Quercetin ameliorates mitochondrial dysfunction and mitigates methamphetamine-induced anxiety-like behavior

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24 **ABSTRACT**

25 Methamphetamine (MA) abuse results in neurotoxic outcomes, including increased
 26 anxiety and depression, during both MA use and withdrawal. Although numerous studies
 27 have reported an association between MA exposure and anxiety, the underlying
 28 mechanism remains elusive. In this study, escalating dose of MA was used to establish an
 29 MA-treated mouse model presenting anxiety behavior. RNA seq was then performed to
 30 profile the gene expression patterns in the hippocampus (HIPP). Differentially expressed
 31 genes (DEGs) were identified and function enrichment analysis was conducted to explore
 32 the underlying mechanisms. Quercetin as an mitochondria protector was used *in vivo* and
 33 *in vitro*. The C57BL/6J mice were co-treated with 50 mg/kg Quercetin and escalating MA.
 34 Anxiety behavior was evaluated by utilizing the elevated plus maze and the open field test.
 35 Transmission electron microscopy and immunohistochemistry were conducted to study the
 36 pathology of MA-induced anxiety . The effects of MA and Quercetin on astrocytes were
 37 investigated by fluorescence staining, transmission electron microscopy, flow cytometry,
 38 and oxygen consumption rate. Western blot and qPCR were performed to analyze altered
 39 protein and gene levels of HIPP in mice and astrocytes. The results demonstrated that
 40 fourteen upregulated differentially expressed genes were identified and significantly enriched
 41 in signaling pathways related to psychiatric disorders and mitochondrial function.
 42 Interestingly, we found that quercetin was able to alleviate MA-induced anxiety-like
 43 behavior by improving neuron number and mitochondria injury. Mechanistically, quercetin
 44 can mitigate aberrant mitochondrial morphology and mitochondrial dysfunction not only by
 45 decreasing the levels of total cytoplasmic reactive oxygen species (ROS),
 46 mitochondria-derived ROS (mtROS), and mitochondrial membrane potential (MMP), but
 47 also increasing the oxygen consumption rate (OCR) and mitochondrial ATP production *in*
 48 *vitro*, indicating Quercetin ameliorated MA-induced anxiety-like behavior by modifying
 49 mitochondrial morphology and function. Furthermore, quercetin reversed OPA1 and DRP1
 50 expression in astrocytes, and mitigated astrocyte activation and the release of inflammatory
 51 factors, which can trigger neuronal apoptosis and synaptic loss. Taken together, we
 52 provided evidence showing that MA can induce anxiety-like behavior via the induction of
 53 oxidative stress and mitochondrial dysfunction. Quercetin exerted antipsychotic activity

54 through mitochondrial modulation, suggesting its potential for further therapeutic
55 development in MA-induced anxiety.

56

57 **Keywords: methamphetamine, anxiety, mitochondrial dysfunction, oxidative stress,**
58 **quercetin**

59 INTRODUCTION

60 As a highly addictive psychostimulant drug, methamphetamine (MA) abuse is an
61 increasingly common worldwide phenomenon, resulting in significant physical, behavioral,
62 cognitive, and psychiatric outcomes (Homer et al., 2008; Meredith et al., 2005;
63 Glasner-Edwards et al., 2014). Epidemiological studies have shown that amphetamine-type
64 stimulants represent the most widely used illicit drugs in the world after cannabis, with ≤51
65 million global users between the ages of 15 and 64 years (Wearne et al., 2018). Among
66 abusers, 72%–100% experience MA-induced psychotic reactions (Srisurapanont et al.,
67 2003; Smith et al., 2009), and 30.2% of chronic MA users are diagnosed with anxiety
68 (Hellem et al., 2016).

69 Human neuroimaging studies have shown that MA users experience significant
70 changes in multiple brain regions, including the orbitofrontal cortex, striatum, amygdala,
71 hippocampus, and insula, which are involved in a variety of functional networks, including
72 the salience network, limbic system, and frontostriatal circuit (May et al., 2020; Uhlmann et
73 al., 2016). Evidence has demonstrated that MA administration can evoke changes in
74 behavior, synaptic transmission, and volume in the hippocampus and cortex (Golsorkhdan
75 et al., 2020; Uhlmann et al., 2016), resulting in the dysregulation of neurotransmitters and
76 their receptors in these regions (Uhlmann et al., 2016), often accompanied by oxidative
77 stress, apoptosis, and autophagy (Huang et al., 2017).

78 A limited number of studies have preliminarily demonstrated that both oxidative stress
79 and mitochondrial dysfunction may play roles in the pathology of anxiety (Chang et al.,
80 2007; Kohno et al., 2018). Excessive dopamine induced by MA is thought to trigger the
81 overproduction of reactive oxygen species (ROS) by the mitochondria and relevant
82 enzymes, exacerbating neurodegenerative diseases (Shin et al., 2017), suggesting that
83 mitochondrial functional processes may play major roles in the brain abnormalities
84 associated with MA-induced anxiety. Increasing experimental evidence has supported the
85 existence of a link between mitochondrial dysfunction, brain dysfunction, and
86 neuropsychiatric disorders (Pei et al., 2018; Wallace et al., 2017; Filiou et al., 2019).
87 Suboptimal mitochondrial function would be vulnerable to the stress-associated depletion of

88 the brain's energy resources, resulting in the development of psychiatric disorders (e.g.,
89 anxiety and depression) (Morava et al., 2013). However, the mechanisms underlying the
90 etiology of MA-induced anxiety remain poorly understood.

