Quinolinic Acid Induces Alterations in Neuronal Subcellular Compartments, Blocks Autophagy Flux and Activates Necroptosis and Apoptosis in Rat Striatum

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Received: 18 March 2022 / Accepted: 29 July 2022 / Published online: 18 August 2022
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Abstract
Quinolinic acid (QUIN) is an agonist of \( N \)-methyl-\( d \)-aspartate receptor (NMDAr) used to study the underlying mechanism of excitotoxicity in animal models. There is evidence indicating that impairment in autophagy at early times contributes to cellular damage in excitotoxicity; however, the status of autophagy in QUIN model on day 7 remains unexplored. In this study, the ultrastructural analysis of subcellular compartments and the status of autophagy, necroptosis, and apoptosis in the striatum of rats administered with QUIN (120 nmol and 240 nmol) was performed on day 7. QUIN induced circling behavior, neurodegeneration, and cellular damage; also, it promoted swollen mitochondrial crests, spherical-like morphology, and mitochondrial fragmentation; decreased ribosomal density in the rough endoplasmic reticulum; and altered the continuity of myelin sheaths in axons with separation of the compact lamellae. Furthermore, QUIN induced an increase and a decrease in ULK1 and p-70-S6K phosphorylation, respectively, suggesting autophagy activation; however, the increased microtubule-associated protein 1A/1B-light chain 3-II (LC3-II) and sequestosome-1/p62 (SQSTM1/p62), the coexistence of p62 and LC3 in the same structures, and the decrease in Beclin 1 and mature cathepsin D also indicates a blockage in autophagy flux. Additionally, QUIN administration increased tumor necrosis factor alpha (TNF\( \alpha \)) and receptor-interacting protein kinase 3 (RIPK3) levels and its phosphorylation (p-RIPK3), as well as decreased B-cell lymphoma 2 (Bcl-2) and increased Bcl-2-associated X protein (Bax) levels and c-Jun N-terminal kinase (JNK) phosphorylation, suggesting an activation of necroptosis and apoptosis, respectively. These results suggest that QUIN activates the autophagy, but on day 7, it is blocked and organelle and cellular damage, neurodegeneration, and behavior alterations could be caused by necroptosis and apoptosis activation.

Keywords Quinolinic acid · Excitotoxicity · Autophagy impairment · Necroptosis · Apoptosis

Introduction
Excitotoxicity is a common event in the physiopathology of a variety of neurological disorders [1, 2]. Moreover, there is evidence supporting an alteration in autophagy pathway induced by excitotoxicity, which is associated with brain tissue damage [3, 4]; however, there is discrepancy about how this impairment causes tissue damage. The evidence supports an association of brain tissue damage and overactivation of autophagy [5], but also supports that a decrease in autophagy leads to brain tissue damage [4].

Macroautophagy is a degradative pathway of cellular components, where long-lived proteins and organelles are sequestered through the formation of a double-membrane...
(autophagosome) and then fused with lysosomes, forming an autolysosome, where the material (cargo) is degraded [6].

The macroautophagy, hereafter autophagy, is a complex pathway that initiates with the formation of phagophore, induced by the activation of Unc51-like autophagy activating kinase 1 (ULK1) protein kinase complex, and its translocation to the initiation sites, such as endoplasmic reticulum membrane [7]. Adenosine monophosphate (AMP)-activated protein kinase (AMPK) phosphorylates ULK1 protein in Ser317, activating the ULK1 complex [8], that recruits and activates the class III phosphatidylinositol 3-kinase complex I (PI3KC3-C1) lipid kinase complex, promoting the formation of phosphatidylinositol 3-phosphate (PtdIns3P), the recruitment of some proteins to the phagophore membrane [9], and the maturation of phagophore to autophagosome. Additionally, the sequestosome-1 SQSTM1/p62 (p62) protein, an autophagy receptor, binds to ubiquitinylated proteins and organelles that will be degraded by autophagy and directs to the autophagosome through its interaction with microtubule-associated protein 1A/1B-light chain 3-II (LC3-II) protein, recruiting the material into the autophagosome. Autophagosomes bind to the lysosomes, which contain degradative proteases such as cathepsin D, an aspartic lysosomal protease, promoting protein degradation in autolysosomes [10].

In addition to the impairment in autophagy pathway due to excitotoxic process, there is evidence supporting the activation of necroptosis as a consequence of excitotoxicity, inducing cellular damage and neuronal death [11, 12]. Necroptosis is a regulated cellular death variant of necrosis that is activated by specific death receptors such as tumor necrosis factor receptor 1 (TNFR1), inducing assembly of necrosome, composed by proteins such as receptor-interacting protein kinase 1 (RIPK1) and receptor-interacting protein kinase 3 (RIPK3). The binding of TNFR1 to tumor necrosis factor alpha (TNFα), its ligand, promotes the autophosphorylation of RIPK1 that phosphorylates to RIPK3, which induces mixed lineage kinase domain-like protein (MLKL) phosphorylation. Phosphorylation of MLKL translocates it to cellular membrane forming a pore, inducing cell lysis and morphological characteristics of necrotic cell death. Necroptosis is observed in different neurological disorders [11, 13]. Furthermore, there is evidence indicating that necroptosis participates in axonal degeneration [11], neuronal death in excitotoxic models [12], and impairment in autophagy pathway, due to the translocation of MLKL to autolysosomal membranes, decreasing degradation [14].

Quinolinic acid (QUIN) is an active metabolite produced in the kynurenine pathway, the major route of tryptophan catabolism [15, 16]. However, as part of the neuroinflammatory processes, it is produced and released by infiltrating macrophages and activated microglia [16, 17]. Striatal and intracerebroventricular administration of QUIN has been used as a model for the study of the underlying mechanism of excitotoxicity in the striatum and hippocampus [18, 19].

There is evidence indicating that QUIN administration activates autophagy at early times (12 h after its administration) in vivo [20]. In contrast, when QUIN was incubated by 24 h in vitro, in primary neuron and astrocyte culture, the expression of Beclin 1 decreases and that of cathepsin D increases [10], suggesting inhibition [4] and activation [21] of autophagy at the same time. However, on day 7, the role of QUIN on autophagy and the contribution of impaired autophagy to brain tissue damage remain unexplored. Additionally, to the impairment of autophagy due to QUIN administration at early times, there is evidence indicating that QUIN induces other types of cellular death such as apoptosis and necrosis. Morphological analyses in vivo suggest that QUIN induces a rapid cytoplasmic disintegration, characteristic of death by necrosis in the lesion core [22], but apoptotic cells are observed in areas surrounding the lesion core [23]. However, other types of cellular death such as necroptosis in brain tissue, after QUIN administration, are still poorly studied. Our hypothesis is that the autophagy, necroptosis, and apoptosis will participate in the neuronal damage in areas surrounding the lesion core at later times. The aim of this study was to explore the effect of QUIN on morphological alterations in subcellular compartments, on the autophagy flux and necroptosis and apoptosis activation on day 7 in the rat striatum to determine if these pathways are involved in a secondary damage generating by QUIN administration.

