 3-AP, suggesting that the action of AMPK seems to result in a possible neuroprotection on the cerebellar ataxia model.

Introduction

The term *taxi* (from Greek, coordination) characterizes the main function performed by the cerebellum. It is applied to the motor function of the limbs, trunk, eyes, and bulbar musculature. Thus, the term ataxia is used when motor coordination is lost [1]. Ataxias can be functionally classified in five different categories. Cerebellar ataxia (I), when the cerebellum and/or its efferent and afferent pathways are affected; sensitive (II) when the proprioceptive pathways are affected; frontal (III), a rare form related to the involvement of the cerebellar-frontal pathway; thalamic (IV), due to damage to the cerebellar-thalamocortical circuit; and vestibular (V), resulting from labyrinth dysfunction [2]. Individuals affected by cerebellar ataxia present classic signs, such as gait and balance alteration, dysmetria, dysdiadochokinesia, cerebellar tremor, dyssynergia, movement decomposition and dysarthria, characterized as scant

48 speech. In addition, signs, and symptoms, such as hypotonia, stuttering, pendulum reflexes,
49 altered eye movements and nystagmus can also appear [3]. However, ataxia does not result only
50 from cerebellar lesions; interference with somatosensory transmission to the cerebellum, either
51 by spinocerebellar injury or by peripheral disorders, can also produce symptoms of ataxia [4, 5].
52 The areas that show the cerebellar lesion can be diagnosed by observing a deficit in the
53 individual motor activity. Midline and intermediate cortical lesions, for example, cause gait
54 disturbance and postural instability [6, 7].

55 A considerable number of animal models with neurological mutations, in addition to
56 those that mimic the phenotype of cerebellar ataxias, have been developed [8]. It is important to
57 shed light in the mechanisms of cerebellar dysfunction, either for the evaluation of therapy,
58 targeting deleterious pathway, or even for the screening of chemical compounds. However,
59 significant differences between animal models and humans regarding the aspects involving
60 cerebellar ataxias remains a barrier to the development of new drugs [2, 9].

61 Metformin is a biguanide drug widely prescribed as an oral antihyperglycemic agent and
62 recommended as the drug of choice for type 2 diabetes (T2D) according to recent European-
63 American clinical guidelines [10]. The main effect of metformin is linked to the mild and
64 transient inhibition of complex I of the mitochondrial respiratory chain [11, 12], which
65 consequently results in a decrease in the cellular energy state, leading to the activation of AMPK
66 [13]. As a result, there is a decrease in the hepatic glucose synthesis (primarily through the
67 inhibition of gluconeogenesis and, to a minor scale, glycogenolysis), and an increase in the
68 insulin-stimulated glucose uptake in skeletal muscle and adipocytes [14–16].

69 In addition to its hypoglycemic effects, metformin has also shown effects on immune
70 cells and inflammatory processes [17–19]. There are evidence showing AMPK regulating
71 metabolic pathways in immune cell function, such as in macrophages, T cells, and dendritic cells
72 (DCs). Macrophages and DCs from AMPKα1-deficient mice produced higher levels of pro-
73 inflammatory cytokines and decreased production of the anti-inflammatory cytokine in response
74 to TLR and CD40 stimulation when compared with cells from wild type mice [20]. Studies using
75 RNA interference (RNAi), or adenovirus have shown that AMPK activation prevents
76 lipopolysaccharides (LPS)-induced pro-inflammatory cytokines, as well as fatty acid synthesis
77 [21–23]. Other studies indicate that AMPK activation has a potential therapeutic effect on the
78 neuroinflammation in central nervous system (CNS) [24–26]. Activation of AMPK blocked
79 interferon gamma (IFN-γ)-induced gene expression, including chemokine (C-C motif) ligand 2
80 (CCL2), tumor necrosis factor alpha (TNF-α), C-X-C motif chemokine ligand 10 (CXCL10), and
81 induced the enzyme nitric oxide synthase (iNOS) in primary astrocytes and microglia, through
82 signal transducer and transcription activator modulation 1 (STAT1) [27]. AMPK suppression in
83 primary astrocytes increased STAT1 expression, leading to the synthesis of pro-inflammatory
84 cytokines and chemokines. It was also reported that during encephalomyelitis, AMPK signaling
85 was downregulated in the brain at the onset and peak of the disease, correlating with increased
86 expression of IFN-γ and CCL2 in the CNS [27, 28]. Studies in animal models showed that the
87 AMPK activation exerted a neuroprotective function, attenuating the pro-inflammatory response
88 [29]. However, there are not evidence regarding the molecular mechanisms involving the
89 activation of AMPK in neurodegenerative pathologies. Therefore, to investigate the role of
90 AMPK activation in the molecular mechanisms in cerebellar ataxia is crucial to have the sense to
91 seek new intervention strategies during the development of the disease.