91 Research has suggested that the treatment of co-occurring psychiatric disorders,
92 including depression and anxiety, may also be important for preventing relapses (Su et al.,
93 2017; Glasner-Edwards et al., 2010). A substantial amount of literature has demonstrated
94 the efficacy of both first- and second-generation antipsychotic drugs for the treatment of
95 psychotic symptoms associated with MA-induced depression and anxiety (Chiang et al.,
96 2019; Shoptaw et al., 2009; Wang et al., 2016). However, adverse events are frequently
97 reported in these studies. Quercetin, which is a flavonoid-type secondary metabolite found
98 in foods and medicinal plants, is presumed to have antioxidant, anti-inflammatory,
99 immunoprotective, and anti-carcinogenic effects and was shown to mitigate anxiety-like
100 behaviors in mice by modulating oxidative stress and monoamine oxidase activity (Dhiman
101 et al., 2019), the preventing antioxidant enzyme impairment, regulating serotonergic and
102 cholinergic neurotransmission, and decreasing neuroinflammation and neuronal apoptosis
103 (Samad et al., 2018; Kosari-Nasab et al., 2019). Quercetin has been confirmed to be safe
104 when used as a single compound in dietary supplements in both animal and human studies,
105 and adverse effects following supplemental quercetin intake have rarely been reported
106 (Andres et al., 2018). Despite evidence that quercetin serves as an oxidative stress and
107 inflammatory modulator, no research has examined the effects of quercetin on anxiety-like
108 behaviors induced by chronic MA.

109 In this study, we aimed to determine whether quercetin intervention following chronic
110 MA use can mitigate anxiety-like behaviors by exploring the underlying molecular
111 mechanisms and targeted brain regions, combined with RNA-sequencing (RNA-seq). We
112 first constructed an animal model of MA-induced anxiety. The HIPP were isolated, and
113 RNA-seq was performed to identify differentially expressed genes (DEGs) between the MA
114 treated and control groups. Subsequently, the potential underlying pathways were analyzed
115 by Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, and the identified DEGs
116 were validated by quantitative reverse transcription-polymerase chain reaction (qPCR).
117 Finally, in view of the functional enrichment, we assessed the antianxiety effects of

118 quercetin in MA-treated mice to explore the underlying mechanism. These findings will
119 contribute to a better understanding of the molecular mechanisms of anxiety induced by MA
120 and allow for the therapeutic potential of quercetin against MA-induced anxiety to be
121 assessed.

122 MATERIALS AND METHODS

123 Animals and treatment

124 Mice were housed with a 12-h light/dark cycle (lights on at 7:00 AM). Behavioral testing is
125 performed between 9:00 AM and 6:00 PM. The experimental mice are transferred to the
126 behavioral testing room 30 min before the first trial to allow them to habituate to the room
127 conditions. All procedures were approved by the Committee on Ethics in the Use of
128 Animals from Kunming Medical University (CEUA no. kmmu2021227). MA was dissolved
129 in sterile saline to a concentration of 1 mg/ml as a stock solution. Quercetin was
130 purchased from Sigma-Aldrich Company (Sigma-Aldrich, MO, USA) and was first
131 dissolved in polyethylene glycol (PEG, Sigma-Aldrich), at a final concentration of 50
132 mg/kg in 20% PEG with 0.9% saline. Adult male C57BL/6 mice which weighed from 22 to
133 25 g were randomly divided into three groups (n = 12 each group): control, MA-treated,
134 and MA + quercetin (Q)-treated. The control group received normal saline injection
135 intraperitoneally; the MA group received escalating MA doses, as described in a previous
136 study (Manning et al., 2016), at 5, 10, and 15 mg/kg during the first, second, and third
137 weeks, respectively; and the MA + Q group received escalating MA doses and quercetin
138 treatment, administered with one dose at 50mg/kg daily for 4 days during the first week
139 and 5 days in the second week, as described in a previous study (Zhang et al., 2019). On
140 the 22nd day, the open field (OFT) and Elevated Plus Maze (EPM) tests were used to
141 examine the motor activity and anxiety levels, respectively. After behavioral testing, the
142 animals were sacrificed immediately, and subsequent experiments were conducted.

143 Conditioned place preference test (CPP)

144 The CPP apparatus consisted of two compartments: one had black and white striped
145 walls, a white floor, and a black ceiling; the other had black and white checkered walls, a
146 black floor, and a white ceiling. The two compartments were separated by a removable
147 board. Behavioral subjects were habituated for 5 min and placed in the experimental
148 environment for adaptation for 3 days before the CPP test.

149 Pre-test, conditioning, and a test were included in the MA CPP session. During the
150 pre-test phase, the mice were placed in the middle of the conditioning apparatus and

151 allowed to freely explore the full extent of the CPP apparatus for 15 min. The time spent in
152 each chamber was measured. Mice that spent >65% (>585 s) or <35% (<315 s) of the total
153 time (900 s) on one side were eliminated from subsequent CPP experiments (Zhou et al.,
154 2019). Conditioning was conducted on mice confined to one chamber for 30 min, which was
155 paired with an intraperitoneal (i.p.) MA injection on days 1, 3, 5, and 7, and on days 2, 4,
156 and 6, the mice were confined to the other chamber for 30 min, which was paired with an i.p.
157 saline injection. For the CPP test, mice were released from the middle part of the CPP
158 apparatus and allowed to freely explore both chambers for 15 min. The CPP score was
159 calculated by subtracting the time spent within the saline-paired side from that spent on the
160 MA-paired side. Mouse behavior was analyzed using the ANY-maze video tracking system
161 (Stoelting Co.).