Materials and Methods

Animals

Forty-five male Wistar rats (280–320 g) from the bioterium of Faculty of Medicine of the National Autonomous University of Mexico were kept under controlled conditions of temperature (25 ± 3 °C), humidity (50 ± 10%), and lighting (12-h light/dark cycles). Animals had free access to standard commercial rat chow diet (laboratory rodent diet 5001; PMI Feeds, Inc., Richmond, IN, USA) and water ad libitum.

All procedures with animals were carried out strictly according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and to the Norma Oficial Mexicana NOM-062-ZOO-1999. The experimental procedures were approved by the Institutional and Local Committee for the Care and Use of Laboratory Animals on the Ethical Use of Animals from Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, INNN project 44/15. During the experiments, all efforts were made to minimize animal suffering.
Reagents
QUIN, apomorphine, paraformaldehyde (PFA), glutaraldehyde, osmium tetroxide, sodium cacodylate, lead citrate, uranyl acetate, acrylamide, bis-acrylamide, ammonium persulfate, glycine, sodium dodecyl sulfate, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, aprotinin, leupeptin, Triton® X-100, Folin & Ciocalteu’s reagent, bovine serum albumin (BSA), phosphatase inhibitor cocktail 3, rabbit anti-p62 antibody (P0067), and mouse anti-α-tubulin antibody (T9026) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-p62 (ab101266), rabbit anti-RIP3 (RIPK3, ab56164), rabbit anti-RIP3 (RIPK3, ab62344), and rabbit anti-RIP3 (phospho S232, p-RIPK3, ab195117) antibodies were obtained from Abcam (Cambridge, MA, USA). Mouse anti-MAP LC3β (LC3-I/LC3-II, G-9; sc-376404), mouse anti-TNFα (52B83; sc-52746), mouse anti-RIP (RIPK1, C-12; sc-133102), mouse anti-BECN1 (Beclin 1, E-8; sc-48341), and mouse anti-cathepsin D (C-5; sc-377124) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit anti-p-ULK1 Ser317 (12,753), rabbit anti-ULK1 (8359), mouse anti-p-p70-S6K Thr389 (9206), rabbit anti-p-S6K (9202), rabbit anti-p-JNK Thr183/Tyr185 (4671), and rabbit anti-JNK (9252) were obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-Bax (GTX61026) was from Gene Tex (Irvine, CA, USA). Mouse anti-B-cell lymphoma 2 (Bcl-2, 14–6992) was from eBioscience, Inc. (San Diego, CA, USA). Donkey-anti-mouse IgG horseradish peroxidase conjugate (715–035-150) and donkey-anti-rabbit IgG horseradish peroxidase conjugate (711–035-152) antibodies were from Jackson ImmunoResearch Laboratories, Inc. (Jennersville, PA, USA). Fluoro-Jade B, polyvinylidene fluoride (PVDF) membrane, Immobilon Western kit, and DPX mounting medium were obtained from Millipore (Bedford, MA, USA) and Universal L Kit SAB-System horseradish peroxidase (HRP) was from Dako (Carpinteria, CA, USA). All the other reagents were obtained from known commercial sources.

Experimental Design
Animals were randomly divided into three groups (n = 15) as follows: (1) SHAM group, (2) 120 nmol of QUIN (QUIN120), and (3) 240 nmol of QUIN (QUIN240). Animals from the SHAM group received a unilateral striatal injection of isotonic saline solution (ISS) whereas the animals from QUIN120 and QUIN240 groups received a unilateral striatal injection of QUIN equivalent to 120 nmol and 240 nmol, respectively. All animals were sacrificed at 7 days after ISS or QUIN administration [24], and samples (striatum or perfused whole brain) were collected.

Animal were further separated in four independent groups (3–4 rats/group) and were used for the evaluation of (1) rotation behavior by apomorphine administration, neurodegeneration by Fluoro-Jade B staining, and cellular and histological damage by hematoxylin and eosin (H&E) staining (n = 4); (2) subcellular compartment alterations by transmission electron microscopy (TEM; n = 3); (3) protein localization by immunohistochemistry (n = 4); and (4) protein levels by western blot (n = 4, Fig. 1).

Fig. 1 Experimental design. Rats were divided into three groups and administered in the right striatum with 1 µL of isotonic saline solution (ISS) (SHAM group) or quinolinic acid 120 nmol or 240 nmol (QUIN120 and QUIN240 groups, respectively). Rotation behavior was evaluated at day 6 after ISS or QUIN administration. Horizontal dotted arrow indicates the group of animals subjected to rotation behavior evaluation. Four independent groups of animals were sacrificed at day 7 after ISS or QUIN administration, as indicated with the four horizontal arrows in color, and the samples (striatum or perfused brain) were collected. The number of animals (n) used in each independent group is indicated. FJ-B Fluoro-Jade B, H&E hematoxylin and eosin, TEM transmission electron microscopy, IHC immunohistochemistry, WB western blot (figure created with Inkscape 0.91).
Striatal Administration

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.), placed in a stereotaxic equipment (Stoelting Co., Woo Dale, IL, USA) and administered in the right striatum with 1 µL of ISS for the SHAM group or QUIN (equivalent to 120 nmol or 240 nmol) for QUIN120 and QUIN240 groups, using a 10-µL Hamilton microsyringe (Hamilton Co., Reno, NV, USA). The administration was carried out according to the following stereotaxic coordinates: +0.5 mm anterior to bregma, −2.6 mm lateral to bregma, and −4.5 mm ventral to dura [25].

Rotation Behavior

Rotation behavior was evaluated according to a previous report [24]. Six days after ISS or QUIN injection, animals were administered with apomorphine (1 mg/kg, s.c.) and placed in individual acrylic box cages. Five minutes later, the number of ipsilateral rotations was counted for 1 h. One rotation was defined as a complete 360° turn. Data were expressed as the total number of turns in 1 h.

Samples for Histological Analysis

Seven days after ISS or QUIN injection, animals were euthanized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with ISS plus heparin (5 U/mL) followed by 4% PFA in 0.2 M phosphate buffer (pH 7.4). Brains were removed and post-fixed in 4% PFA for 48 h and subsequently dehydrated in ethanol and xylene solutions and embedded in Paraplast plus paraffin (McCormick Scientific, St. Louis, MO, USA). Coronal sections of 5 µm were obtained in a HM 325 rotatory microtome (Thermo Fisher Scientific, Inc., Waltham, MA, USA) every 100 µm, covering a total distance of 300 µm. These sections were used in Fluoro-Jade B, H&E, and immunohistochemistry assays.

Fluoro-Jade B Staining

Neurodegeneration was evaluated using Fluoro-Jade B staining. Sections were deparaffinized in xylene for 15 min and placed in absolute ethanol for 2 min, followed by 1% NaOH–80% ethanol solution for 5 min and 70% ethanol solution for 2 min, and rinsed with distilled water for 2 min. Sections were immersed in 0.06% KMnO4 solution for 10 min and rinsed with distilled water for 2 min. Finally, samples were stained with 0.0004% Fluoro-Jade B for 20 min, washed with distilled water, dried at 50 °C for 15 min, immersed in xylene, and mounted with DPX mounting medium. Five fields in the striatum localized in areas surrounding the lesion core of the injection were counted with a Leica microscope (Cambridge, UK) using a ×40 objective. Results were expressed as the number of positive cells to Fluoro-Jade B per field.

