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ORIGINAL PAPER

Interleukin 3 Inhibits Glutamate-Cytotoxicity in Neuroblastoma Cell Line

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Abstract

Interleukin 3 (IL-3) is a well-known pleiotropic cytokine that regulates the proliferation and differentiation of hematopoietic progenitor cells, triggering classical signaling pathways such as JAK/STAT, Ras/MAPK, and PI3K/Akt to carry out its functions. Interestingly, the IL-3 receptor is also expressed in non-hematopoietic cells, playing a crucial role in cell survival. Our previous research demonstrated the expression of the IL-3 receptor in neuron cells and its protective role in neurodegeneration. Glutamate, a principal neurotransmitter in the central nervous system, can induce cellular stress and lead to neurotoxicity when its extracellular concentrations surpass normal levels. This excessive glutamate presence is frequently observed in various neurological diseases. In this study, we uncover the protective role of IL-3 as an inhibitor of glutamate-induced cell death, analyzing the cytokine's signaling pathways during its protective effect. Specifically, we examined the relevance of JAK/STAT, Ras/MAPK, and PI3 K signaling pathways in the molecular mechanism triggered by IL-3. Our results show that the inhibition of JAK, ERK, and PI3 K signaling pathways, using pharmacological inhibitors, effectively blocked IL-3's protective role against glutamate-induced cell death. Additionally, our findings suggest that Bcl-2 and Bax proteins may be involved in the molecular mechanism triggered by IL-3. Our investigation into IL-3's ability to protect neuronal cells from glutamate-induced damage offers a promising therapeutic avenue with potential clinical implications for several neurological diseases characterized by glutamate neurotoxicity.

Keywords IL-3 · Glutamate cytotoxicity · Neuroprotection · Apoptotic cell death

Introduction

Glutamate is one of the most important neurotransmitters in the Central Nervous System (CNS) [1] that modulate different functions and plasticity in the mammalian brain [2]. However, high concentrations of glutamate produce several deleterious effects on neuronal metabolism, survival, and neurogenesis [1], this effect is known as "glutamate neurotoxicity," and it is characterized by a time-dependent cell damage leading to cell death [3, 4]. The changes in neuronal metabolism produced by the increase in extracellular glutamate concentrations cause atrophy and necrosis in neurons, alterations in cellular matrix protein synthesis, and increased oxidative stress, inhibiting neurogenesis processes [4]. In recent decades, it has been widely studied that glutamate neurotoxicity is involved in the pathophysiology of certain neurological diseases [5] as amyotrophic lateral sclerosis (ALS) [6], stroke, Alzheimer's disease, Parkinson's disease [7, 8] and Huntington's disease [9–12]. The excess of glutamate can cause cell damage and/or death and includes two stages [5]. Acute neuronal inflammation is the signature mark of the first stage. It depends on the absorption of extracellular Na^+ and the Cl^- uptake by the cell, which causes plasma membrane depolarization, leading to the opening of Ca^{2+} channels and inducing the second stage, which is characterized by neuronal degeneration [7, 13]. In this second stage, the massive extracellular Ca^{2+} flux, together

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with some release of free Ca^{2+}, causes an increase of Ca^{2+} concentration in cellular compartments, initiating a cascade leading to cell death [14, 15].

Trophic factors are proposed as candidates for treating neurodegenerative diseases given the neuroprotective effects they exert on different cell populations [16–18]. Neurotrophic factors are proteins that promote the survival and differentiation of several neuronal cell types, regulating the formation and establishment of synaptic connections and gene expression through interaction with specific cell receptors [19]. In the nervous system, particularly in its development, apoptosis appears to be triggered by the deprivation of specific trophic factors [20]. Lack of activation of an induced transduction pathway by trophic factors leads to caspases` activation by complex mechanisms [21]. These trophic factors play important roles during development, controlling the process of programmed cell death and regulating the final number of neurons and connections in the nervous system. Cytokines are one of the largest trophic factors family. IL-3 is a pleiotropic cytokine whose main function is to regulate the proliferation and differentiation of precursor cells of hematopoietic lineage and the activation of mature leukocytes [22]. The receptor for IL-3 consists of a heterodimer composed of a subunit-specific for this cytokine called IL-3Rα of 60–70 kDa, which is capable of binding it with a low affinity but without being able to transduce signals by itself into the cell and a subunit of 130–140 kDa, called βc, that is common for IL-3, IL-5, and GM-CSF [23], which it is necessary to bind the cytokine with high affinity and it is responsible for the activation of the signaling cascade. Both α and β subunits belong to the cytokine receptor superfamily type I and contain conserved extracellular domains called Modules of Cytokine Receptors (CRMs). The binding of IL-3 to its receptor triggers several signaling pathways that exert cell survival. Among these signaling cascades are the Akt/PKB pathway, the ERK/MAPK pathway, and the JAK/STAT pathway [23, 24].

Importantly, this cytokine fulfills many relevant functions for brain cells [25–28]. However, it has been barely studied under normal conditions, much less under conditions of neuronal damage. Previous studies from our laboratory have shown that IL-3 induces survival and inhibits the apoptotic process against neurotoxicity caused by Aβ peptides (beta-amyloid) in primary culture of cortical neurons and that activation of the PI3 K/Akt signaling pathway is essential in this effect. In addition, it shows that IL-3 inhibits the process neurodegenerative induced by Aβ maintaining microtubule dynamics by inhibiting modifications that the tau protein undergoes in response to this toxic agent [29, 30].