162 **Open Field Test (OFT)**

163 Each experimental animal was placed in the corner of the open field apparatus (50 ×
164 50 × 40 cm³, SANS Co., Jiangsu, China), which consisted of a white plastic floor and wall.
165 The OFT performance was recorded using a video camera attached to a computer and
166 controlled by a remote device. The total distance traveled (cm) and time spent in the center
167 area (20 × 20 cm²) were recorded during a 5-minute test period. After each trial, the whole
168 open field apparatus was cleaned with 75% ethyl alcohol to efficiently remove odor to
169 prevent any bias based on olfactory cues. The mouse behavior was analyzed using the
170 ANY-maze video tracking system (Stoelting Co.).

171 **Elevated plus maze test (EPM)**

172 The EPM consisted of two open arms (30 × 5 cm) and two enclosed arms of the same
173 size, with 15 cm white plastic walls. The four arms were connected by a central square (5 ×
174 5 cm) (SANS Co., Jiangsu, China). The arms were elevated 55 cm above the floor. Each
175 experimental mouse was placed in the central square of the maze, facing one of the
176 enclosed arms. The number of entries into each arm and the time spent in the open arms
177 were recorded during a 5-min test period. When a mouse falls from the maze, the data are
178 excluded. After each trial, all arms and the center area were cleaned with 75% ethyl alcohol,
179 as previously described. All experimental data were collected and analyzed described.

180 **RNA preparation, library construction, and sequencing**

181 Total RNA was isolated using RNA-Bee reagent, following the manufacturer's protocol.
182 RNA purity was determined using the NanoPhotometer spectrophotometer (IMPLEN, CA,
183 USA), and the concentration was determined using the Qubit RNA Assay Kit (Life
184 Technologies, CA, USA). Samples with RNA integrity values >7.0 were used for the
185 following experiments, which were assessed by the RNA Nano 6000 Assay Kit of the
186 Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

187 Sequencing libraries were prepared using the NEB Next Ultra RNA Library Prep Kit
188 (Illumina, USA), according to the manufacturer's recommendations, which were described
189 in our previous research (Sun et al., 2020). In brief, mRNA was purified using poly-T
190 oligo-attached magnetic beads, fragmentation was performed using divalent cations, and
191 library quality was assessed on the Agilent Bioanalyzer 2100 and qPCR. The clustering of
192 the index-coded samples was performed on an acBot Cluster Generation System using
193 TruSeq PE Cluster Kitv3-cBot-HS (Illumina, San Diego, CA, USA). The library preparations
194 were then sequenced on an Illumina Hiseq platform, and paired-end reads were generated.

195 **Quantification and differential expression analysis of mRNA**

196 As mentioned in Sun et al. (Sun et al., 2020), the reference genome index was built,
197 and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5.
198 Feature Counts v1.5.0-p3 was used to count the reads numbers. The expected number of
199 fragments per kilobase of transcript sequence per millions (FPKM) of base pairs sequenced
200 was calculated based on the gene length, and read counts were mapped to the gene.

201 Differential expression analysis ($n = 3$ per group) was performed using the DESeq2 R
202 package (1.16.1). P -values were adjusted using the Benjamini and Hochberg approach for
203 controlling the false discovery rate (FDR), and $p < 0.05$ and $\log_2$ fold-change > 2 were
204 considered to be significant DEGs. DEGs enrichment in the KEGG pathways was assessed
205 using the web tool Metascape (<http://metascape.org>).

206 **Immunofluorescence staining and imaging**

207 Mice were deeply anesthetized and perfused with 25 ml ice-cold PBS, followed by 25
208 ml 4% ice-cold paraformaldehyde (PFA) in PBS. Brains were removed and dehydrated with

209 15% and 30% sucrose at 4°C. The fixed brains were sliced into 30-µm-thick sagittal slices
210 using a Leica CM1950. Slices were permeabilized in 1.2% Triton X-100 in PBS for 15 min
211 and subject to incubation in blocking solution. Slices were incubated with primary
212 antibodies for NeuN (1:200, Abcam), glial fibrillary acidic protein (GFAP, 1:500, Abcam) for
213 24 h at 4°C, followed by incubation with species-matched and Alexa Fluor conjugated
214 secondary antibodies raised in rabbit (1:5000, Invitrogen) for 2 h at room temperature.
215 4',6-Diamidino-2-phenylindole (DAPI, 1:1000, Invitrogen) was incubated after secondary
216 antibody incubation for 15 min at room temperature. Slices were mounted and coverslipped
217 using VECTASHIELD H-1000 mounting medium and scanned on a Nikon C2 confocal
218 microscope using NIS-Element software.

219

220 **H&E staining and Electron microscope imaging**

221 The brain tissues were fixed in 4% paraformaldehyde (PFA) immediately after sacrifice.
222 H&E staining was performed on 5µm paraffin sections using standard H&E staining
223 protocol which were described previously (Sun et al., 2020). The thin sections were made
224 with an ultramicrotome, stained by OsO₄. Electron microscopy and ultrastructural studies
225 were performed on a transmission electron microscope (JEM-1400Flash).