H&E Staining

Cellular and histological damage was evaluated using H&E staining. Sections were deparaffinized in xylene and hydrated in solutions of decreasing ethanol concentration and distilled water. Sections were stained with hematoxylin for 1 min, then rinsed with distilled water and stained with eosin for 1 min. Finally, samples were dehydrated in solutions of increasing ethanol concentration and xylene and mounted with DPX mounting medium. Five fields in the striatum localized in areas surrounding the lesion core of the injection were counted with a Leica microscope (Cambridge, UK) using a ×40 objective. Results were expressed as the percent of damaged cells per field.

Immunohistochemistry

Histological localization of p62, MAP LC3β (LC3), and RIPK3 was evaluated by immunohistochemistry. Sections were deparaffinized in xylene and hydrated in solutions of decreasing ethanol concentration and distilled water. Sections were permeabilized with phosphate-buffered saline (PBS) plus 0.2% Triton® X-100 for 1 h, boiled in 10 mM sodium citrate buffer (pH 6.0) plus 0.2% Triton® X-100 for 1 h, and cooled at room temperature for 1 h. The peroxidase endogenous activity was inactivated with 1% hydrogen peroxide (H2O2) for 15 min. Sections were blocked with 2.5% BSA for 1 h at room temperature and then were incubated with rabbit anti-p62 (1:500; ab101266) antibody overnight and mouse anti-MAP LC3β (1:25) and rabbit anti-RIP3 (1:200; ab62344) antibodies during 48 h at room temperature. Sections were incubated with Universal L Kit SAB-System HRP according to the manufacturer’s instructions. Finally, sections were incubated with 3,3'-diaminobenzidine, counterstained with hematoxylin, and covered with DPX mounting medium. Sections in the striatum area were visualized in a Nikon E 200 microscope (Nikon, Melville, NY, USA) using ×40 objective. Results were expressed as representative micrographs of each experimental group.

TEM

Subcellular compartment alterations were evaluated by TEM. Seven days after QUIN injection, rats (n = 3 per group) were deeply anesthetized with pentobarbital (100 mg/kg, i.p.), and transcardially perfused with cool ISS followed by 4% PFA and 1.5% glutaraldehyde in Sörensen’s buffer (46 mM NaH2PO4, 154 mM Na2HPO4, 154 mM NaCl, pH 7.2). Brains were removed, and the areas of interest were dissected and fragmented, post-fixed in the same solution for
24 h, immersed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH = 7.2), and subsequently embedded in epoxy resin. Ultrathin Sects. (90 nm) of the striatal regions were obtained, contrasted with 3% lead citrate Reynolds and 1% uranyl acetate, and examined with a transmission electron microscope (FEI Tecnai BioTWIN, Hillsboro, OR, USA). The ultrastructural analysis was carried out in neurons localized in the areas surrounding the lesion core of the injection. For quantification, the number of well-preserved mitochondria and rough endoplasmic reticulum (RER) was counted in 2 randomly chosen neurons from each rat, considering three animals for experimental group. Results were expressed as the well-preserved mitochondria or RER.

With the aim to determine two different proteins (LC3, p62), we did double-marker immunoelectron microscopy using antibodies labeling with two different sizes of gold particles. Tissue fragments from the same areas of interest were fixed in the same buffer for 2 h at 4 ºC, dehydrated in graded ethyl alcohol, and embedded in LR White hydrosoluble resin (London Resin Cp., Hampshire, UK). Thin sections from 70 to 90 nm were placed on nickel grids. The grids were incubated overnight at 4 ºC with specific mouse anti-LC3 antibodies (1:10) in PBS. After rinsing with PBS, the grids were incubated for 2 h at room temperature with goat anti-mouse IgG conjugated to 10-nm gold particles (1:20) in PBS. After repeated rinsing with PBS, a second overnight incubation was carried out with a rabbit polyclonal specific antibody against p62 (1:200; ab101266), followed by goat anti-rabbit IgG labeled with 6-nm gold particles (1:20) in PBS. The grids were stained with uranium salts and analyzed in the electron microscope.

Western Blot

Protein levels of p-ULK1, ULK1, p-p70-S6K, p70-S6K, Beclin 1, preprocathepsin D, procathepsin D, cathepsin D, MAP LC3β (LC3-1/LC3-II), p62, Bcl2, Bax, p-JNK, JNK, TNFα, RIPK1, RIPK3, and p-RIPK3 were evaluated by western blot. Seven days after ISS or QUIN administration, the right striatum of animals was collected and homogenized in 500 µL of cold lysis buffer (10 mM Tris HCl (pH 7.6), 15 mM NaCl, 0.25 mM sucrose, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin A, 1 mM PMSF, phosphatase inhibitor cocktail 1 ×, and 1% Triton® X-100) and centrifuged at 10,621 × g for 20 min at 4 ºC. The supernatant was collected, and the protein concentration was measured by Lowry method [26]. Fifty micrograms of supernatant was loaded in 10%, 12%, or 15% of polyacrylamide gel, and proteins were separated and transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk and incubated with primary mouse antibody anti-TNFα (1:500), mouse anti-RIP (1:500), rabbit anti-ULK1 (1:1000), mouse anti-β-actin (1:500), mouse anti-MAP LC3β (1:500), rabbit anti-p62 (1:1000; P0067), mouse anti-Bcl-2 (1:600), rabbit anti-Bax (1:600), rabbit anti-p-ULK1 (1:1000), mouse anti-β-actin (1:500), rabbit anti-p70-S6K (1:1000), rabbit anti-p70-S6K (1:1000), rabbit anti-p-JNK (1:1000), rabbit anti-JNK (1:1000), and mouse anti-α-tubulin (1:10,000) overnight followed by the incubation of the secondary donkey antibody anti-mouse IgG horseradish peroxidase conjugate (1:10,000) or donkey antibody anti-rabbit IgG horseradish peroxidase conjugate (1:10,000). Membranes were revealed using an Immobilon Western kit (Millipore Co., Billerica, MA, USA), and images were obtained with a photodocumenter (Vilber Lourmat, Eberhardzell, Germany). Images were analyzed using the ImageJ software (ImageJ v1.52a; NIH, Bethesda, MD, USA), and the data were expressed as relative optical density (OD) of the protein/α-tubulin.

Statistical Analysis

All data are presented as the mean ± SD. Data were analyzed by one-way ANOVA and Tukey’s post hoc test except for results of apoptosis that were analyzed by Student’s t test using Prism 5.0 software (GraphPad, San Diego, CA, USA). P values of <0.05 were considered to be statistically significant.

Results

QUIN Induces Rotation Behavior Impairment and Brain Tissue Damage

QUIN is an endotoxin that induces excitotoxicity and increases oxidative stress, leading to tissue and cellular damage. Seven days after intrastriatal administration of QUIN, the number of ipsilateral turns was significantly increased compared to the SHAM group (Fig. 2). Western blot analysis showed a significant decrease in the levels of Beclin 1, p70-S6K, and Bcl-2 and an increase in the levels of JNK and RIPK3 in the QUIN group compared to the SHAM group.