In this work, we explored the protective effect of IL-3 in neuronal cells, preventing or minimizing glutamate toxicity. Our principal aim was to test if IL-3 protects against glutamate-induced cellular damage in N2a cells and characterize the signaling pathways involved in this protective function of IL-3. Protecting neuronal cells against glutamate-induced damage by IL-3 could be a promising therapeutic strategy.

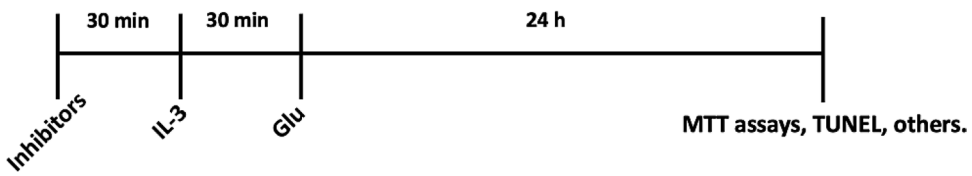
Materials and Methods

Cell Culture and Treatments

N2a cell line derived from rat neuroblastoma (ATCC CCL-131™) was obtained from American Type culture collection (Manassas, USA). It was grown in Dulbecco's Modified Eagle (DMEM) medium supplemented with 10% fetal bovine serum (from Hyclone, Logan, UT, USA), 100 U/mL penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (from Hyclone, Logan, UT, USA), 1mM Sodium Pyruvate, and essential amino acids (from Corning, NY, USA), in a humified environment with 5% CO_2 at 37 °C. Neurite outgrowth was promoted by changing the cells to DMEM medium plus 0.25% serum, and the addition of 5 mM db-cAMP. After 48 h treatment, cells exhibited a differentiated morphology with long neuritic processes [31].

After 48 h of treatment with 5 mM db-cAMP, the cells were pre-incubated with IL-3 (3 nM and/or 6 nM), followed by incubation with 7 mM glutamate for 24 h. To determine the role of different signalling pathways in the activity of IL-3, the cells were treated with different specific inhibitors 30 min before incubation with IL-3. The inhibitors used in this study were 10 µM AG490, 50 µM LY294002, 10 µM AKT4 inhibitor, PD98059 20 µM.

The timeline of the treatments is as follows:



Western Blot Analysis

Cells were cultured and treated with Glutamate (Merck KGaA, Darmstadt, Germany) in presence or absence of IL-3 (R&D Systems, Minneapolis. USA), for different time periods and concentrations. Then, cells were lysed in Lysis buffer (50 mM Tris-HCl pH 7,5; 150 mM NaCl; 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA; and protease inhibitors: 100 µg/mL PMSF, 0.08mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin and 1.4 mM E-64) for 15 min on ice. After this time, the plate was scraped with a stripper. The lysates were centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant containing the proteins and stored at -20 ° C for later quantification. Protein concentration was determined using the Bradford assay. Protein extracts were resolved by SDS–PAGE, 100 or 60 µg per lane, on a 10% or 12% polyacrylamide gel (and transferred into Nitrocellulose Membrane, 0.45 µM (Bio-Rad Laboratories). Next, the membrane was blocked with 5% skim milk, and incubated with 1:1000 dilutions of primary antibodies against IL-3 subunits (anti-IL3Rα and βc; Santa Cruz Biotechnology, CA, USA), p-JAK2 (#3771), JAK2 (#3230), p-Akt (#4060), Akt (#4691), p-ERK (#4370), and ERK (#4695); (All Cell Signaling Technology, Inc., Danvers, MA, USA), tubulin (CP06, Calbiochem, USA) was used as loading control. The reaction was developed using anti-rabbit or anti-mouse HRP-conjugated antibodies (1:5000, ThermoFisher Scientific, Waltham, Massachusetts, USA) and the enhanced chemiluminescence western blot analysis system was visualized by Syngene G: BOX equipment.

Indirect Immunofluorescence

The cells were grown on covers-slips coated with poly-L-Lysine in culture plates, then the cells were exposed to different stimuli such as glutamate and IL-3 alone or combined to different concentrations and times. The medium was removed, washed with 1X PBS, and fixed with 4% paraformaldehyde for 15 min. The cells were permeabilized and blocked for 30 min with blocking solution (BSA 1%, skim milk 5%, 1X PBS) with 0.3% Triton X-100. The cells were incubated with the primary antibodies of interest diluted 1: 100 in the same blocking solution overnight in a humid chamber at 4 °C. Cells were washed and incubated with anti-rabbit, anti-goat or anti-mouse IgG– Alexa 488 (1:300, Invitrogen) with propidium iodide (1.7 mg/ml, Sigma Aldrich). The samples were mounted using the fluorescence mounting medium DAKO and visualized with an inverted Olympus FluoView 1000 confocal microscope.