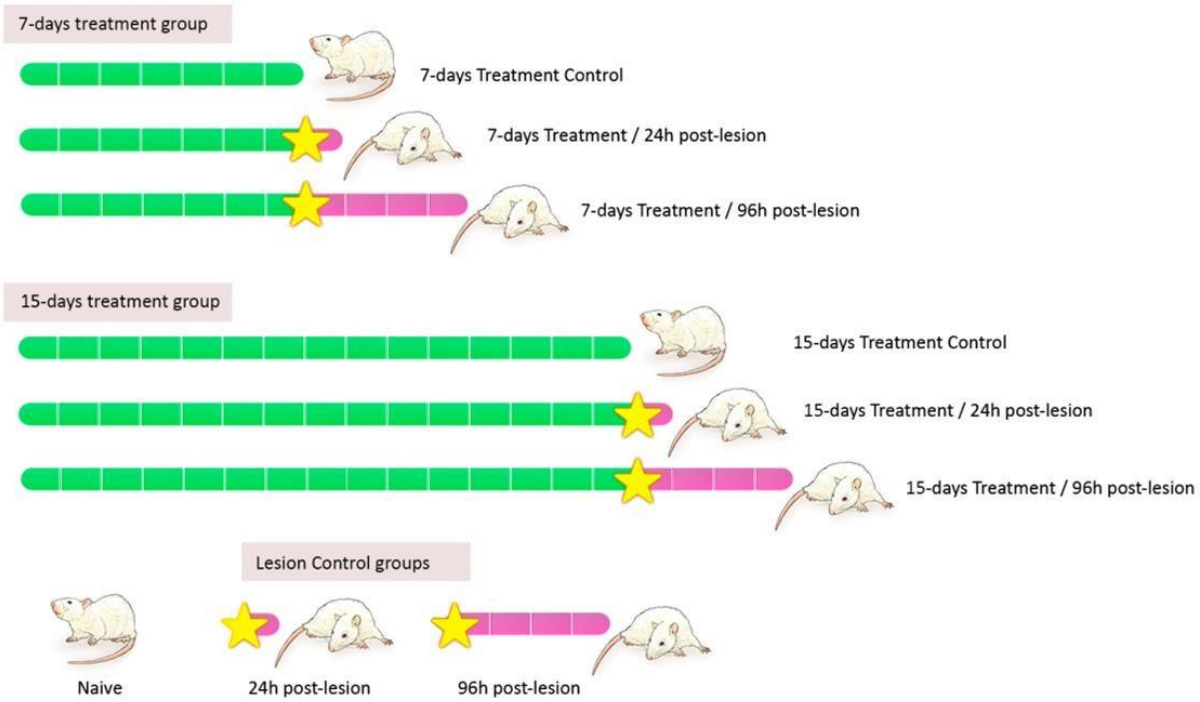
Material and Methods

1. Animals and Experimental Design

All experiments were conducted according to the standards of the National Council for Control of Animal Experimentation and approved by the Animal Care Committee of the Health Sciences Center, Federal University of Rio de Janeiro (CEUA protocol, 2016/03). The study complied with the "Principles of Laboratory Animal Care" generated by the National Society for Medical Research and the United States, and the National Academy of Sciences Guide for the Care and Use of Laboratory Animals. Males Wistar rats (4-5 weeks of age) were housed in a temperature-controlled room (23°C) on a 12/12-hour light/dark cycle, with food and water *ad libtum*.

Twenty-seven rats were divided into 9 experimental groups. The first group was injected with 0.9% saline as a control (CONT). Three other groups were pre-treated with metformin for 7 consecutive days, with a dose of 150 mg/kg [30], intraperitoneal (IP), once a day: Group 2 were pre-treated with metformin (7D); Group 3was pre-treated with metformin,injected with 3-AP and euthanized 24 hours after the injection (7D3AP 24h). Group 4 was pre-treated with metformin, injected with 3-AP and euthanized 96 hours after the injection (7D3AP 4D). The same approach was used with animals pre-treated with metformin for 15 consecutive days. Group 5 was pre-treated with metformin; Group 6 was pre-treated with metformin, injected with 3-AP, and euthanized 24 hours after the injection (15D3AP 24h). Group 7 was pre-treated with metformin, injected with 3-AP, and euthanized 96 hours after the injection (15D3AP 4D). The last 2 groups were not pre-treated with metformin but were injected with 3-AP, euthanized 24 hours (3AP 24h) and 96 hours after the injection (3AP4D). Figure 1 shows the distribution and treatment of

115 each group. The animals were euthanized in a CO_2 chamber followed by cervical dislocation,
116 and the blood was harvested by cardiac puncture, in the presence of 3% sodium citrate, used as
117 an anticoagulant agent. Then, the cerebellum (CE) and brain stem (BS) were harvested. Blood
118 was centrifuged at 2500 x g for 15 minutes to collect the plasma, which was stored at –80ºC.
119 Tissues were immediately placed in 1.5 mL cryotubes and immersed in liquid nitrogen for
120 further analysis. Animals injected only with saline were used as controls, and tissues and blood
121 were preserved followed the same criteria as treated animals.



122 **Fig.1** Schematic figure with the distribution and treatment of each group. Twenty-seven rats
123 were divided into 9 experimental groups. The green bar represents the animals treated with
124 metformin and the pink bar the animals that were injured with 3AP. The star represents the
125 neurotoxin injection

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127

128 **2. Injury Model**

129 Rats were submitted to a single IP injection of 3-AP (Sigma Chemicals, St. Louis, MO,
130 USA), dissolved in 0.9% saline at a dose of 65 mg/kg, and euthanized 24 and 96 hours after the
131 injection [31]. Animals injected only with saline solution were euthanized and used as a control
132 in parallel with the experimental animals.

133 **3. Protein Determination**

134 Plasma and tissue protein contents were determined using a modified Lowry method
135 [32]. Bovine serum albumin (0.1 g%) was used as a standard.