Fig. 2 Rotation behavior induced by quinolinic acid (QUIN). Animals were administered in the right striatum with 1 µL of isotonic saline solution (SHAM group) or QUIN (120 nmol or 240 nmol) and, 6 days after, were administered with apomorphine (1 mg/kg, s.c.). The number of ipsilateral turns was recorded for 1 h. Data are expressed as the mean ± SD of four animals per group. **P < 0.01 vs SHAM group.
Fig. 3 Histological striatal tissue damaged after quinolinic acid (QUIN) administration. Animals were administered in the right striatum with 1 µl of isotonic saline solution (SHAM group) or QUIN (120 nmol or 240 nmol). Brains were collected and fixed after 7 days of the striatal injury, coronal Sects. (5 µm) were obtained, and Fluoro-Jade B or hematoxylin and eosin (H&E) staining was performed. Representative micrographs of each group for Fluoro-Jade B staining at ×40 (a, upper panel), H&E staining at ×40 (a, middle panel), and panoramic H&E staining at ×4 (a, lower panel) are shown. White arrows show positive cells to Fluoro-Jade B (a, upper panel), and bold arrows show damaged cells (a, middle panel). Black rectangles indicate the analyzed zone at ×40 (a, lower panel). The percentage of positive cells to Fluoro-Jade-B per field of five random fields (b) and the percent of damaged cells per field (c) in the striatum are presented in the graphs. Damaged neurons were identified by pyknotic nucleus and shrinking cytoplasm. Representative image of the administration site in the striatum is shown in d. Black arrow indicates the syringe trajectory. Data are expressed as the mean ± SD of four animals per group. ***P < 0.001 vs SHAM group.
damage. QUIN120 tends to increase the number of ipsilateral turns per hour at day 6; however, only QUIN240 increases significantly the number of ipsilateral turns compared to SHAM group (Fig. 2; ANOVA: $P = 0.0027, F (2, 7) = 15.54$; Tukey's post hoc test: SHAM vs QUIN120 [$P = 0.1113$]; SHAM vs QUIN240 [$P = 0.0021$]).

The impairment in rotation behavior in QUIN groups could be related with an increase in the neurodegeneration and tissue and cellular damage induced by QUIN at day 7. To explore this, we evaluated neurodegeneration and histological damage. The SHAM group shows a mean of 4.06 positive cells per field to Fluoro-Jade B (Fig. 3a (upper panel) and b; ANOVA: $P < 0.001$, $F (2, 77) = 111.1$) and 23.23% of damaged cells (Fig. 3a (middle panel) and c; ANOVA: $P < 0.001$, $F (2, 69) = 132.3$) that could be associated with the administration of ISS or the mechanical damage induced by the syringe during the striatal administration.

QUIN increases the number of cells in neurodegeneration (positive cells to Fluoro-Jade B) in a dose–response way with respect to SHAM group (Fig. 3a, upper panel).

**Fig. 4** Mitochondrial alterations induced by quinolinic acid (QUIN) in striatal neurons of rat. Animals were administered in the right striatum with 1 µL of isotonic saline solution (SHAM group) or QUIN (120 nmol or 240 nmol). Brains were collected and fixed 7 days after intrastriatal administration and transmission electron microscopy were performed. a Low-power representative micrograph of normal neuron from the SHAM group. b High-power micrograph of mitochondria indicated in the inset of a with normal structure. c Cytoplasm of neuron from the QUIN120 group shows wide dispersed and abnormal organelles. d High-power micrograph from swollen mitochondria indicated in the inset of e shows dilated cristae. e Cytoplasm of neuron from the QUIN240 group shows abnormal spherical mitochondria. f High-power micrograph of spherical mitochondria circumscribed in the inset of e shows abnormal dilated cristae with effacement of the matrix. Bold arrows show dilatation of mitochondrial cristae. g The number of well-preserved mitochondria comparing the SHAM group with the QUIN120- and QUIN140-treated groups. 2 randomly chosen neurons per animal, and three rats per group were used. Data are expressed as the mean ± SD. *$P < 0.05$ and ***$P < 0.001$ vs SHAM group. Mt mitochondria
The number of positive cells to Fluoro-Jade B in QUIN120 and QUIN240 groups was 45.68 and 57.17, respectively (Fig. 3b; ANOVA: \( P < 0.001, F (2, 77) = 111.1 \); Tukey’s post hoc test: SHAM vs QUIN120 \( P < 0.0001 \); SHAM vs QUIN240 \( P < 0.0001 \)). Besides, QUIN increases the percent of damaged cells per field in a dose–response way at 7 days (Fig. 3a, middle panel) to 68.84% and 86.56% in QUIN120 and QUIN240 groups, respectively (Fig. 3c; ANOVA: \( P < 0.001, F (2, 69) = 132.3 \); Tukey’s post hoc test: SHAM vs QUIN120 \( P < 0.0001 \); SHAM vs QUIN240 \( P < 0.0001 \)). Furthermore, interstitial edema and the loss of neuropil integrity can be observed with the dose of 120 of QUIN, but it is more evident in the QUIN240 group (Fig. 3a, middle and lower panels).

**QUIN Induces Damage in Mitochondria and RER**

We examined the effect of QUIN (120 nmol and 240 nmol) on cellular organelles at 7 days post-lesion. Neurons from the SHAM group show well-preserved mitochondria, with

![Fig. 5](image-url)

**Fig. 5** Rough endoplasmic reticulum (RER) alterations induced by quinolinic acid (QUIN) in striatal neurons of rat. Animals were administered in the right striatum with 1 µL of isotonic saline solution (SHAM group) or QUIN (120 nmol or 240 nmol). Brains were collected and fixed 7 days after intrastriatal administration and transmission electron microscopy were performed. (a) Low-power representative micrograph of normal neuron of the SHAM group, with abundant and well-developed RER. (b) High-power micrograph of the inset area of (a) shows well-developed RER with numerous ribosomes. (c) Neuron from the QUIN120 group show dispersed organelles with scarce RER. (d) High-power micrograph of RER indicated in the inset of (c) shows fragmented RER (arrow) with almost complete disappearance of ribosomes. (e) Micrograph of neuron from the group QUIN240 shows the cytoplasm with scarce organelles. (f) High-power micrograph of the inset in (e) shows RER constituted by thin and fragmented membranes (arrow) without ribosomes. (g) The number of well-preserved RER cisterna around the nucleus comparing the SHAM group with the QUIN120- and QUIN240-treated groups, 2 randomly chosen neurons per animal, and three rats per group were studied. Data are expressed as the mean ± SD. **\( P < 0.01 \) vs SHAM group.
intact crests (Fig. 4a and b). Nonetheless, in neurons of animals from the QUIN groups, the organelles are disorganized in the cytoplasm. QUIN120 produces neuronal swelling and dilatation of mitochondrial cristae compared to the SHAM group (Fig. 4c and d). Moreover, QUIN240 increases the subcellular abnormalities compared with the QUIN120 group; small and spherical-like morphology of mitochondria with extensive cristae dilatation is observed (Fig. 4e and f). Likewise, QUIN240 induces complete effacement of cristae and absence of intermembranal space of mitochondria with matrix effacement (Fig. 4f). This was confirmed by counting well-preserved mitochondria in randomly chosen neurons. In comparison with the SHAM group, QUIN120 produced a decrease of mitochondria with normal morphology, which was even lower in QUIN240-treated animals (Fig. 4g; ANOVA: \( P = 0.0005, F (2, 15) = 13.15 \); Tukey’s post hoc test: SHAM vs QUIN120 \( P = 0.0205 \); SHAM vs QUIN240 \( P = 0.0004 \)).