MTT Assays for Cellular Viability

Cells were cultured in 96-well plates, being exposed to glutamate as a stressor, IL-3 or both at different times and concentrations in the presence or absence of inhibitors; 10 µM AG490, 50 µM LY294002, 10 µM AKT4 inhibitor, PD98059 20 µM. After being subjected to the different treatments, we added 10 µL of 0.5 mg/ml MTT (3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide; thiazolyl blue) for 24 h at 37 °C. Subsequently, the cells were lysed with lysis solution (dimethylformamide 50% SDS 20%) and incubated overnight at 37 °C. The absorbance was recorded in an ELISA reader with a 540 nm filter. The results were expressed as a percentage of control (cells stimulated with vehicle).

Detection of Apoptotic Cells by TUNEL kit

Cells were cultured on cover-slips coated with poly-L-Lysine in culture plates to carry out the different treatments for 24 h. To measure DNA fragmentation, cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed using the *in sit*u Cell Death detection kit as described by the manufacturer (Roche, Basel. Switzerland) kit was used according to the maker's indications to detect fragmentation of DNA. The visualization of the nuclei with the fragmented DNA was performed by fluorescence microscopy.

RNA Isolation and Quantitative Reverse Transcription Real-Time PCR (RT-qPCR)

Cells were treated with 7mM glutamate, 3nM IL-3 or both, in the presence or absence of inhibitors; 10 µM AG490, 50 µM LY294002, 10 µM AKT4 inhibitor, PD98059 20 µM at 24 h. The total RNA was isolated from cells with Trizol (Life Technologies; Carlsbad, California, USA) following the manufacturer's instructions. RNA was quantified by spectrophotometric analysis using the Infinite® 200 NanoQuant equipment (TECAN, Männedorf, Switzerland). Sample concentration and purity were determined by 260/280 nm UV absorbance. Total RNA isolated from cells was used for RT-qPCR assay to quantify mRNA expression. cDNAs were synthetized using iScript kit (BIO-RAD; Hercules, California, USA) and the qPCR analysis was performed using the Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies, Stratagen, Santa Clara, California, USA). RT-qPCR result was evaluated by StepOne v2.3 software (StepOne Plus equipment, Thermo Scientific, Waltham, Massachusetts, USA). The expression analysis was evaluated by relative quantification using Comparative Ct Method ($2^{-\Delta\Delta Ct}$) [32] and was normalized respect to β- actin. The

primer sequences are specific for Mus musculus and the theoric Tm was analyzed by AmplifX Software. Primers used are: (1) Bcl-2 expression, sense sequence: 5′-AGCATGCGACCTCTGTTTGAT-3′ and antisense: 5′-GGTTTGTCGACCTCACTTGT-3′, (2) Bax expression, sense sequence: 5′-TGCTAGCAAACTGGTGCTCA-3′ and antisense: 5´-CTTGGATCCAGACAAGCAGC-3´, and β-actin expression, sense sequence: 5′-TACCACCATGTACCCAGGCA-3′, antisense sequence: 5′- CTCAGGAGGAGCATTGATCTTGAT − 3′.

To determine the expression of IL-3 receptor, the PCR was performed using 5U/ul DNA polymerase (Invitrogen, Thermo Fisher, Massachusetts, USA) using IL-3rα sense: 5′- ACGGCACACGGGAAGATATCAGAA-3′ antisense: 5′-ATACGTGTTCCCTGAGCATCCGTT-3′ IL-3rβ sense: 5′-ACAACGACTACCCAGCCACATCA-3′ antisense: 5′-AGGCCTGTCTGGTTGGAATGAGAA-3′. For each reaction the protocol was as follow: IL-3rα and IL-3rβ: 94 °C for 1 min, and 30 cycles (94 °C for 50 s, 55 °C for 1 min, 72 °C for 1 min), and finally at 72 °C for 5 min.

Statistic Analysis

Statistical significance was evaluated using the student's t-test or by analysis of variance (ANOVA followed by the Bonferroni test, One-way ANOVA), using the GraphPad program Prism 9.0 (San Diego, CA, USA)., the value of p is indicated in each case.

Results

IL-3 Receptor is Expressed in N2a Cells

We previously demonstrated the presence of IL-3 receptors in cortical neurons [29]. Here, the first analysis was to determine the expression of IL-3 receptors in N2a cells. The protein levels of IL-3 receptor, α and βc subunits were detected by immunodetection analysis using specific antibodies. In Fig. 1A, it is possible to observe a band with a molecular weight of 56 kDa corresponding to the α subunit and a band with a molecular weight of 125 kDa corresponding to the βc subunit of the IL-3 receptor. To determine the expression levels of both α and βc subunits, an RT-PCR was performed with specific primers for each subunit (Fig. 1B). An amplification product of the expected size for each subunit was detected. The sizes correspond to 437 bp and 348 bp for the α and β subunits, respectively. Thus, both IL-3 receptor subunits are present in this cell model and are detectable at the protein and mRNA levels.

The presence of the IL-3 receptor was also corroborated in N2a cells by indirect immunofluorescence analysis, in which the cells were incubated with specific antibodies against the α and β subunit of the receptor following the procedure described in the methods section. In Fig. 1C, we can visualize that both receptor subunits are expressed in this cell line evenly distributed throughout all cells.