226 **Measurement of ATP, mitochondrial membrane potential (MMP), and reactive oxygen 227 species (ROS)**

228 Intracellular ATP was determined using a firefly luciferase-based ATP assay kit (Beyotime,
229 Beijing, China) based on a fluorescence technique. In brief, astrocytes (1×10^4) were
230 plated in 96-wells, and appropriate drug treatments were applied for 48 h. Opaque-walled
231 96-well plates with culture media (50 µL) were prepared. Luminescence test solution (50
232 µL) was added and incubated for 30 min and then measured using a luminescence
233 microplate reader. Mitochondria-derived ATP was measured after treatment with 300 mM
234 iodoacetic acid (IAA, Sigma-Aldrich, MO, USA). IAA was added to half of the wells and
235 the cells were then incubated at 37 °C and 5% CO₂ for 60 min. After 30 min, 1 mM
236 oligomycin was added to half of the wells containing IAA and incubated for 30 min to
237 abolish all ATP production and confirm that the ATP levels in the presence of IAA were

238 produced by the mitochondrial ATP synthase. Subsequently, the media was removed
239 from the wells and cellular ATP was measured using ATP assay kit (mentioned before).

240 JC-1 assay was conducted to analyze the MMP using the JC-1 mitochondrial
241 membrane potential assay kit (Beyotime, Beijing, China). In brief, astrocytes (2×10^5) were
242 plated in 6-well plates and, after appropriate drug treatments, stained with 10 μ M JC-1 for
243 20 min. JC-1 exhibits double fluorescence staining, either as red fluorescent J-aggregates
244 (530 nm excitation/590 nm emission, as P3) at high potentials or as green fluorescent
245 J-monomers (490 nm excitation/530 nm emission, as P2) at low potentials; Flow cytometric
246 analysis was performed by fluorescence-assisted cell sorting (FACS) after JC-1 staining
247 detected changes in MMP and the value was calculated. The relative proportion of red and
248 green fluorescence was used as an index of change in membrane potential.

249 For ROS generation measurements, primary astrocytes were plated in 96-wells, and
250 appropriate drug treatments were applied for 48 h. Then, cells were loaded with the ROS
251 probe 70-dichlorodihydrofluorescein diacetate (DCFDA, 2 μ M) for 40 min at RT in the dark.
252 The mROS assay was conducted using the MitoSOX red mitochondrial superoxide
253 indicator (Mercury Drive, Sunnyvale, CA). Cells were measured by FACS at 488 nm
254 excitation.

255 **Oxygen consumption rate (OCR) analysis**

256 Astrocytes were plated at 7.5×10^4 /well in seahorse assay plates and treated with their
257 respective treatments at 37°C and 5% CO₂ and for 24 h. Mitochondrial oxygen consumption
258 rate (OCR) was measured by extracellular flux (XF) assay (Seahorse XFP analyzer, Agilent
259 Technologies, Santa Clara, CA), according to manufacturer's procedures. Briefly, cells were
260 incubated in a CO₂-free environment for 1 h, and OCR was measured every 3 min for the
261 next 90 min. First, OCR was acquired in basal conditions (20 mM glucose), followed by in
262 the presence of 1.5 μ M oligomycin (ATP synthase inhibitor), with 3.5 μ M carbonyl
263 cyanide-p-trifluoromethoxy phenylhydrazone (FCCP), and, finally, with 0.5 μ M
264 rotenone/antimycin A.

265 **Quantification of gene expression assay**

266 Quantification of gene expression was performed by ABI 7500 Sequence Detection System

(Applied Biosystems, Foster City, CA, USA). The qPCR assays were performed as described in our previous study (Chen et al., 2016). The primers used for qPCR are shown in **Table S1**. The mRNA levels were determined by qPCR in triplicate for each of the independently prepared RNA samples, and mRNA levels were normalized against the levels of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression.

Western blotting analysis

Total protein was extracted from astrocytes or brain tissue and quantified by bicinchoninic acid (BCA) protein assay kit (Pierce, USA) and used for immunoblotting analysis, as described previously. Briefly, the blot was incubated with a specific primary antibody overnight (anti- GFAP, 1:1000, Abcam; anti-MFN2, 1:200, Abcam; anti-OPA1, 1:200, Abcam; anti-DRP1, 1:200, Abcam), followed by incubation with HRP-conjugated secondary antibody. The bands were detected with a chemiluminescence detection kit (Millipore Co., MA, USA) and scanned using the iBright FL1500 chemiluminescence imaging system (Thermo Fisher Scientific, USA).

Statistical analysis

The results are presented as the mean $\pm$ standard error of the mean (SEM). For comparisons between two or multiple groups, the Student's t-test or one-way analysis of variance (ANOVA) analysis was conducted, respectively. Significance is indicated by asterisks: * p <0.05, ** p <0.01, *** p <0.001.

RESULTS

Repeated MA administrations induce anxiety-like behavior in mice

To identify an optimal concentration for the generation of an MA-addicted mouse model, we tested three different MA concentrations (2.5, 5, and 10 mg/kg, i.p.). Mice are trained to associate the MA reward with the paired context during training for the MA CPP (**Figure 1A**). A preference for the MA-paired side indicates the expression of a reward–context associated memory, which was assessed by measuring the time that an animal spent on the MA-paired side in the CPP apparatus. After four sessions of MA CPP training, mice

295 treated with 5 mg/kg MA showed a significant preference for the MA-paired side. The 2.5
296 mg/kg MA group (**Figure 1B**, $p < 0.05$) also showed a significant preference for the
297 MA-paired side. However, 6 of 8 mice died in the 10 mg/kg MA-treated group. Therefore,
298 we chose 5 mg/kg MA as the initial concentration to establish a subsequent mouse model
299 (**Figure 1C**).