Otherwise, in the perinuclear cytoplasm of the SHAM group, abundant well-developed RER and free ribosomes are observed (Fig. 5a and b), a characteristic consistent with its high protein synthesis activity. However, QUIN administration induces morphological alterations of RER in striatal neurons such as fragmentation and thinning of RER membrane.
as well as a decrease of ribosomes (Fig. 5c–f). Rats of the QUIN120 group show fewer free ribosomes and lesser ribosomes in RER, as well as fragmented RER (Fig. 5c and d), and QUIN240 induces more accentuated subcellular abnormalities such as fragmented and thinner RER cisternae with scarce attached ribosomes (Fig. 5e and f). This observation was confirmed by counting well-preserved RER cisterna around the nucleus of randomly chosen neurons. In comparison with the SHAM group, the QUIN120 group showed a lesser number of well-preserved cisterna groups of RER, which was even lower in QUIN240 (Fig. 5g; ANOVA: $P=0.0048$, $F(2, 15)=7.8$; Tukey’s post hoc test: SHAM vs QUIN120 [$P=0.5313$]; SHAM vs QUIN240 [$P=0.0044$]).

QUIN Induces Axonal Damage

We found that QUIN induces cellular damage as well as mitochondrial and RER damage; for that reason, we evaluated the effect of QUIN on the axons of striatal neurons. In the SHAM group, we observed intact axons with well-preserved myelin sheaths and axoplams and some normal mitochondria and numerous microtubules (Fig. 6a, b, and g).

QUIN induces alteration in the axon morphology and components, such as myelin layer discontinuity, separation of the compact myelin sheaths, and abundant fragmentation with disappearance of neurofilaments and microtubules. Rats of the QUIN120 group show axon dilatation, neurofilament

![Fig. 7](image-url)

**Fig. 7** Effect of quinolinic acid (QUIN) on cellular localization of phosphotyrosine-independent ligand for the Lck SH2 domain of 62 kDa (p62) and microtubule-associated protein 1A/1B-light chain 3 (LC3) proteins by immunohistochemistry at light and electron microscopy levels. Animals were administered in the right striatum with 1 µL of isotonic saline solution (SHAM group) or QUIN (120 nmol or 240 nmol). Brains were collected and fixed 7 days after intrastriatal administration and immunohistochemistry were performed. Representative micrographs ($\times 40$) of the lesion core in the striatum of each group for p62 (upper panel) and LC3 (lower panel) are shown. Bold arrows show positive cells to p62 (upper panel) and LC3 (lower panel). Scarc, almost negative p62 immunostaining is detected in the SHAM group; in contrast, numerous neurons show p62 immunostaining in the QUIN120 group, whereas the strongest p62 immunostaining is observed in swollen and shrinking neurons from the QUIN240 group. Weak LC3 immunostaining is exhibited by striatal neurons from the SHAM group. Neurons from the QUIN120 group show mild LC3 immunoreactivity, and stronger LC3 immunostaining is exhibited by neurons in the QUIN240 group. Subcellular detection of LC3 (big black dots, gold particle size 10 nm) and p62 (small dots, gold particle size 6 nm) in cytoplasmic vacuole of neuron (left bottom panel) and RER (right bottom panel), from QUIN240- and QUIN120-treated rats, respectively, by immunoelectron microscopy using specific antibodies labeled with colloidal gold. Both molecules coexist in the same structures
**Fig. 8** Effect of quinolinic acid (QUIN) on protein level involved in the autophagy pathway. Animals were administered in the right striatum with 1 µL of isotonic saline solution (SHAM group) or QUIN (120 nmol or 240 nmol). Right striata were collected at day 7 after intrastratial administration and western blotting were performed. Representative images of western blot and densitometric quantification of phosphorylated ribosomal protein S6 kinase beta-1/ribosomal protein S6 kinase beta-1 (p-p70-S6K(Thr389)/p70-S6K) ≈ 70 kDa/70 kDa (a), phosphorylated Unc51-like autophagy activating kinase 1/Unc51-like autophagy activating kinase 1 (pULK1(Ser317)/ULK1) ≈ 140 kDa/140 kDa (b), Beclin 1 ≈ 60 kDa (c), phosphotyrosine-independent ligand for the Lck SH2 domain for the Lck SH2 domain of 62 kDa (p62) ≈ 62 kDa (d), microtubule-associated protein 1A/1B-light chain 3-II/I (LC3II/LC3I) ≈ 14 kDa/16 kDa (e), procathepsin D ≈ 52 kDa (f), preprocathepsin D ≈ 46 kDa (g), and cathepsin D ≈ 34 kDa (h) are shown. Data are expressed as the mean±SD of four animals per group. *P<0.05, **P<0.01, and ***P<0.001 vs SHAM group. OD optical density.
and QUIN240 groups were observed and compared to those showed strong immunostaining (Fig. 7). The double-marker immunostaining was more frequently in shrink neurons in the QUIN240 group. In both groups, QUIN120 and QUIN240, the cytoplasmic staining of p62 and LC3 compared to the SHAM group. In the SHAM group, the detection of p62 and LC3-II/LC3-I ratio increase only in the QUIN240 group (Fig. 8d; ANOVA: $P < 0.0001$, $F(2, 18) = 29.86$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0002$, SHAM vs QUIN240 $P = 0.0015$) and the levels of cathepsin D were unchanged after QUIN treatment (Fig. 8f; ANOVA: $P = 0.0012$; Fig. 8e; ANOVA: $P < 0.0001$, $F(2, 18) = 17.18$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0094$; SHAM vs QUIN240 $P = 0.9940$); however, a decrease in the maturation of cathepsin D was observed, since the levels of procathepsin D show a decrease in the QUIN240 group (Fig. 8g; ANOVA: $P = 0.0019$, $F(2, 7) = 17.49$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0517$, SHAM vs QUIN240 $P = 0.0015$) and the levels of cathepsin D decrease with both doses of QUIN (Fig. 8h; ANOVA: $P = 0.0002$, $F(2, 8) = 23.12$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0005$; SHAM vs QUIN240 $P < 0.0001$). Moreover, p62 levels and LC3-II/LC3-I ratio increase only in the QUIN240 group (Fig. 8c; ANOVA: $P < 0.0001$, $F(2, 16) = 23.12$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0005$; SHAM vs QUIN240 $P < 0.0001$). Finally, the levels of procathepsin D were unchanged after QUIN treatment (Fig. 8f; ANOVA: $P = 0.9424$, $F(2, 9) = 0.0597$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.9707$; SHAM vs QUIN240 $P = 0.9940$); however, a decrease in the maturation of cathepsin D was observed, since the levels of preprocathepsin D decreased in the QUIN240 group (Fig. 8g; ANOVA: $P = 0.0019$, $F(2, 7) = 17.49$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0517$, SHAM vs QUIN240 $P = 0.0015$) and the levels of cathepsin D decrease with both doses of QUIN (Fig. 8h; ANOVA: $P = 0.0002$, $F(2, 8) = 29.86$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0029$; SHAM vs QUIN240 $P = 0.0002$), suggesting a decrease in autophagy flux. These results suggest that QUIN240 (mainly) increases the activation of autophagy at early stages, but it promotes an autophagy flux blockade at late stages on the pathway.