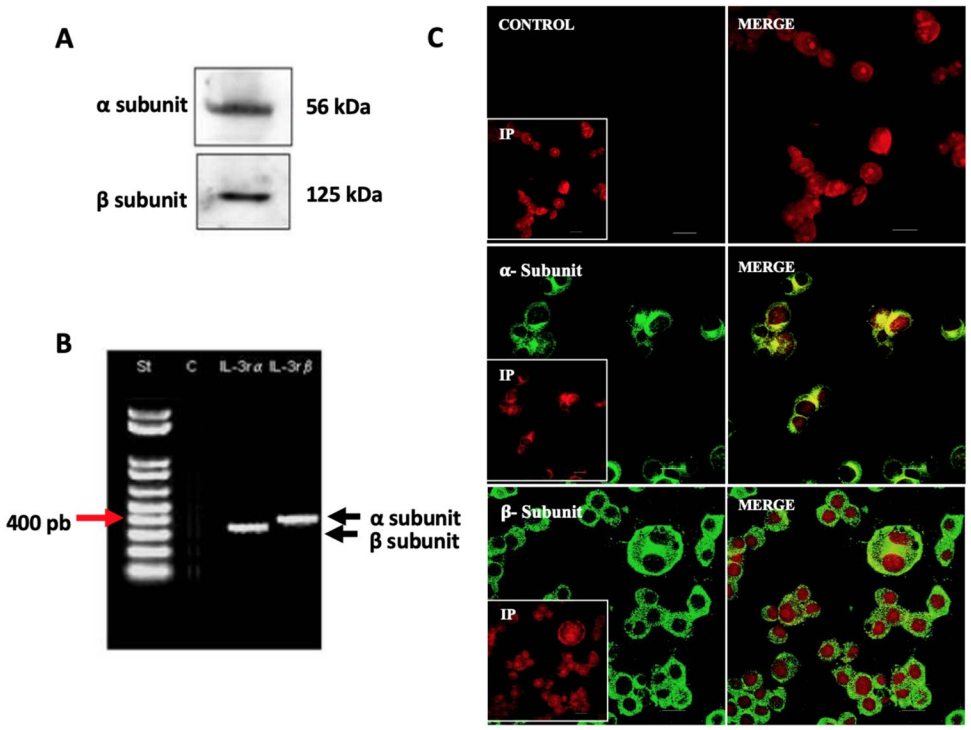


Fig. 1 Expression of IL-3 receptor in N2a cell line. **A** Western blot analysis using specific antibodies for alpha (IL-3α) and beta (IL-3β) subunits for IL-3 receptor. **B** Analysis of gene expression for IL-3 subunits α and β. **C** Indirect immunofluorescence visualized by microscopy confocal of IL-3 receptor subunits, in green (IL-3r α and IL-3r β), and nuclei were stained with propidium iodide (IP), in red. Negative control was performed in the absence of primary antibodies for the respective subunits. Results are representative of three independent experiments. Scale bar, 20 μM