136 **4. Lipid Extraction and Quantification**

137 Lipid extraction was performed using the method of Bligh and Dyer [33] with
138 modifications. Samples of CE and BS were added to a lysis buffer (50 mm Tris-HCl, pH 7.4,
139 containing 1% NP-40, 250 mM NaCl, 5 mM EDTA and 50 mM NaF), homogenized, centrifuged
140 at 2500 x g for 5 minutes to pellet, and the supernatant collected. Then, samples were subjected
141 to protein determination using a modified Lowry method [32]. After that, samples were
142 standardized to 10 mg (protein) and then subjected to lipid extraction. Samples were added to
143 conical glass tubes, containing a solution of chloroform-methanol-water at the proportion of
144 2:1:0.8 mL, and subjected to intermittent shaking for 2 hours (5 minutes/each). Then, the tubes
145 were centrifuged at 230 x g for 20 minutes at 4 °C in a refrigerated centrifuge (Hitachi, Ltd, TO,
146 Japan). The supernatant was collected and added 1 mL of water and 1 mL of chloroform. After
147 stirring, two phases (aqueous and organic) raised up. The tubes were centrifuged again at 230 x g
148 for 20 minutes at 4ºC. The organic phase (containing the lipids) was removed and dried under a

149 nitrogen gas storm. Extracted lipids were analyzed by Thin Layer Chromatography (TLC), using
150 the solvents hexane - diethyl ether - acetic acid as mobile phase, in the proportion of 60:40:1v/v,
151 respectively. To visualize the lipid classes, silica plates were immersed for 10 seconds in a
152 carbonization solution consisting of 8% $CuSO_4$ and 10% H_3PO_4 (v/v) and heated at 110 °C for 20
153 minutes [34]. The plates were then analyzed by densitometry using the ImageMaster TotalLab
154 software (TotalLab, Newcastle, England, UK).

155 **5. Immunoblotting**

156 Samples of CE and BS were homogenized in lysis buffer (same as used for lipid
157 extraction) containing a protease and phosphatase inhibitor cocktail (Sigma Chemicals, St. Louis,
158 MO, USA), and subjected to protein determination by a modified Lowry method [32]. Then, 60
159 μg/protein of each sample was mixed with sample buffer containing 1% sodium dodecyl sulfate
160 (SDS) and 0.5% β-mercaptoethanol, boiled for 4 minutes, and then applied to the bis-acrylamide
161 gel (7.5%). Proteins separated by electrophoresis at a constant voltage of 100V for 1.5 hours
162 were transferred to a nitrocellulose membrane at a constant amperage of 250 mA for 90 minutes
163 (GE Healthcare Life Sciences, Marlborough, MA, USA), and then blocked for 1 hour with TBS-
164 T (20 mM Tris-HCl, 500 mM NaCl and 0.1% Tween 20, pH 7.5), containing 3% bovine serum
165 albumin. Subsequently, the membranes were incubated overnight with the mouse monoclonal
166 anti-beta actin (1:1000, Santa Cruz, CA, USA), polyclonal rabbit anti-phospho AMPK (1:500,
167 Santa Cruz, CA, USA), and mouse polyclonal anti-AMPK (1:500, Santa Cruz, CA, USA). After,
168 the membranes were washed three times with TBS-T and incubated for 90 minutes with rabbit or
169 mouse polyclonal antibody conjugated to horseradish peroxidase (HRP) (1:20,000, Santa Cruz,
170 CA, USA). Immunoreactive proteins were visualized by chemiluminescence using an ECL kit
171 (ECL - Amersham Pharmacia, GE Healthcare Life Sciences, Marlborough, MA, USA) and the

172 Image Quant LAS 4000 device (GE Healthcare Life Sciences, Marlborough, MA, USA).
173 Immunoreactive bands were analyzed by densitometry using the ImageMaster TotalLab software
174 (TotalLab, Newcastle, England, UK).

175 **6. Fatty Acid Analysis by Gas Chromatography Mass Spectrometry (GC-MS)**

176 Extracted lipids from cerebellum and brain stem (up to 5 mg) were dissolved in 1 mL
177 toluene in a conical glass tube and 1% sulfuric acid in 2 mL methanol was added. The mixture
178 was left overnight at 50ºC. Subsequently, 1 mL water containing 5% sodium chloride was added
179 and the esters were extracted with hexane using Pasteur pipettes. The solvent was removed in a
180 nitrogen gas storm. Samples were resuspended in 100 µL heptane. A GC-mS Shimadzu
181 equipment, model GP2010 Plus, with an HPU (5% phenyl - methylpolysiloxane), Agilent (25 m
182 x 0.20 mm x 0.33 µm) column was used. The injector was maintained at 250°C, with split flow.
183 The column oven temperature was raised to 40-160°C with a heating rate of 30°C/minute, 160-
184 233°C with a heating rate of 1°C/minute, and 233-300° C, with a heating rate of 300°C/minute,
185 held for 10 minutes. Helium was used as the carrier gas with a linear velocity of 32.9 cm/second.
186 One µL of sample was injected into the chromatograph. For detection by mass spectrometry, a
187 detector containing an electron ionization source (EI-70 eV) and a quadruple mass analyzer,
188 operated in scans from 40 to 440 c.u., were used. The interface was maintained at 240°C and the
189 ion source, at 240°C. The identification of the constituents of the mixture was made by
190 comparing their mass spectra with those of the NIST05 library, contained in the mass
191 spectrometer computer, as well as their retention times with the Supelco 37 Component FAmE
192 mix (sigma) standard.

193 **7. Cytokine Analysis**

194 The analysis of IL-10, TNF-α and IL-6 was performed using the plasma from animals,
195 following the manufacturer`s protocol (R&D, Systems Corporation, Minneapolis, MN). Briefly,
196 96-well microplates (A-2, Costar) were sensitized with anti-cytokine monoclonal antibodies
197 diluted in PBS and incubated for 24 hours at room temperature. The plates were blocked with
198 PBS + 4% bovine serum albumin (SAB-Sigma Chemicals, St. Louis, MO, USA) and incubated
199 for 2 hours at room temperature. After three washes with PBS + 0.05% Tween-20, the plasma
200 samples and the samples from standard curve were added. The plates were incubated for 1 hour
201 at room temperature and an anti-cytokine antibody conjugated to biotin was added to the plates
202 and incubated for 1 hour. After three washes with PBS + 0.05% Tween-20, streptavidin
203 peroxidase from the kit was added and incubated for 20 minutes at room temperature. After
204 further washing, 3, 3', 5, 5' tetramethylbenzidine substrate (TmB, Zymed) was added to the plates
205 and the reaction was stopped by adding 1 M sulfuric acid. Plates were read using an ELISA
206 microplate reader at 450 nm.