300 Mice with 3-week MA-treated mice demonstrated a fear of entering the open arms of
301 the EPM test (**Figure 1D**) with fewer numbers of entries to open zone and less time (** $p <$
302 0.01) and distance(* $p < 0.05$) spent in the open zone. Consistently, MA treatments also
303 displayed decreased locomotor activity and spent significantly less time in the center of the
304 OFT than control mice (**Figure 1E**, * $p < 0.01$), suggesting that we successfully generated
305 an MA-treated mouse model with anxiety-like behaviors.

306

307 **RNA-seq revealed differentially expressed genes in the HIPP in the MA-treated** 308 **mouse model**

309 To better elucidate the molecular features of MA-induced anxiety-like behaviors in mice,
310 we performed RNA-seq to investigate DEGs between control and chronic MA-treated mice
311 in HIPP. Hierarchical clustering analysis was performed and displayed in **Figure 2A**.
312 Forteen upregulated genes were identified between the control and MA-treated groups,
313 which were displayed in **Supplementary Table 1**.

314 To investigate the altered signaling pathways involved in mice presenting with
315 MA-induced anxiety-like behavior, we performed KEGG enrichment analyses. DEGs
316 between the control and MA-treated groups were mainly enriched in signaling pathways
317 associated with protein folding (chaperone-mediated protein folding, response to unfolded
318 protein, chaperone cofactor-dependent protein refolding, 'de novo' posttranslational protein
319 folding, 'de novo' protein folding, cellular response to unfolded protein), synaptic plasticity
320 and synaptic transmission (regulation of synaptic plasticity, positive regulation of synaptic
321 transmission), rhythmic processes (regulation of synaptic plasticity, positive regulation of
322 synaptic transmission), protein phosphorylation (negative regulation of phosphorylation,
323 negative regulation of protein phosphorylation, peptidyl-threonine dephosphorylation),
324 oxidative stress, and the intrinsic apoptotic signaling pathway (**Figure 2B**). As Mitochondria

couple with the endoplasmic reticulum (ER) are important in protein folding (Marchi, et al. 2014). These results suggested mitochondrial and ER play an important role in MA-induced anxiety and might be promising therapeutic targets.

Furthermore, as immediate early genes (IEGs) have been reported to be involved in methamphetamine use and the development of neuropsychiatric disorders (McCoy et al., 2011; Gallitano et al., 2020), we used three samples from each group to validate four selected DEGs classified as IEGs (*Fos*, *Egr1*, *Arc* and *Nr4a1*). The results revealed that, in general, MA upregulated all the four IEGs identified, in accordance with the RNA-seq results (Figure 2D).

Administration of quercetin ameliorates anxiety-like behaviors in an MA mouse model

Quercetin has been reported to mitigate anxiety-like symptoms in a lipopolysaccharide-induced mouse model of anxiety (Lee et al., 2020; Samad et al., 2018) and has been reported as a mediator of mitochondrial function and ER stress (Khan et al., 2016). Therefore, we evaluated the effects of quercetin on MA-induced behavioral phenotypes and the role played by quercetin in mitochondrial functional modifications in the present study (Figure 3A).

In the EPM test, the time spent and the number of entries into the open arms were significantly reduced in the MA-treated group compared with those in the control group (Figure 3B, $p < 0.05$). Conversely, when compared with the control group, the time spent and number of entries into the closed arms were significantly elevated in the MA-treated group (Data not shown). However, mice treated with MA+ and quercetin showed the significant restoration of the time spent and the number of entries into the open arms compared with those for the single MA-treated group (Figure 3B, $p < 0.05$), with no significant differences observed between the control group and the MA+Q group (Figure 3B, $p > 0.05$).

The OFT results revealed that a single administration of MA significantly reduced the number of times MA-treated mice crossed in the central zone compared to the saline-treated group (Figure 3C, $p < 0.05$), but no significant difference in the number of crossings in the peripheral zone were observed (Data not shown). Quercetin combined

355 with MA treatment significantly enhanced the number of central zone crossings compared
356 with the single MA-treated group (**Figure 3C**, $p < 0.05$).

357

358 **Quercetin alleviates neuronal injury and decreases astrocyte activation induced by** 359 **MA *in vivo***

360 Most of previous researches have focused on MA-induced neuronal injury.
361 Nevertheless astrocytes have been shown to participate in anxiety development through
362 the synaptic pruning of neurons (Çalışkan et al., 2020), and they were reported to be
363 activated in an MA-treated animal model, resulting in morphological and phenotypic
364 changes (Zhou et al., 2019). To assess the change of neurons and astrocytes in anxiety
365 induced by MA, we first assessed the numbers of neuron by immunostaining for NeuN and
366 pathological alterations by H&E staining. And then to evaluate astrocytes activation by
367 immunostaining and western blotting for GFAP. The results demonstrated MA
368 administration markedly decreased the numbers of neurons (**Figure 4A**), numerous
369 impaired neurons with karyopyknosis, cell gaps, and debris in dentate gyrus (**Figure 4B**).
370 Furthermore, after MA treatment GFAP expression increased (**Figure 4G**, $p < 0.05$) with a
371 larger area of astrocytes (**Figure 4C-E**, $p < 0.01$). Quercetin rescued the decreasing of
372 neuron numbers and neuron damage, and attenuated the activation of astrocytes (**Figure**
373 **4C-E**, $p < 0.01$), suggesting that MA treatment facilitated neuronal loss and astrocytes
374 activation, whereas quercetin reversed these cellular phenotypes.