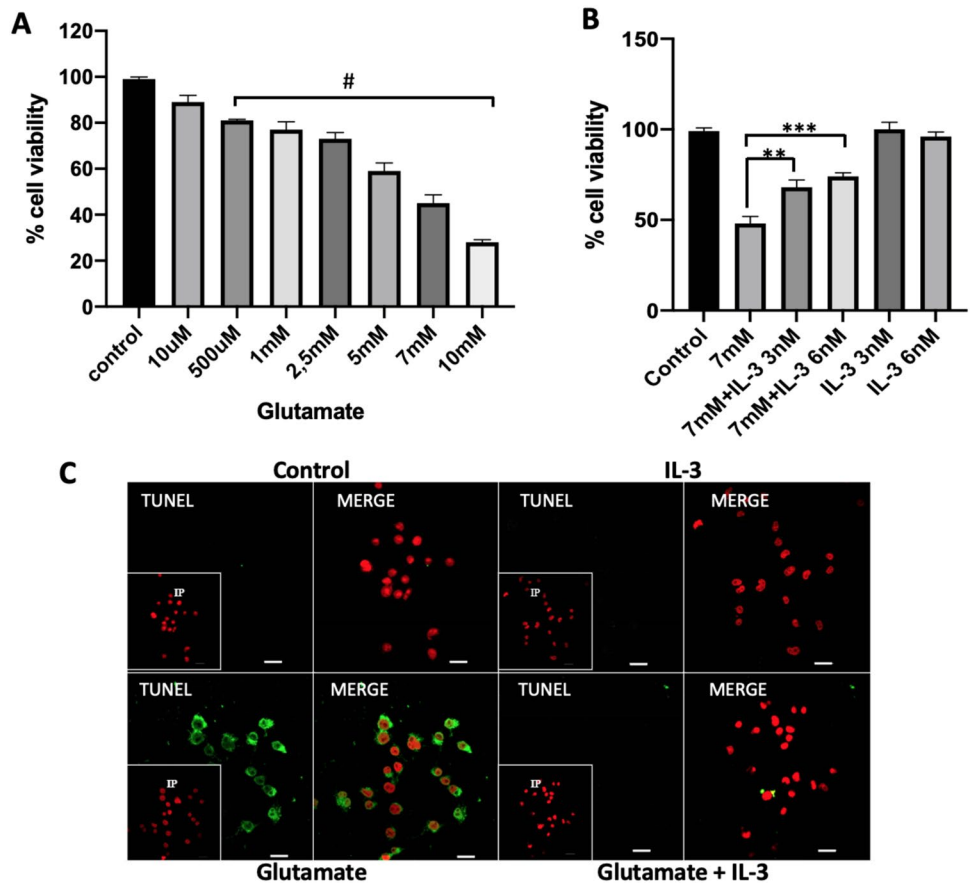


Fig. 2 Effect of glutamate and IL-3 on N2a cell viability. **A** Cells were incubated for 24 h with different concentrations of glutamate, and cell viability was measured using MTT assay. **B** Cells were pre-incubated with two different concentrations of IL-3 (3 nM and 6 nM) for 30 min and then maintained in the presence or absence of 7 mM glutamate for 24 h; cell viability was measured using MTT assay. **C** Cells were previously incubated with 3 nM IL-3 for 30 min, then treated with 7 mM glutamate for 24 h, and the effect on DNA fragmentation was measured by TUNEL assay, and nuclei were stained with propidium iodide (IP) in red. Results are representative of three independent experiments. Data are presented as mean± S.E. (# $p < 0.0001$ comparing to the control; *** $p < 0.0001$ and ** $p < 0.001$ comparing to 7 mM glutamate treatment). Scale bar 20 µM

Effect of Glutamate and IL-3 on N2a Cells Viability

Cell viability was analyzed with different glutamate concentrations at 24 h to determine the cytotoxic effect in this cell line. Figure 2A shows a decrease in cell viability in a dose-dependent manner when N2a cells were treated with increasing glutamate concentrations. The minimum glutamate concentration to produce a percentage of cell death statistically significant to the control was 500µM. At 7 mM, the cell death was around 50%, that why this concentration was used in subsequent assays. Then the role of IL-3 on cell cytotoxicity induced by glutamate was analyzed; Fig. 2B shows that 3 nM and 6 nM of IL-3 significantly recover in the 7 mM glutamate-induced cell death. From 3 nM IL-3 the inhibitory effect of induced cell death by glutamate is statistically significant. These data strongly suggest that IL-3 would play a role as an inhibitor of glutamate-induced cell death.

An analysis of the fragmented DNA, using the TUNEL kit, was performed to corroborate the effect of IL-3 on glutamate-induced cell death. N2a cells were preincubated with 3 nM IL-3 at a concentration of 3nM and then treated with 7mM glutamate for 24 h. Later, the cells were visualized through fluorescence microscopy (Fig. 2C). The results showed that glutamate induces DNA fragmentation in many cells (Fig. 2C, bottom-left panel). However, the mark was decreased in cells preincubated with IL-3 (Fig. 2C, bottom-right panel). When incubating only with IL-3, TUNEL-positive cells were not observed (Fig. 2C, see TUNEL in the right-top-right panel). Therefore, these results show the protective effect that produces the cytokine against high glutamate concentrations.

Effect of IL-3 on Glutamate-Induced Apoptosis

Activation of caspase 3 was analyzed by indirect immunofluorescence to determine if IL-3 can specifically inhibit the apoptosis process triggered by glutamate. Figure 3A shows that the positive signal for active-caspase 3 appears with the treatment using 7mM glutamate, and the signal decreases pre-incubating the cells with IL-3. This activation, or positive signal, is not present in cells without stimuli or in cells treated with IL-3 alone. Quantification of the active-caspase 3 positive signal is represented in