207 **8. Statistical Analysis**

208 All results were expressed as mean ± standard error. Statistical analyzes were performed
209 using the GraphPad Prism software (v. 8.0, GraphPad Inc., CA, USA). Values for each group
210 were compared by Student's t-test and one-way ANOVA followed by Tukey's multiple
211 comparison test. Differences were considered statistically significant when $p \leq 0.05$.

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213 **Results and Discussion**

214 **1. Metformin increases AMPK activation in the cerebellum and brain stem**

215 The neurotoxin 3-AP, which was used to generate a cerebellar ataxia model in this work,
216 causes cerebellar neurodegeneration by an alteration in the respiratory chain in mitochondria
217 [35]. Thus, the hypothesis about a possible modulation of the AMPK in this condition was
218 raised, since it is a central regulator of energy homeostasis, which coordinates the metabolic
219 pathways and, therefore, balances the supply of nutrients with the demand for energy, through
220 the AMP/ATP ratio [36]. Our results demonstrated that pre-treatment with metformin for 7 and
221 15 days was able to maintain the phosphorylation levels of AMPK in CE and BS and was higher
222 in rats injured with 3-AP 24h and four days pos lesion (Figure 2, groups 7D 24h; 7D 4D; 15D
223 24h and 15D 4D compared to control groups). We also observed a reduction of ataxia signs
224 when rats were pre-treated with metformin and injected with 3-AP (data not shown). Different
225 studies have already described that AMPK has a potent neuroprotective effect, attenuating the
226 neuronal death induced by glucose deprivation, chemical hypoxia, glutamate and amyloid β
227 peptide, in cultured hippocampal neurons [37]. Furthermore, metformin reduced neuronal
228 apoptosis in cultured cortical neurons exposed to ethanol [38]. Pre-treatment with metformin,

229 two weeks before global cerebral ischemia, inhibited inflammatory responses and attenuated cell
230 death in the rat hippocampus, in an AMPK-dependent manner [39]. Furthermore, treatment with
231 metformin for 3 weeks before permanent occlusion of the middle cerebral artery reduced infarct
232 volume and improved neurological deficit [40]. Taken together, our results suggest that AMPK
233 activation has a potential role in mediating the prevention of cerebellar ataxia in rats.

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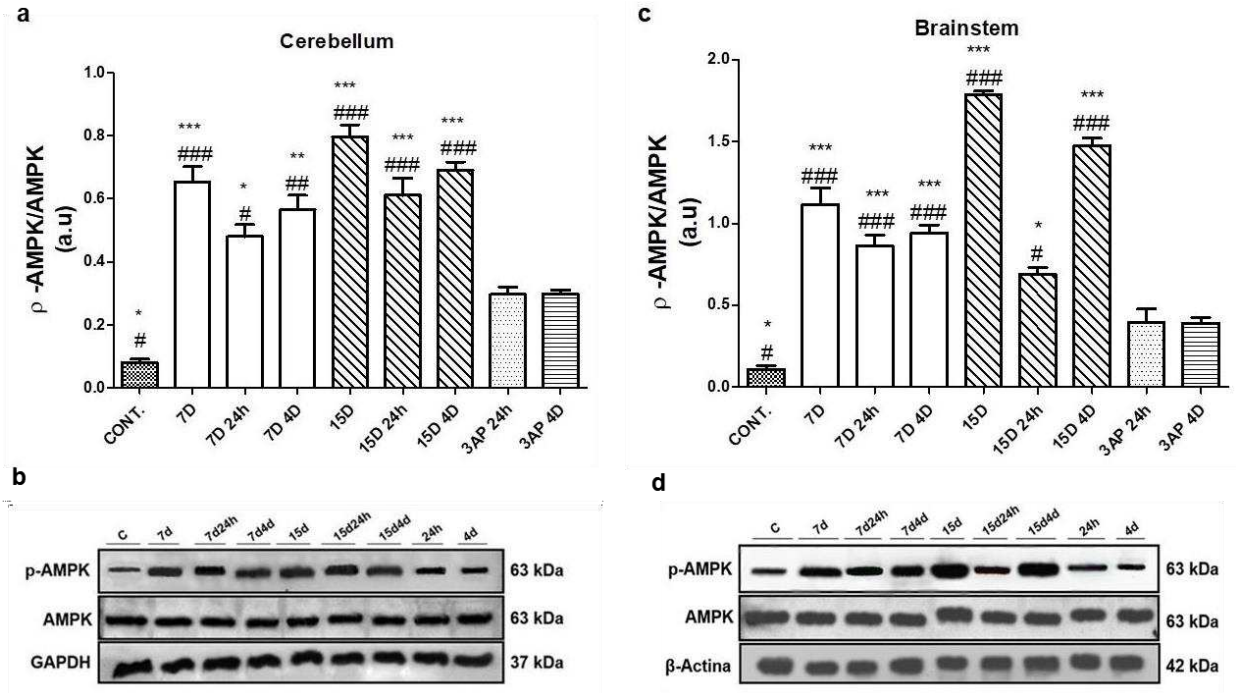

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238 **Fig.2** Metformin promotes AMPK activation in the cerebellum and brainstem. AMPK activation
239 (phosphorylation of T172) and total AMPK protein level were assessed by western blotting. The