375 We also evaluated mitochondrial morphology in the HIPP using electron microscopy.
376 The control group presented with relatively long, tubular mitochondria, whereas the
377 mitochondria in the HIPP of MA-treated mice were relatively more fragmented and swollen,
378 with the loss of cristae (**Figure 4D**), indicating MA-induced damage to mitochondria in HIPP.
379 Quercetin treatment was able to reinstate mitochondrial morphology toward long tubular
380 mitochondria with intact cristae in MA-treated mice (**Figure 4D**).

381

382 **Quercetin ameliorates mitochondrial dysfunction and aberrant morphology in** 383 **astrocytes *in vitro***

384 To assess the effect of MA on mitochondria *in vitro*, we conducted experiments in
385 astrocytes. We found that when astrocytes were exposed to MA, tubular mitochondria
386 shortened in length and swelled in width, forming large, spherical structures (**Figure 5A**),
387 and similar morphological changes in mitochondria were viewed using mitotracker (**Figure**
388 **5B**). quercetin treatment rescued abnormal mitochondrial morphology in astrocytes treated
389 with MA (**Figure 5A-B**).

390 In addition, mitochondria have been considered to serve as the intracellular source of ROS

in animal cells, and when confronting with oxidative stress, mitochondrion is the primary target attacked by ROS, and also produces excessive amounts of ROS due to the damage to enzymes in the electron transport chain. Therefore, we first evaluated the total ROS by DCFDA and the mitochondrial ROS by MitoSOX staining and quantified the fluorescence intensity in MA-treated astrocytes with or without quercetin treatment. The results showed that both the total ROS and mitochondrial ROS were markedly increased in MA treated group, which were rescued by quercetin (**Figure 5D**). A series of redox reactions creates an electrochemical gradient through the mitochondrial electron transport chain, which drives the synthesis of ATP (Mitchell et al., 1961) and generates the MMP. In this study, we evaluated the ability to generate total ATP and mitochondria-derived, and measured the MMP to represent mitochondrial function. The results revealed that the quercetin supplementation of MA-treated astrocytes markedly elevated both the total and mitochondria-derived ATP levels (**Figure 5E**) and reversed the observed decrease in MMP in MA-treated astrocytes (**Figure 5F**). We used an XF24 metabolic bioanalyzer to assess the effects of quercetin supplementation on OCR and glycolytic flux rates in MA-treated astrocytes and found that quercetin markedly reinstated the decrease in the OCR induced by MA (**Figure 5G**).

408

409 DISCUSSION

In the present study, we found that quercetin attenuated anxious symptoms and pathology, and modified mitochondria in both a mouse model and cultured astrocytes treated with MA. These findings, for the first time, suggested that quercetin could inhibit the progression of anxiety induced by MA by modulating mitochondrial function and morphology in the central nervous system. These results indicate that quercetin represents a potential therapeutic medicine for future development and support the hypothesis that changes in mitochondrial function mediate anxiety progression.

We found that MA-induced anxiety-like behavior in mice, as previously described (Ru et al., 2019). Consistent with our findings, Iwazaki et al (Iwazaki et al., 2008) have demonstrated synaptic plasticity- and synaptic transmission-, oxidative stress-, and

intrinsic apoptotic-related signaling pathway were dysregulated, implying that mitochondria as a target organelle in MA-induced anxiety. Mitochondria, which are capable of generating secondary ROS and amplifying initial oxidative insults, are both a source and a target of oxidative stress (Song et al., 2017). Mitochondrial dysfunction can also be triggered by the accumulation of misfolded proteins (Saito et al., 2018) and failed to maintain synaptic ion homeostasis and synaptic plasticity due to decreased ATP production and overloaded Ca^{2+} concentrations (Fang et al., 2015). Furthermore, mitochondria function which play an important role in apoptosis via the intrinsic apoptotic program was identified to be dysregulated in frontal cortex in MA-treated rat (Iwazaki et al., 2008). The parameters about mitochondria function were aberrant in our results. Based on these, we speculate that mitochondria represent a primary target organelle of MA treatment in the HIPP.

Furthermore, four identified IEGs (*Fos*, *Egr1*, *Arc*, and *Nr4a1*) in our animal model, which were reported to change in chronic and acute MA-treated mice (Cheng et al., 2015; Akiyama et al., 2008; McCoy et al., 2011), were validated by qPCR in the transcriptomics analysis of chronic MA-treated mouse brains. The changes observed in these genes by qPCR and western blot were consistent with our RNA-seq results. Nr4a1 (also known as TR3 or NGFI-B) is an orphan member of the nuclear receptor superfamily, which migrates from the nucleus to the mitochondria, where it binds to Bcl-2 to induce apoptosis and cause the release of cytochrome c (Liu et al., 2008). According to previous reports, Nr4a1 can evoke cellular oxidative stress and disrupt ATP generation (Zhang et al., 2018), and the expression and activity of Nr4a1 are sustained by chronic stress in animal models and in human studies of neuropathologies sensitive to the buildup of chronic stress (Jeanneteau et al., 2018). Nr4a1 has been deemed to be important for regulating metabolic and morphological aspects of neuronal functions by modifying the expression of several mitochondrial regulatory genes, including *Mfn1*, *Mfn2*, *Fis1* and *OPA1* (Jeanneteau et al., 2018), which can result in mitochondrial dysfunction and behavioral phenotypes. Thus, we speculated that quercetin might modulate genes associated with mitochondrial function via downregulating Nr4a1. We then detected Mfn2, OPA1 and Drp1 expression at both the protein and mRNA levels after quercetin treatment. As