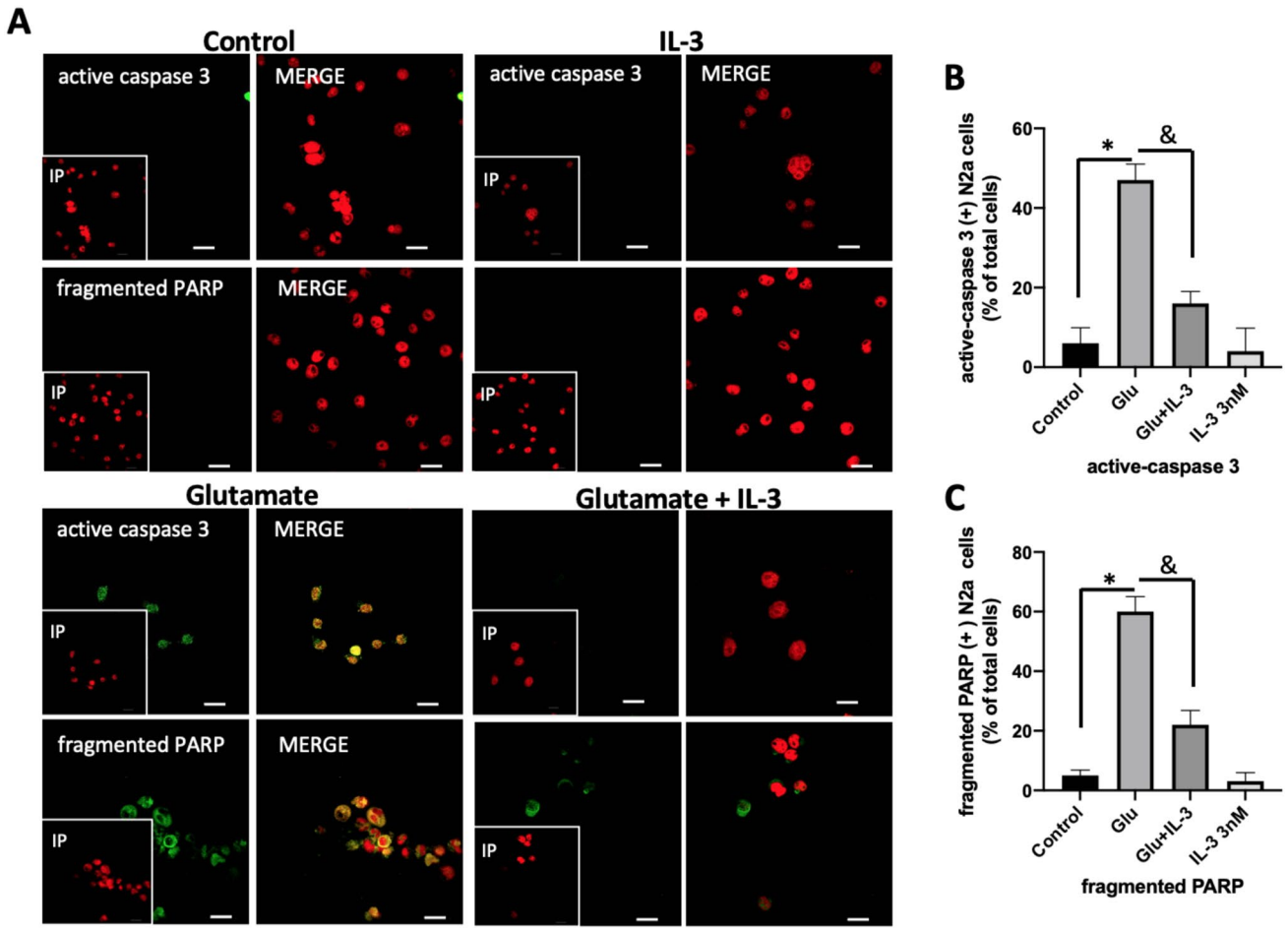


Fig. 3 Protective effect of IL-3 on glutamate-induced apoptosis. **A** Cells were stimulated with 7 mM glutamate in the presence and absence of 3 nM IL-3. Cells were fixed and stained using anti-active-caspase 3 or anti-cleaved- PARP antibodies as apoptotic markers (green). As a negative control, the cells were treated with vehicle. The result was visualized by fluorescence microscopy, and nuclei were stained with propidium iodide (IP), in red. **B** Graph represents the quantitative analysis of positive cells for active-caspase 3. **C** Graph represents the quantitative analysis of positive cells for fragmented PARP. Results are representative of three independent experiments. Data are presented as mean± S.E. (*$p < 0.001$ comparing to control and & $p < 0.001$ comparing to glutamate treatment). Scale bar, 20 µM

Fig. 3B. At the same time, indirect immunofluorescence was also used to analyze the presence of one of the principal caspase 3 substrates, PARP (Fig. 3A); the positive signal indicates the existence of PARP fragmented. The results show a clear positive signal in N2a cells stimulated with glutamate, and as we expected, this signal decreases in cells preincubated with IL-3. A quantification of PARP fragmented positive signal is represented in Fig. 3C.

Activation of JAK2 in N2a Cells Against Stimulation with IL-3

IL-3 can activate several signal transduction pathways in its target cells; one of them is the JAK/ STAT pathway [33]. The role of JAK2 kinase in IL-3 receptor the response of IL-3 receptor is critical, and it is considered the beginning of all responses generated by IL-3. We tested if this kinase is phosphorylated in the presence of IL-3 in N2a cells through immunofluorescence assays and Western blot analysis of phosphorylated JAK2. Immunofluorescence analysis (Fig. 4A) shows that this kinase is activated early, at 10 min, remaining phosphorylated for up to 60 min. Also, Western blot analysis (Fig. 4B) corroborates the immunofluorescence assays. A rapid activation of JAK2 at 2 min, increasing the phosphorylation twice to the control levels, and presenting a pick of activation at 30 min with an increase of 5,2 times to control levels; this phosphorylation remains for up to 60 min (4,3 times to control levels). These results demonstrate that IL-3 can activate JAK2 in N2a cells. Next, we studied the involvement of this signal transduction pathway in the protective effect of IL-3 in this model. The cells were treated with a