240 animals were divided into 9 experimental groups: CONT -control; 7D - pretreated with
241 metformin for 7 days; 7D 24h- pretreated with metformin for 7 days and euthanized 24 hours
242 after injury; 7D 4D - pretreated with metformin for 7 days and euthanized 4 days after injury;
243 15D - pretreated with metformin for 15 days; 15D 24h - pretreated with metformin for 15 days
244 and euthanized 24 hours after injury; 15D 4D - pretreated with metformin for 15 days and
245 euthanized 4 days after injury; 3AP 24h - euthanized 24 hours after injury; 3AP 4D- euthanized 4
246 days after injury. Bands were analyzed by densitometry and displayed as bar histograms. Results
247 are representative of three independent experiments. Data represent mean ± SD. *** P<0.05
248 versus control (One-way ANOVA)

249249

250 **2. AMPK activation modulates the lipid profile in cerebellar ataxia**

251 Different lipid classes play important roles in the neuronal cell population: they can be
252 used as energy substrates by astrocytes, used as building blocks in the cellular structural
253 machinery, as well as bioactive molecules [41]. Different groups have studied the composition of
254 lipids in the brain and the most lipid classes found in this structure are grouped as sphingolipids,
255 glycerophospholipids and cholesterol, being present in very close proportions [42–44]. They are
256 involved in the development, maintenance and in many other cellular processes in the brain, such
257 as synaptogenesis, neurogenesis, and nerve impulse conduction [45, 46]. Therefore, any
258 modulation on lipid metabolism in the brain results in a change of lipid composition and
259 proportion in intracellular compartments, which is seen as a common biomarker for different
260 neuronal disorders, such as Alzheimer's, Parkinson's, and Huntington's diseases, as well as in
261 schizophrenia [45, 47]. Here, we characterized the lipid classes in CE and BS under different
262 experimental conditions. Figure 3b shows the lipid profile found in the BS. Four main lipid

263 classes were present in this structure. While esterified cholesterol showed no statistically
264 significant difference between the control and experimental groups, total phospholipids and free
265 cholesterol showed a significant increase after pre-treatment with metformin for 15 days.
266 Cholesterol, a vital lipid class constituent of the nervous system, plays an important role both
267 during development and adulthood [42]. In humans and rodents, cholesterol is actively
268 synthesized in the CNS during the first few weeks after birth and, at this stage, any disruption on
269 its synthesis can lead to neurodegenerative disorders, including Huntington´s disease,
270 Parkinson´s disease, Alzheimer´s disease and other atypical cognitive deficits [48, 49]. Some
271 studies emphasize the comparison of the main brain regions affected with changes in cholesterol
272 metabolism. For example, in Niemann-Pick type C disease, the Purkinje cells of the cerebellum
273 are most severely affected, due to accumulation of cholesterol in late endosomes and/or
274 lysosomes preferentially causing these neurons to die rather than neurons in other parts of the
275 brain. Other neurodegenerative pathologies also have the characteristic of neuronal degeneration
276 in different regions of the brain. Data such as these raise the interest in investigating the different
277 mechanisms of cholesterol homeostasis and their impact on the survival of neuronal types [50].
278 Hence, it is known that cholesterol is crucial for cellular processes, such as glial cell
279 proliferation, neurite outgrowth, microtubule stability, synaptogenesis, and myelination [51].
280 However, the induction of ataxia by the neurotoxin 3-AP did not change the concentrations of
281 cholesterol ester, showing that there was no involvement of this lipid class in the induction or
282 prevention of cerebellar ataxia. Similar results were observed in the cerebellum (Figure 3a).

283 Fatty acids represent a lipid class that is crucial for all mammalian cells [52]. They
284 exhibit a variety of biological functions to maintain vital cellular processes at different levels,
285 serving as energetic substrates, sustaining the structural integrity of cell membranes, and acting

286 as cell signaling molecules [53]. Fatty acids actively participate both in the development of the
287 nervous system, during embryonic and postnatal life, and on its maintenance during adulthood
288 until aging [54–56]. Therefore, the quantification of free fatty acids in CE and BS (Figure 3a and
289 3b) was important to evaluate the effects of AMPK activation in ataxia. Our results showed a
290 significant reduction of free fatty acids only in the animals pre-treated with metformin and
291 injured with 3-AP, when compared to the injured groups only, suggesting a role of the AMPK in
292 the fatty acid metabolism Indeed, different reports have suggested that AMPK activation
293 modulates lipid synthesis and degradation through the phosphorylation of key substrates. Li and
294 colleagues [57] showed that the sterol regulatory element-binding protein-1c (SREBP1c), a key
295 transcription factor involved in fatty acid and triacylglycerol synthesis, was directly
296 phosphorylated and consequently supressed by AMPK. Furthermore, AMPK can further reduce
297 hepatic lipid content by suppressing SREBP1c expression through a decrease in the mammalian
298 target of rapamycin (mTORC) activity [58], an important mediator for the cell growth and
299 metabolism regulation [59]. Other studies have shown that triacylglycerol synthesis is inhibited
300 by pre-treatment with AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside)
301 compound, a direct activator of AMPK, by reducing the incorporation of fatty acids into
302 triacylglycerol [60]. As we did not observe differences in the triacylglycerol content between rats
303 pre-treated with metformin and injured with 3-AP and control groups, the reduction seen in the
304 content of fatty acids could be due to an increase of fatty acid oxidation or in the fatty acid
305 synthesis. More experiments would be necessary to answer it. In addition, animals pre-treated
306 with metformin and subsequently injured with 3-AP did not show signs of ataxia, and, at the
307 same time, they showed modulation of free fatty acid metabolism, suggesting that AMPK
308 activation is intrinsically related to ataxia.