450 anticipated, quercetin reversed the MA-induced changes in Nr4a1, OPA1 and Drp1
451 expression at both the protein and mRNA levels (**Supplementary Figure 1**), but failed to
452 rescued Mfn2 expression downregulated by MA-treated. Overall, quercetin was capable
453 of targeting mitochondria to alleviate neuronal impairments and the anxiety-like behaviors,
454 probably through the Nr4a1–OPA1/ Drp1-mitochondrial morphology and functional
455 pathway.

456 In this article, we demonstrated that chronic MA exposure resulted in the
457 dysregulation of mitochondrial morphology and mitochondrial dysfunction, suggesting
458 that the modification of mitochondrial function and morphology might represent a
459 potential strategy for alleviating anxiety-like behaviors induced by MA. Accumulating data
460 has highlighted the contributions of brain mitochondria and bioenergetics to the
461 development of psychiatric disorders and stress-related pathologies (Manji et al., 2012;
462 Einat et al., 2005). The fundamental role played by mitochondria in the synthesis of the
463 primary excitatory neurotransmitter (glutamate) and the inhibitory neurotransmitter
464 (γ -aminobutyric acid, GABA) suggest that the mitochondrial adaptations observed in the
465 context of anxiety may contribute to an imbalance in neural excitation and inhibition,
466 which is thought to underlie several neuropsychiatric disorders (Filiou et al., 2019).
467 Mitochondria-targeting drugs have been reported to show good outcomes in
468 anxiety-related studies in both humans and rodents (Filiou et al., 2019). Quercetin is a
469 bioactive compound with diverse pharmacologic effects that have been reported to exert
470 several beneficial effects, including neuroprotective effects (Costa et al., 2016), the
471 regulation of the sleep–wake cycle (Kambe et al., 2010), and the optimization of
472 mitochondrial function (Houghton et al., 2018). In this study, quercetin supplementation
473 significantly mitigated MA-induced anxiety-like behavior by improving mitochondrial
474 morphology and function, alleviating neuronal injury, both *in vivo* and *in vitro*. In summary,
475 mitochondrial damage and intracellular ROS production are closely related (Cao et al.,
476 2017) and metabolic disturbance (Li et al., 2015), eventually leading to mitochondrial
477 dysfunction (Xie et al., 2016). The damaged mitochondria accumulation induced ,synaptic
478 loss, and neuron apoptosis (de la Mata et al., 2017), underscoring the importance of
479 mitochondria in the development of psychotic disorders. Here, we also identified the

novel pharmacological efficacy of quercetin in the treatment of anxiety induced by MA.

MA exposure facilitated mitochondrial dysfunction and morphological abnormalities (Valian et al., 2019), which can trigger various innate immune signaling pathways in a cell-intrinsic or -extrinsic manner (Bader et al., 2020), resulting in the release of inflammatory factors. In this study, we found that MA treatment accelerated astrocyte activation, accompanied by the elevation of interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF) α in the HIPP and MA-treated astrocytes (**Supplementary Figure 2**).

Astrocytes serve as crucial regulators of the innate and adaptive immune responses, which play critical roles in neuroinflammation (Colombo et al., 2016). According to the previous research, astrocytes can dispose of and recycle damaged mitochondria released by neurons, and release healthy extracellular mitochondrial particles to neuron (Hayakawa et al., 2016). As a major source of glycogen and lactate, astrocytes provided neurons with additional energy and play key metabolic roles in the CNS (Shirakawa et al., 2010). Moreover, astrocyte dysfunction has been shown to facilitate the pathogenesis of neurological and psychiatric disorders (Tian et al., 2018). Quercetin is a bioactive compound with antioxidant and anti-inflammatory properties (Khan et al., 2016) and was able to rescue mitochondrial dysfunction and morphological abnormalities and reduce the concentration of IL-1 β , IL-6, and TNF α in astrocytes treated with MA (**Figure 5 and Supplementary Figure 2**).

Malfunctioning mitochondria in astrocytes trigger various innate immune signaling pathways in a cell-intrinsic or -extrinsic manner (Bader et al., 2020), resulting in the release of inflammatory factors. These results suggested that MA treatment accelerated astrocyte activation and facilitated the release of inflammatory factors, which can trigger neuronal apoptosis and synaptic loss (Garwood et al., 2011; Çalışkan et al., 2020). Moreover, the excessive accumulation of damaged mitochondria, combined with astrocyte activation and the increase in inflammatory factors, induces neurotoxicity and promotes neuronal apoptosis (Nicholls et al., 2004), which initiates a vicious cycle that aggravates the anxiety process (Çalışkan G et al., 2020; Garwood et al., 2011). These findings also confirmed the neuroprotective activity of quercetin, through rebalancing neuroinflammation levels and modulating astrocytes activation.