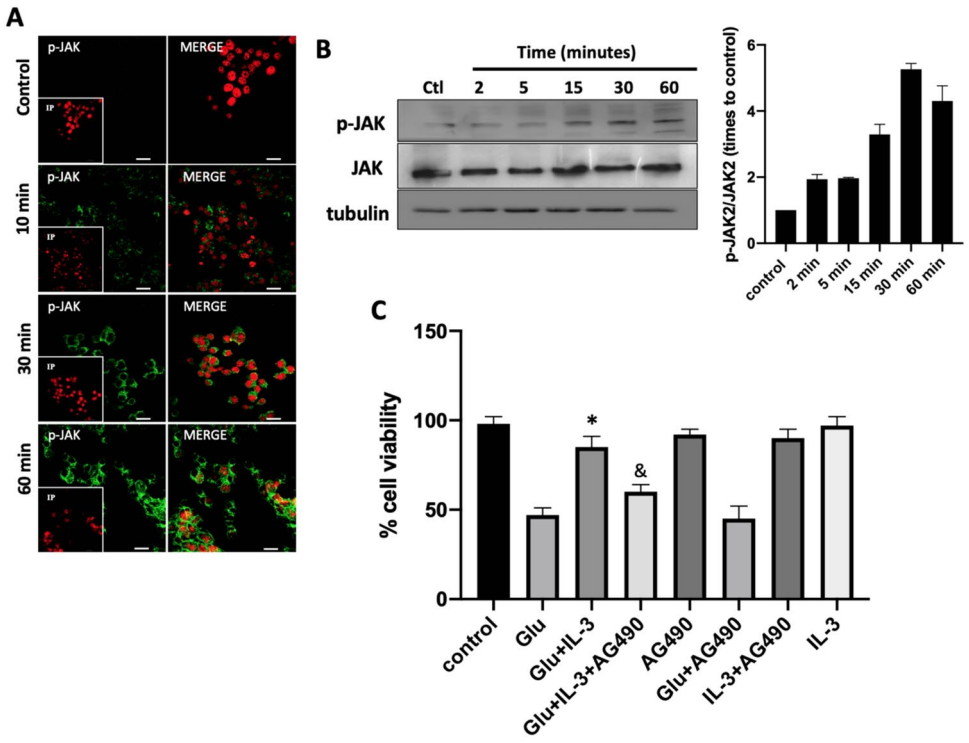


Fig. 4 Role of JAK/STAT signaling pathway. **A** Indirect immunofluorescence of N2a cells treated with 3 nM IL-3 for different times (from 0 to 60 min), using anti-p-JAK2 (green). The nuclei were stained with propidium iodide (IP; red). Scale bar, 20 µM. **B** Western blot analysis of total proteins from cells treated with 3 nM IL-3 during different time periods, using specific antibodies anti-p-JAK2 and anti-JAK2. Antibody against tubulin was used as a loading control. As a negative control, the cells were treated with vehicle. Graph represents the quantitative analysis of p-JAK2 normalized to total JAK2. **C** Cells were pre-incubated 30 min with 10 µM AG490, then treated with 3 nM IL-3 and 7 mM glutamate for 24 h. Cell viability was measured using MTT assay. Results are representative of three independent experiments. Data are presented as mean± S.E. (*$p < 0.001$ comparing to Glutamate treatment, and & $p < 0.001$ comparing to glutamate with IL-3 treatment)

specific inhibitor, AG490, 30 min before incubation with IL-3, and the effect of the inhibitor was analysed using the MTT method. Figure 4C clearly shows that by using the inhibitor AG490 to inhibit the JAK2 pathway, the protective effect induced by IL- 3 is blocked. We can also observe that AG490 alone does not significantly affect cell viability (Fig. 4C).

Akt Activation in N2a Cells with IL-3 Stimulation

Another essential pathway activated by IL-3 in hematopoietic cells is the PI3 K/Akt pathway. This signaling pathway is widely studied in cell viability. Immunofluorescence and Western blot analyses were performed to detect phosphorylated Akt protein to determine if IL-3 activates the Akt pathway in N2a cells. In Fig. 5A, we can observe the protein phosphorylation at 10 min after stimulating the cell culture using immunofluorescence. Using western blot analysis, we see an increase in phosphorylation at the protein level at 15 min of 3,8 times to control levels, and then a decrease in the phosphorylation at 60 min (just 1,4 times to control) (Fig. 5B). Then, we studied the involvement of this signaling pathway in the protective effect of IL-3. The inhibitors used were LY294002, a specific inhibitor of PI3 K, and AKT IV inhibitor, a specific inhibitor of Akt. Figure 5C and D show that the PI3 K/Akt pathway also would have an essential role in the effect of IL-3. Specifically, the pre-incubation with its inhibitor LY290024 completely blocks the protective effect of IL-3 on the viability of N2a cells under stress by glutamate (Fig. 5C). Since Akt is downstream of PI3 K, viability analysis was also performed with a specific inhibitor for Akt, AKT IV inhibitor (Fig. 5D), to analyze the participation of Akt protein in the protective effect of IL-3 against high glutamate concentrations. The results show that pre-incubation with these inhibitors completely blocks the protective effect of IL-3 on cell viability, decreasing cell viability, suggesting that Akt is an important protein for the effect of IL-3 on the viability induced by IL-3 in the face of stress caused by high glutamate concentrations.