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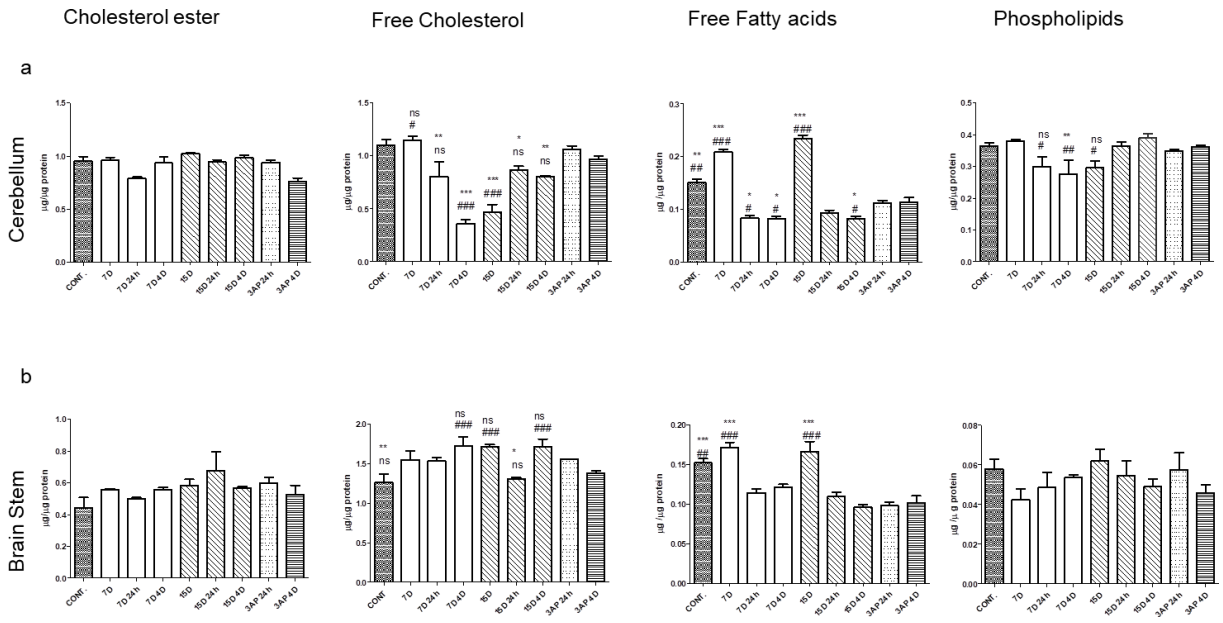

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311 **Fig.3** Quantitative analysis of cholesterol ester, free cholesterol, free fatty acids and
312 phospholipids in the cerebellum and brainstem by TLC. The animals were divided into 9
313 experimental groups: CONT -control; 7D - pretreated with metformin for 7 days; 7D 24h-
314 pretreated with metformin for 7 days and euthanized 24 hours after injury; 7D 4D - pretreated
315 with metformin for 7 days and euthanized 4 days after injury; 15D - pretreated with metformin
316 for 15 days; 15D 24h - pretreated with metformin for 15 days and euthanized 24 hours after
317 injury; 15D 4D - pretreated with metformin for 15 days and euthanized 4 days after injury; 3AP
318 24h - euthanized 24 hours after injury; 3AP 4D- euthanized 4 days after injury

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320 **3. Activation of AMPK by metformin modulates saturated and unsaturated fatty acids**

321 Besides the role of free fatty acids discussed above, they also play a central role in
322 neuroinflammation [61]. Alterations found in the content of free fatty acids, instigated us to

323 identify and classify free fatty acids regarding their role in cerebellar ataxia. The results showed
324 a significant decrease in the levels of saturated fatty acids, such as palmitic and stearic acid, in
325 ataxic animals pre-treated with metformin, when compared to the injured animals only (Figure
326 4). The involvement of these saturated fatty acids in the central nervous system is still unclear.
327 Gupta et al. [62] showed that the presence of palmitic and stearic acid induced the release of pro-
328 inflammatory cytokines, such as TNF-α and IL-6 in astrocytes. Similar results were reported in a
329 study on Parkinson's disease [63]. On the other hand, linoleic and arachidonic acid, products of
330 the pro-inflammatory pathway, were found at high levels in injured animals without prior AMPK
331 activation. The opposite was observed in the amount of polyunsaturated fatty acids (PUFAs)
332 involved in the anti-inflammatory pathway, such as α-linolenic and docosapentaenoic acid, when
333 animals were pre-treated with metformin and then injured with 3-AP (Figure 4). Since all groups
334 had comparable levels of essential fatty acids, we suggest that AMPK activation modulates
335 inflammatory pathways during cerebellar ataxia induced by 3-AP. The fatty acids AL and ALA
336 share the same converting enzyme to initiate their respective pathways, suggesting that the
337 modulation of fatty acids observed in both pathways is due to the activation of AMPK, and a
338 possible action of these molecules in the converting enzymes of these fatty acids. Therefore, here
339 we emphasize the importance of these analyses to help elucidate how fatty acids are related to
340 both AMPK activation and cerebellar ataxia. The molecular mechanisms involved in the
341 modulation exerted by this enzyme in inflammatory pathways are still unknown, requiring a
342 detailed molecular approach.