**QUIN Blocks Autophagy Flux**

There is evidence indicating that excitotoxicity impairs autophagy pathway, and since we observed that QUIN induced mitochondrial damage, which could be associated to impairment in autophagy, we explore the status of autophagy flux. First, we performed an immunohistochemical analysis of p62 and LC3. In the SHAM group, the detection of p62 and LC3 is low in striatal cells (Fig. 7). QUIN120 increases the cytoplasmic immunostaining of p62 and LC3 compared to the SHAM group. Additionally, QUIN240 increased even more the staining of p62 and LC3. Interestingly, the positive labeling to p62 was commonly observed in the nucleus of neuronal cells in the three groups, particularly in the QUIN240 group. In both groups, QUIN120 and QUIN240, the immunostaining was more frequently in shrink neurons with pyknotic nuclei, and numerous swollen cells also showed strong immunostaining (Fig. 7). The double-marker immunogold technique showed some cytoplasmic vacuoles and fragments of RER with coexistence of both p62 and LC3 (Fig. 7), and immunolabeling to p62 was also observed in the cytoplasm forming clusters with lesser LC3 detection (Fig. 7).

The increase in p62 and LC3 levels could be an indicative in autophagy impairment; for that reason, we evaluated the autophagy pathway at different levels by western blot. Phosphorylation state of ULK1 in Ser317 and p-70S6K in Thr389 was measured as indicative of activation of autophagy pathway. An increase in ULK1 phosphorylation and a decrease in p70S6K phosphorylation levels in QUIN120 and QUIN240 groups were observed and compared to those

in the SHAM group, suggesting an increase in autophagy (Fig. 8a; ANOVA: $P < 0.0001$, $F(2, 9) = 44.04$; Tukey’s post hoc test: SHAM vs QUIN120 $P < 0.0001$; SHAM vs QUIN240 $P < 0.0001$; Fig. 8b; ANOVA: $P = 0.0112$, $F(2, 15) = 6.147$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0196$; SHAM vs QUIN240 $P = 0.0389$). Surprisingly, Beclin 1 levels decrease after QUIN administration (120 nmol and 240 nmol) compared with the SHAM group (Fig. 8c; ANOVA: $P < 0.0001$, $F(2, 16) = 23.12$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0005$; SHAM vs QUIN240 $P < 0.0001$). Moreover, p62 levels and LC3-II/LC3-I ratio increase only in the QUIN240 group (Fig. 8d; ANOVA: $P = 0.0006$, $F(2, 8) = 21.82$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.9157$; SHAM vs QUIN240 $P = 0.0012$; Fig. 8e; ANOVA: $P < 0.0001$, $F(2, 18) = 17.18$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.1909$; SHAM vs QUIN240 $P < 0.0001$). Finally, the levels of procathepsin D were unchanged after QUIN treatment (Fig. 8f; ANOVA: $P = 0.9424$, $F(2, 9) = 0.0597$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.9707$; SHAM vs QUIN240 $P = 0.9940$); however, a decrease in the maturation of cathepsin D was observed, since the levels of preprocathepsin D decreased in the QUIN240 group (Fig. 8g; ANOVA: $P = 0.0019$, $F(2, 7) = 17.49$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0517$, SHAM vs QUIN240 $P = 0.0015$) and the levels of cathepsin D decreased with both doses of QUIN (Fig. 8h; ANOVA: $P = 0.0002$, $F(2, 8) = 29.86$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.0029$; SHAM vs QUIN240 $P = 0.0002$), suggesting a decrease in autophagy flux. These results suggest that QUIN240 (mainly) increases the activation of autophagy at early stages, but it promotes an autophagy flux blockade at late stages on the pathway.

**QUIN Induces Apoptosis**

It has been reported that the blockage in autophagy flux could be related to an increase in apoptosis or necroptosis [14, 27]. To explore the participation of apoptosis after QUIN treatment, we measured the levels of proapoptotic protein Bax, the anti-apoptotic protein Bcl-2, the Bax/Bcl-2 ratio, and the activation (phosphorylation at Thr183/Tyr185) of JNK. QUIN240 decreases Bcl-2 levels and increases Bax levels, compared to the SHAM group (Fig. 9a; $t$ test: $P = 0.0116$; Fig. 9b; ANOVA: $P = 0.0123$, $F(2, 8) = 8.022$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.9869$; SHAM vs QUIN240 $P = 0.0166$). Moreover, the Bax/Bcl-2 ratio (Fig. 9c; $t$ test: $P = 0.0287$) and the activation of JNK (Fig. 9d; ANOVA: $P = 0.0003$, $F(2, 8) = 26.44$; Tukey’s post hoc test: SHAM vs QUIN120 $P = 0.2919$; SHAM vs QUIN240 $P = 0.0003$) increase in the QUIN240 group, suggesting an activation of apoptosis pathway after 7 days. This was in agreement with the
electron microscopy study that showed numerous nervous cells with condensed nucleus and almost preserved cytoplasm that correspond to apoptotic cells (Fig. 9e–g).

**QUIN Induces Necroptosis**

Also, in this work, we explored the activation of necroptosis pathway as a mechanism of tissue damage since alterations in axons and blockage in autophagy flux was observed after QUIN injection. The expression of RIPK3 protein, one of the principal indicators of necroptosis, was evaluated at 7 days. In the SHAM group, few cells showed slight immunostaining while the QUIN-treated group showed numerous positive cells (Fig. 10a). In the QUIN120 group, the main positive mark to RIPK3 is located in the cytoplasm and axons of shrunk and swollen neurons. In the QUIN240 group, there are more RIPK3 immunostained in damaged nervous cells with cytoplasmic and nuclear immune-reactivity, and endothelial cells also showed intense immunostaining (Fig. 10a).