509

510 **CONCLUSION**

511 In conclusion, our study indicated that MA damaged brain cells, including neurons
512 and astrocytes, in the HIPPO by altering mitochondrial metabolism, energy, and
513 morphology in astrocytes, which can cause the development of neuropsychiatric
514 disorders, such as anxiety. The oral supplementation of quercetin neutralized the
515 neuropsychiatric status of MA-treated mice, and our findings support the potential of
516 quercetin as a candidate agent for alleviating MA-induced anxiety and support the
517 hypothesis that mitochondria mediate anxiety progression. Further study is necessary to
518 illustrate the contributions and mechanisms of mitochondria in the progression of anxiety
519 induced by MA.

520

521 **DATA AVAILABILITY STATEMENT**

522 The original contributions presented in the study are included in the article and
523 Supplementary Material, further inquiries can be directed to the corresponding authors.

524

525 **ETHICS STATEMENT**

526 All our studies using Kunming mice were approved by the the Committee on Ethics in the
527 Use of Animals from Kunming Medical University (CEUA no. kmmu2021227) and were
528 performed in accordance with Yunnan Administration Rule of Laboratory Animal.

529

530 **AUTHORS CONTRIBUTIONS**

531 FC and JY designed the experiments, participated in the data analysis, and wrote the
532 manuscript; JS and LZ performed the sequence alignment and bioinformatics, YZ, YD, and
533 WT performed the animal experiments, FC, CC, and ZZ performed the cell experiments.
534 HW, YX, and MZ participated in data analysis. JY and KW conceived of the study,
535 supervised the project, and participated in the drafting of the manuscript. All authors read
536 and approved the final manuscript.

537

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549

550 **COMPETING INTERESTS**

551 The authors declare no competing financial interests.

552

553

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748

749 **Figure 1. Repeated MA administrations induce anxiety-like behavior in mice. A.**

750 Timeline of the Conditional place preference (CPP) experimental procedure. B. The
751 movement trajectory of the two groups of mice across compartments (left); CPP scores
752 were assessed as the difference of time spent in the drug-paired compartment between
753 the post and pre-conditioning phases (right). C. Timeline of the model establishment and
754 sample collection. D and E. Anxiety assessment with the elevated plus maze (EPM) and
755 open field test (OFT). Student- t-tests, $*p < 0.05$, $**p < 0.05$.

756

757

758 **Figure 2. RNA-seq revealed differentially expressed genes in the HIPP in the**

759 **MA-treated mouse model. A.** Hierarchical clustering analysis of RNAs with altered
760 expression between the two groups ($p < 0.05$, fold change > 2). Red strip, high relative
761 expression; blue strip, low relative expression. Color intensity reflects the degree of
762 expression increase or decrease. B. Thirty most enriched KEGG classifications of
763 assembled differential genes in HIPP. C. The genes implicated by the Top 5 KEGG
764 signalling pathways in HIPP. D. qPCR validation for RNA-seq data.

765

766

767 **Figure 3. Administration of quercetin ameliorates anxiety-like behaviors in an MA**

768 **mouse model. A.** Timeline of the model establishment and sample collection. B and C.
769 Anxiety assessment with the elevated plus maze (EPM) and open field test (OFT).
770 Student t-tests, $*p < 0.05$, $**p < 0.05$.

771

772 **Figure 4. Quercetin alleviates neuronal injury and decreases astrocyte activation in**

773 **HIPP induced by MA *in vivo*. A.** Immunofluorescence was performed with DAPI (blue) and
774 anti-NeuN (green). B. Hippocampus sections with H&E staining. C. Electron microscopy
775 analysis (magnification, $\times 20,000$). Areas in red boxes are magnified (Insets). D.
776 Immunofluorescence was performed with DAPI (blue) and anti-GFAP (green). E-G. GFAP
777 intensity and area were quantified to evaluate astrocytes reactivity. E. Representative
778 image of GFAP labels astrocytes. F. Area of astrocytes ($\mu\text{m}^2/\text{cell}$) in each group, $*p < 0.05$,

779 ** $p < 0.01$. G. Representative band pattern of the WB of different treatment of astrocytes
780 using antibodies for GFAP and β -Actin (left); summary bar graphs of GFAP and β -Actin
781 levels in different group (right).

782

783 **Figure 5. Quercetin ameliorates mitochondrial dysfunction and aberrant morphology**

784 **in astrocytes *in vitro*.** A. Electron microscopy analysis (magnification, $\times 20,000$). Areas in
785 red boxes are magnified (Insets). B. Representative image of astrocytic mitochondria. Alive
786 astrocytes were incubated with MitoTracker Green as a probe for mitochondria in each group.
787 The percentage of astrocytes for short-shape (blue) and long-shape (green) mitochondria
788 in different group is presented as histograms in C. D. Total ROS (left) and
789 mitochondria-derived ROS (right) production quantification by flow cytometry in each group.
790 D. Quantification of the mitochondrial membrane potential (MMP). F. Quantification of the
791 total (left) and mitochondria-derived ATP (right) by using an ATP quantification kits. G. An
792 analysis of O₂ consumption in astrocytes. The Agilent SeahorseXFe24 analyzer measures
793 OCR at basal and after injection of oligomycin ($3.5 \mu\text{M}$), FCCP ($4 \mu\text{M}$), and antimycin A
794 ($1 \mu\text{M}$)/rotenone ($1 \mu\text{M}$) for three measurement cycles at each step (left). Basal,
795 ATP-linked, maximal, and reserve capacity OCR in each group. * $p < 0.05$, ** $p < 0.01$,
796 *** $p < 0.001$ vs. control, as determined by Student's t test.

797

A **B** **C** **D**