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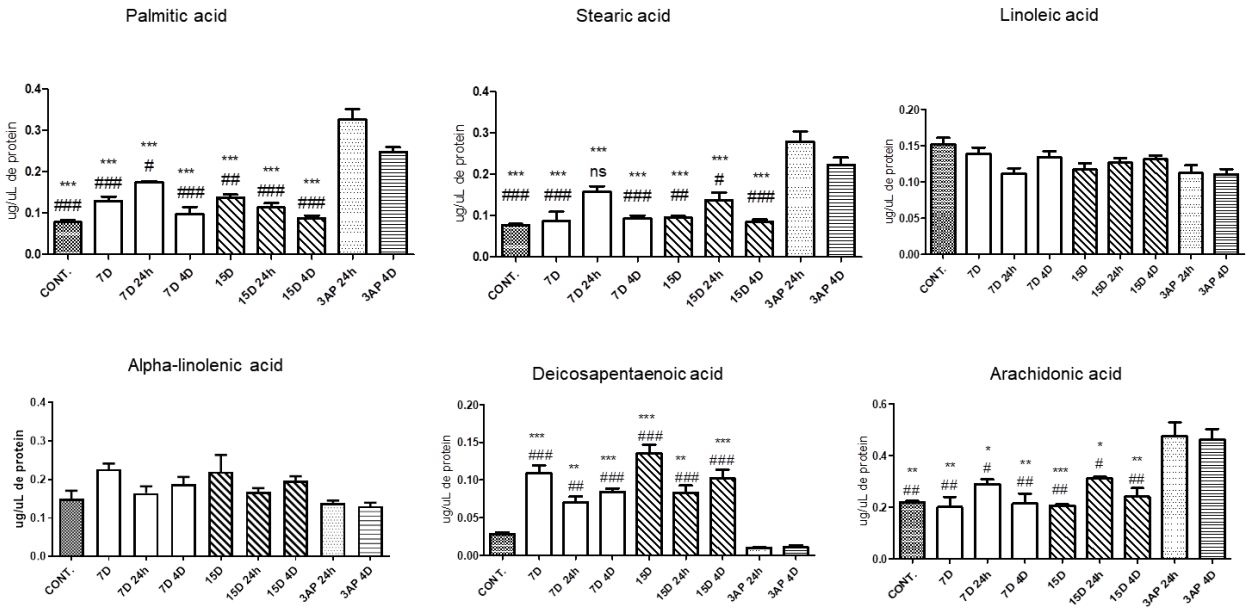

347 **Fig.4** Quantitative analysis of fatty acid in the cerebellum by GC/MS. The animals were divided
348 into 9 experimental groups: CONT -control; 7D - pretreated with metformin for 7 days; 7D 24h-
349 pretreated with metformin for 7 days and euthanized 24 hours after injury; 7D 4D - pretreated
350 with metformin for 7 days and euthanized 4 days after injury; 15D - pretreated with metformin
351 for 15 days; 15D 24h - pretreated with metformin for 15 days and euthanized 24 hours after
352 injury; 15D 4D - pretreated with metformin for 15 days and euthanized 4 days after injury; 3AP
353 24h - euthanized 24 hours after injury; 3AP 4D- euthanized 4 days after injury. Results are
354 representative of three independent experiments. Data represent mean ± SD. *** P<0.05 versus
355 control (One-way ANOVA)

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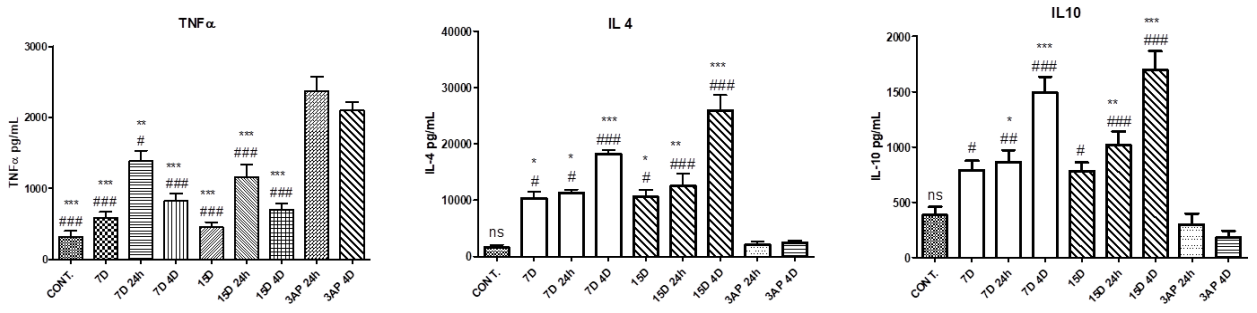
357 **4. Metformin-induced AMPK activation modulates inflammatory cytokines**

358 Neuroinflammation is the main cause of neurodegenerative diseases [29, 64]. Activated
359 glial cells release different pro-inflammatory cytokines, chemokines, and reactive oxygen species
360 (ROS). On the other hand, anti-inflammatory cytokines synthesized by regulatory T cells and
361 neuropeptides secreted by neurons and other cells on CNS protect neurons against
362 neuroinflammation [64]. Evidence confirms that neuroinflammatory processes involving
363 immune cells, glial cells, and neuronal cells are crucial and fundamental for understanding the
364 pathogenesis of a disease, such as the progressive loss of dopaminergic neurons in Parkinson's
365 disease [65–67]. Regarding cerebellar ataxia, our data showed the presence of lipid mediators
366 involved in anti- and pro-inflammatory pathways, possibly under the influence of AMPK
367 activation. Based on this, we verified a possible impact on this modulation on the levels of pro-
368 and anti-inflammatory cytokines. Our results showed levels of TNF-α in the ataxic groups
369 without pre-treatment with metformin significantly higher than those of all other groups (Figure
370 5). The opposite was found regarding anti-inflammatory cytokines, IL-4 and IL-10 (Figure 5).
371 Thus, it`s possible notice that in ataxic groups conditioned to daily pre-treatment with
372 metformin, both groups of animals, with 7 and 15 days of pre-treatment, showed low levels of
373 the pro-inflammatory cytokines and high levels of cytokines. These findings confirm our data on
374 fatty acids analysis involved in inflammation and point to a possible role of these lipid mediators.