The electron microscopy study confirms the extensive cellular damage and cell death, showing necrotic cells with pyknotic nucleus and shrunken cytoplasm or swollen neurons with chromatin disappearance and dilated cytoplasm with few and damaged organelles (Fig. 9f). The activation of the necrototic pathway starts with the binding of TNFα to its cellular receptor, TNFR1, which promotes the phosphorylation of RIPK1, when caspase-8 is inhibited. This phosphorylation activates RIPK1 and induces the subsequent activation of RIPK3. To evaluate the necrototic pathway with more detail, we measured the levels of TNFα, RIPK1, RIPK3, and p-RIPK3 by western blot, with the aim to support our finding in RIPK3 immunohistochemistry, and determined whether necroptosis pathway could be participating in neuronal damage after QUIN administration. TNFα levels tend to increase in the QUIN120 group, but only QUIN240 increases TNFα levels compared to the SHAM group (Fig. 10b; ANOVA: $P = 0.0036, F (2, 15) = 8.353$; Tukey’s post hoc test: SHAM vs QUIN120 $[P = 0.4255]$; SHAM vs QUIN240 $[P = 0.0027]$). QUIN does not modify RIPK1 levels; nonetheless, a tendency to increase is observed with QUIN240 (Fig. 10c; ANOVA: $P = 0.0672, F (2, 16) = 3.211$; Tukey’s post hoc test: SHAM vs QUIN120 $[P = 0.1984]$; SHAM vs QUIN240 $[P = 0.6554]$). Moreover, QUIN240 increases the levels of RIPK3 compared to SHAM group, while QUIN120 only show a tendency to increase RIPK3 levels (Fig. 10d; ANOVA: $P = 0.0266, F (2, 9) = 5.578$; Tukey’s post hoc test: SHAM vs QUIN120 $[P = 0.3354]$; SHAM vs QUIN240 $[P = 0.0215]$). Finally, an increase in p-RIPK3 is observed in the QUIN240 group compared to the SHAM group (Fig. 10e; ANOVA: $P = 0.0017, F (2, 8) = 15.72$; Tukey’s post hoc test: SHAM vs QUIN120 $[P = 0.8763]$; SHAM vs QUIN240 $[P < 0.0038]$).

**Discussion**

QUIN is an important tool for the study of the molecular mechanisms involved in excitotoxicity that leads to cellular damage and death as well as in the search of potential therapeutic targets for neurodegenerative disorders. Excitotoxicity occurs when NMDA receptors are overactivated by agonists such as QUIN leading to cellular death [28].

In this work, QUIN caused a rotation behavior in animals after apomorphine administration in a dose–response manner. Apomorphine, an agonist of dopamine receptors, induces an ipsilateral rotation behavior in animals with unilateral striatal lesion, due to an imbalance in dopamine receptor activity, and lesion size is proportional to the rotation speed [29]. The rotation behavior correlated with the extension of tissue damage observed, since QUIN increased neurodegeneration and cellular damage in a dose–response manner. According with our results, in rats and *Caenorhabditis elegans*, QUIN induces cycling behavior and behavioral deficits, respectively, along with neurodegeneration [24, 30].

The cellular damage observed after QUIN administration could be related with the increase in oxidative stress [16, 31]. Previous reports show that QUIN generates an overproduction of hydroxyl radical and superoxide anion, at 50 min and 2 h, respectively [32, 33]; increases the lipoperoxidation at 2 h and protein oxidation at 4 h [34, 35]; and decreases glutathione levels at 4 h [36].

It has been reported that oxidative stress induced by QUIN causes damage in subcellular compartments such as mitochondria and RER [16, 37]. Here, QUIN induced dilatation of cristae and small and round mitochondrial morphology in a dose–response way. These mitochondrial alterations could be related with Ca$^{2+}$ influx, ROS production [16, 38], and the activation of signaling pathways that promote cell death such as JNK pathway. Santana-Martínez et al. [24] reported an increase in p-JNK (active JNK) levels in mitochondria, which could be associated with apoptosis induction. Additionally, there are reports in vivo indicating that QUIN produces alterations in mitochondrial complexes [16, 39], and mitochondrial dysfunction at 24 h [40] and 21 days post-lesion [39], which could be associated with the morphological alterations observed in mitochondria in this work.

Also, we observed fragmentation and thinning membrane in RER, as well as a decrease of ribosomes in QUIN groups. The damage observed in RER also follows a dose–response effect. Fernandes et al. [41] reported that QUIN decreases the sarco/ER Ca$^{2+}$-ATPase (SERCA)-mediated Ca$^{2+}$ uptake in microsomal fractions of rat striatum, which could be involved in the morphological alterations observed in RER with QUIN. Furthermore, damage in organelles and a decrease in ER were also observed in cells of rat liver in response to 60 nmol of QUIN [42].
Fig. 10 Effect of quinolinic acid (QUIN) on protein levels involved in the necroptosis pathway. Animals were administered in the right striatum with 1 µL of isotonic saline solution (SHAM group) or QUIN (120 nmol or 240 nmol). Brains for immunohistochemistry or right striatum for western blot were collected at day 7 after intrastriatal administration. a Representative micrographs (×40) of the lesion core of each group for receptor-interacting protein kinase 3 (RIPK3) are shown. Bold arrows show positive cells to RIPK3. Slight RIPK3 immunostaining is observed in striatal tissue from the sham group; in comparison, neurons and axons from the QUIN120 group show mild RIPK3 immunoreactivity. Strong RIPK3 immunostaining is showed by damaged striatal neurons of the QUIN240 group. Representative images of western blot and densitometric quantification of b tumor necrosis factor alpha (TNFα) ≈ 40 kDa, c receptor-interacting protein kinase 1 (RIPK1) ≈ 75 kDa, d RIPK3 ≈ 57 kDa, and e phosphorylated RIPK3 (S232) ≈ 57 kDa are shown. Data are expressed as the mean ± SD of four animals per group. *P < 0.05 and **P < 0.01 vs SHAM group. OD optical density.
Moreover, discontinuity and separation of the compact myelin sheaths and abundant fragmentation with disappearance of neurofilaments and microtubules in axons were observed after QUIN administration. There is evidence indicating that QUIN is involved in demyelinating diseases, such as multiple sclerosis [43], and it is toxic to oligodendrocytes [44]. NMDA receptors in compact myelin of oligodendrocytes (part of oligodendrocyte that surrounds the axon cylinder of neurons) in optic nerve cells induce the accumulation of Ca$^{2+}$ in myelin sheaths during ischemia injury causing damage [45]. Additionally, the glutamate-induced excitotoxic process causes axonal degeneration through the activation of the necroptotic pathway [11].

There are reports indicating that excitotoxicity and oxidative stress promotes disruption in autophagy flux and, in consequence, promotes the accumulation of damaged organelles,
increasing cellular damage [4, 46]. Previously, the autophagy flux blockage was observed in excitotoxic models at early times in hippocampal neurons [3] and in the striatum of rats administered with glutamate [47], which has been associated with cellular damage and cellular death [3]. In this work, we observe that QUIN240 increased the levels of p62 and LC3-II and both molecules were confined in cytoplasmic vacuoles, suggesting a blockage in autophagy flux, and this proposal is supported by the reduction in mature cathepsin D and Beclin 1 levels, despite the increase observed in ULK1 phosphorylation and the decrease in p70S6K phosphorylation.