375375



376 **Fig.5** Plasma levels of both proinflammatory and anti-inflammatory cytokines. The animals were
377 divided into 9 experimental groups: CONT -control; 7D - pretreated with metformin for 7 days;
378 7D 24h- pretreated with metformin for 7 days and euthanized 24 hours after injury; 7D 4D -
379 pretreated with metformin for 7 days and euthanized 4 days after injury; 15D - pretreated with
380 metformin for 15 days; 15D 24h - pretreated with metformin for 15 days and euthanized 24
381 hours after injury; 15D 4D - pretreated with metformin for 15 days and euthanized 4 days after
382 injury; 3AP 24h - euthanized 24 hours after injury; 3AP 4D- euthanized 4 days after injury.
383 Results are representative of three independent experiments. Data represent mean ± SD. ***
384 $P<0.05$ versus control (One-way ANOVA)

385 Interestingly, animals that received metformin showed induction of anti-inflammatory
386 pathways, probably supported by the presence of fatty acids involved in anti-inflammation, such
387 as docosapentaenoic acid, which synthesizes mediators that pro-resolve inflammation and
388 possibly induce the release of high levels of anti-inflammatory cytokines, such as IL-4 and IL-
389 10. Therefore, AMPK activation may potentiate the endogenous synthesis of pro-inflammatory
390 mediators, after inducing neurological injury and secondary inflammation. The pro-
391 inflammation-resolving mediators possibly provide a cellular target with approaches to limit
392 inflammatory processes after neurological injury, preventing the emergence of secondary lesions,
393 resulting from the worsening of the neuroinflammatory condition. The potent anti-inflammatory
394 properties of pro-resolving mediators in peripheral diseases [68] and the therapeutic efficacy of
395 their precursors, fatty acids, in neurological disease provide a solid basis for further exploration
396 of their neuroprotective efficacy in neurodegenerative diseases. The endogenous nature of pro-
397 resolution mediators makes them promising candidates for readily accessible therapies, which

398 could alter the inflammatory balance to resolve cellular pathophysiology and limit the extent of

399 injury (Figure 6).

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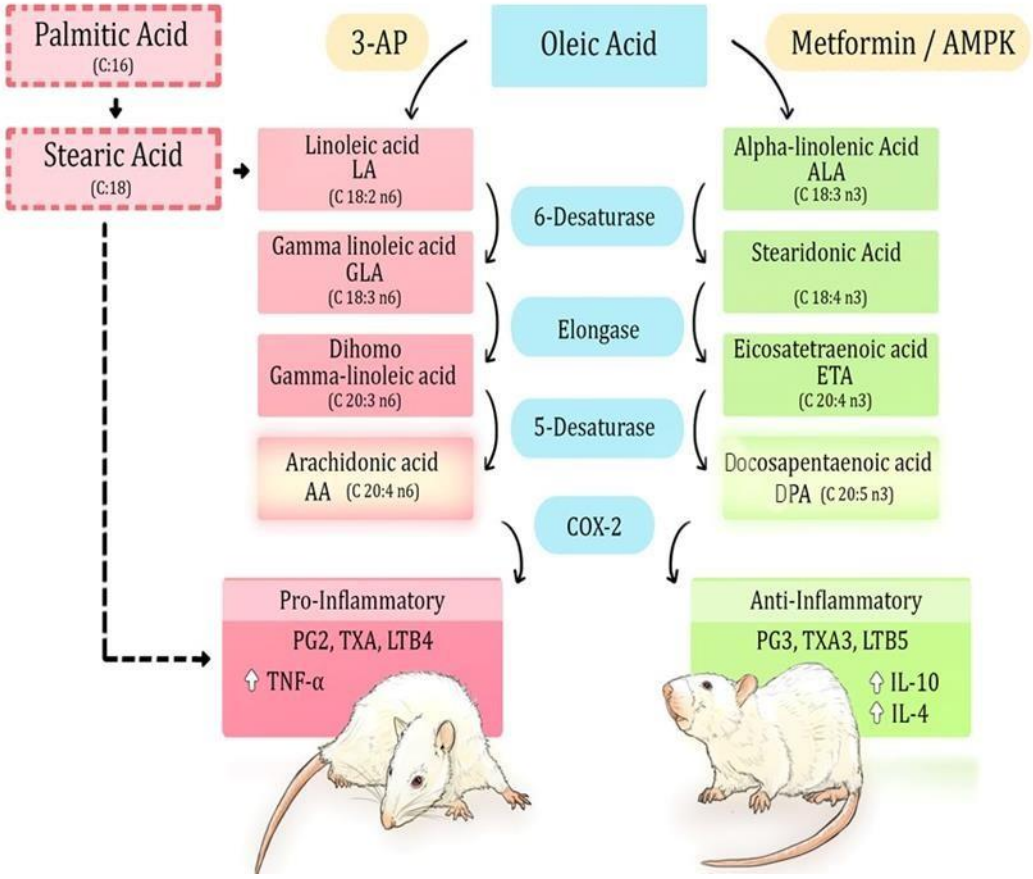

401 **Fig.6** Schematic model for the neuroprotective role of metformin/AMPK pathway against

402 induction of cerebellar ataxia by 3-AP. Our data demonstrates that induction of AMPK activation

403 by metformin inhibits the production of inflammatory cytokines and induces the production pro-

404 infammatory cytokines.

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Author Contribution

All authors contributed to the study conception and design. Material preparation and data collection were performed by Taina Correa Atella, George Kluck, and Jorge Medina. Data analyses were performed by Taina Correa Atella, George Kluck, and Jorge Medina. Supervision was under Georgia Atella and Silvana Allodi. The first draft of the manuscript was written by Taina Correa Atella, George Kluck, Jorge Medina, and Silvana Allodi. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Declarations

Ethics Approval: Not applicable.

Consent to Participate: Not applicable.

Consent for Publication: Not applicable.

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