Phosphorylation of ULK1 at Ser317 by AMPK activates the autophagy, whereas ULK1 phosphorylation at Ser757 by mTORC1 inhibits autophagy [8]. The increase in Ser317 phosphorylation on ULK1 suggests an activation of autophagy; moreover, the decreases in Thr389 phosphorylation in p70S6K, a target of mTORC1, support an activation of autophagy, indicating a decrease in mTORC1 activity. These results suggest an increase in autophagy at early stages; however, we found a decrease in Beclin 1 and accumulation of LC3-II and p62 proteins on day 7, suggesting an impairment of this pathway in later stages, probably at phagophore formation level as suggested by our double-marker immunogold study that showed both proteins confined in cytoplasmic vacuoles or later step such as lysosomal dysfunction.

Beclin 1 is an important protein involved in the nucleation of phagophore, is part of the PI3KC3 complex, and mediates the localization of some proteins important in phagophore formation [48]. The decrease of Beclin 1 is associated with autophagy inhibition [4]. Nguyen et al. [27] reported a decrease in Beclin 1 levels induced by its cleavage by calpain in an excitotoxic and oxidative stress–dependent mechanisms, according with our results. Furthermore, Bieri et al. [49] reported a decrease in Beclin 1 by its cleavage induced by caspase-3 in patients with Alzheimer’s disease, hAAP transgenic mouse model, and excitotoxic model induced by kainic acid. The decrease in Beclin 1 levels observed in this work could be associated with the activation of caspase-3, since we observed a decrease in Bcl-2 and an increase in Bax levels and Bax/Bcl-2 ratio, indicating an activation of intrinsic apoptosis and suggesting the activation of caspase-3. Indeed, we observed many apoptotic cells in our ultrastructural study, supporting apoptosis activation. Moreover, it is important to note that the decrease in Beclin 1 was observed 5 days after kainic acid administration [49], similar to our model (7 days after QUIN injection).

Additionally, the blockage in autophagy flux by QUIN was evident by the accumulation of LC3-II and p62 levels. This blockage could be associated with the decrease in mature cathepsin D levels, an aspartic protease important in protein degradation by lysosomes or by the activation of necroptosis. Cathepsin D decreases after cerebral ischemia [21, 50], promoting lysosomal dysfunction and inducing blockage of autophagy flux, without altering the autophagy initiation [21]; also, there is evidence indicating that knockout mice to cathepsin D presents accumulation of autophagosomes in neurons from brain cortex [51]. Moreover, the decrease of cathepsin D, compared with the decline in other cathepsins involved in lysosomal function such as cathepsin B and L, is the cause of autophagy dysfunction [21, 52]. The decline in mature cathepsin D could be related to the decrease in its maturation process or increase in mature cathepsin D degradation, instead of impairment in its synthesis. We observed that QUIN240 does not alter the pre-cathepsin D levels, while decreasing the maturation process, suggesting that the impairment in its synthesis does not occur. According with our results, Hossain et al. [21] reported that a decrease in cathepsin D levels after cerebral ischemia is not associated with a decline in its synthesis.

The deficiency of cathepsin D is also associated with an increase in apoptosis in retinal photoreceptor cells [52]. Correlating with this report, we observed an increase in Bax levels and Bax/Bcl-2 ratio and a decrease in Bcl-2 levels, as well as an increase in JNK phosphorylation. The decrease in Bcl-2 could be associated with JNK phosphorylation. JNK leads to phosphorylation of Bcl-2 at Ser70 [53] inactivating and promoting its proteasomal degradation and increasing cell apoptosis [54, 55]. This increase in apoptosis could be associated with the
Another possibility involved in the blockage of autophagy flux is the lysosomal dysfunction promoted by necroptosis activation at early times. QUIN induces necrosis and apoptosis death in striatum [22, 23]; however, there is increasing evidence suggesting the participation of other types of cellular death such as necroptosis [11, 12]. Necroptosis initiates with the signaling of TNFα to its receptor, inducing the activation of RIPK1 that activates RIPK3 and, in consequence, activates its late effector (MLKL) [56]. The necroptosis pathway is implied in brain damage after cerebral ischemia [56, 57] and subarachnoid hemorrhage [58] and contributes to axonal degeneration in excitotoxic models [11]. Its activation occurs at early times in brain damage [58] followed by apoptosis induction [56], and its inhibition decreases cerebral damage [57, 59]. Despite the fact that necroptosis is an event that occurs early in brain damage with a major expression of RIPK3 between 24 and 48 h [56, 58], we observed an increase in TNFα, RIPK3, and p-RIPK3 levels at 7 days after QUIN240, suggesting that necroptosis remains active at this time. Feng et al. [17] found an increase in TNFα as a consequence of microglial activation by QUIN, suggesting that TNFα observed in our work could be secreted by microglia, activating necroptosis. On the other hand, the levels of RIPK1 did not change compared to the SHAM group; however, a tendency to increase was observed in the QUIN240 group, suggesting that at this time, the activation of necroptosis decline. As mentioned above, the activation of necroptosis could be involved in the autophagy disruption observed in the QUIN240 group, through lysosomal dysfunction. Phosphorylated MLKL disrupts autolysosomal membrane, decreasing intraluminal lysosomal acidity, promoting autolysosomal dysfunction, and blocking the autophagy flux [14]. For that reason, the blocking in autophagy flux after QUIN administration could be associated with necroptosis activation at early times.

We propose that QUIN induced excitotoxicity and oxidative stress promoting cellular and subcellular compartment damage, which could be exacerbated by the blockage in autophagy flux, through the activation of necroptosis and apoptosis. Moreover, the axonal damage could be related with the activation of necroptosis (Fig. 11).

Conclusion

QUIN induces rotation behavior, neurodegeneration, and morphological alterations in mitochondria, RER, and axons of striatal neurons at 7 days post-lesion, possibly through the impairment in autophagy and an increase in necroptosis and apoptosis pathways, suggesting that the autophagy activation is a dynamic event that remains active for long times and that the necroptosis and apoptosis as the main pathways involve in the neuronal death.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s12035-022-02996-1.

Author contribution Conceptualization: Perla D. Maldonado and Carlos Alfredo Silva-Islas; methods: Carlos Alfredo Silva-Islas, Ricardo Alberto Santana-Martínez, and Juan Carlos León-Contreras; formal analysis and investigation: Carlos Alfredo Silva-Islas; writing or preparation of the original draft: Carlos Alfredo Silva-Islas and Ricardo Alberto Santana-Martínez; writing which included review and editing: Carlos Alfredo Silva-Islas, Diana Barrera-Oviedo, Jose Pedraza-Chaverri, Rogelio Hernández-Pando, and Perla D. Maldonado; funding acquisition: Perla D. Maldonado; resources: Perla D Maldonado; supervision: Jose Pedraza-Chaverri, Rogelio Hernández-Pando, and Perla D. Maldonado. All authors read and approved the final manuscript.

Funding This work was supported by CONACYT (Grant No. A1-S-21433 to PDM).

Data availability The datasets generated during and/or analyzed though the current study are available as supplementary material.

Declarations

Ethics approval and consent to participate This research only involves animals. All procedures with animals were carried out strictly according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and to the Norma Oficial Mexicana NOM-062-ZOO-1999. The experimental procedures were approved by the Institutional and Local Committee for the Care and Use of Laboratory Animals on the Ethical Use of Animals from Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, INNN project 44/15. During the experiments, all efforts were made to minimize animal suffering. Informed consent is not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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