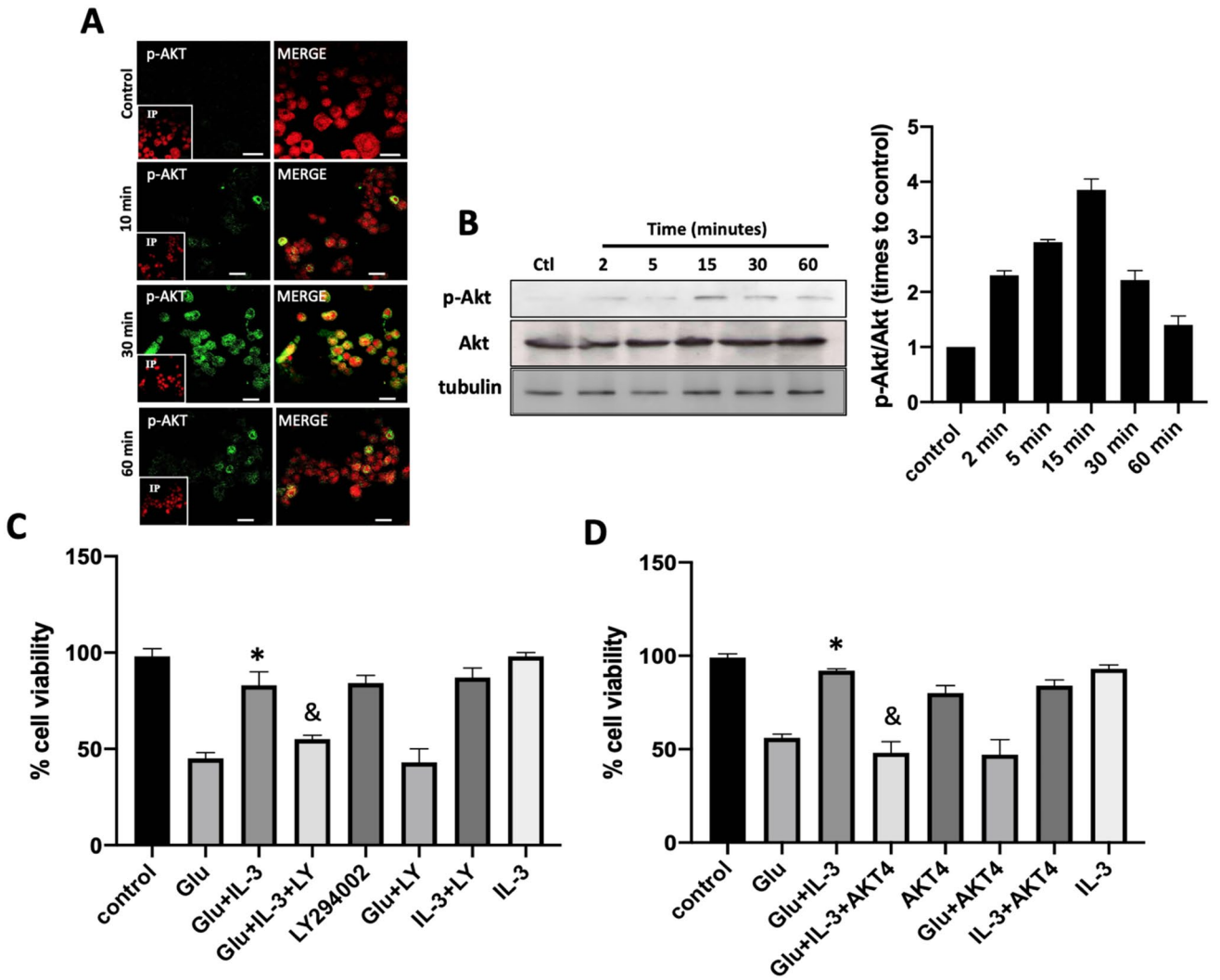


Fig. 5 Role of PI3 K/Akt signalling pathway. **A** Indirect immunofluorescence of N2a cells treated with 3 nM IL-3 for different time (from 0 to 60 min), using anti-p-Akt (green). The nuclei were stained with propidium iodide (IP; red). Scale bar, 20 µM. **B** Western blot analysis of total proteins from cells treated with 3 nM IL-3 during different time periods, using specific antibodies anti-p-Akt and anti-Akt. Antibody against tubulin was used as a loading control. As a negative control, the cells were treated with vehicle. Graph represents the quantitative analysis of p-Akt normalized to total Akt. **C** Cells were pre-incubated 30 min with 50 µM LY294002, then treated with 3 nM IL-3 and 7 mM glutamate for 24 h. Cell viability was measured using MTT assay. **D** Cells were pre-incubated 30 min with 10 µM AKT4 inhibitor, then treated with 3 nM IL-3 and 7 mM glutamate for 24 h. Cell viability was measured using MTT assay. Results are representative of three independent experiments. Data are presented as mean± S.E. (*$p<0.001$ comparing to glutamate treatment, and & $p<0.001$ comparing to glutamate with IL-3 treatment))

Activation of MAPK in N2a Cells After IL-3 Stimulation

The ERK-MAPK pathway is one of the most important for cell proliferation, and the IL-3 receptor transduces survival signals via MAPK in hematopoietic systems. To determine if IL-3 can activate these proteins in N2a cells, we tested the phosphorylation of ERK1 and ERK2 by immunofluorescence and Western blot. Figure 6A shows that IL-3 significantly increases p-ERK after 10 min of treatment. Western Blot analysis (Fig. 6B) indicates that the protein phosphorylation is increased at 2 min after treatment, presenting an increase of 12 times to control phosphorylation levels. This phosphorylation remains up to 60 min of treatment with 38 times to control cells.

Next, the cells were treated with a specific inhibitor for the MAPK pathway by blocking MEK, PD98059, 30 min before incubation with IL-3, and the effect of inhibitors was analyzed using the MTT assay. Figure 6C shows that the treatment with PD98059 has a significative effect on cell survival induced by IL-3 in the presence of glutamate; this

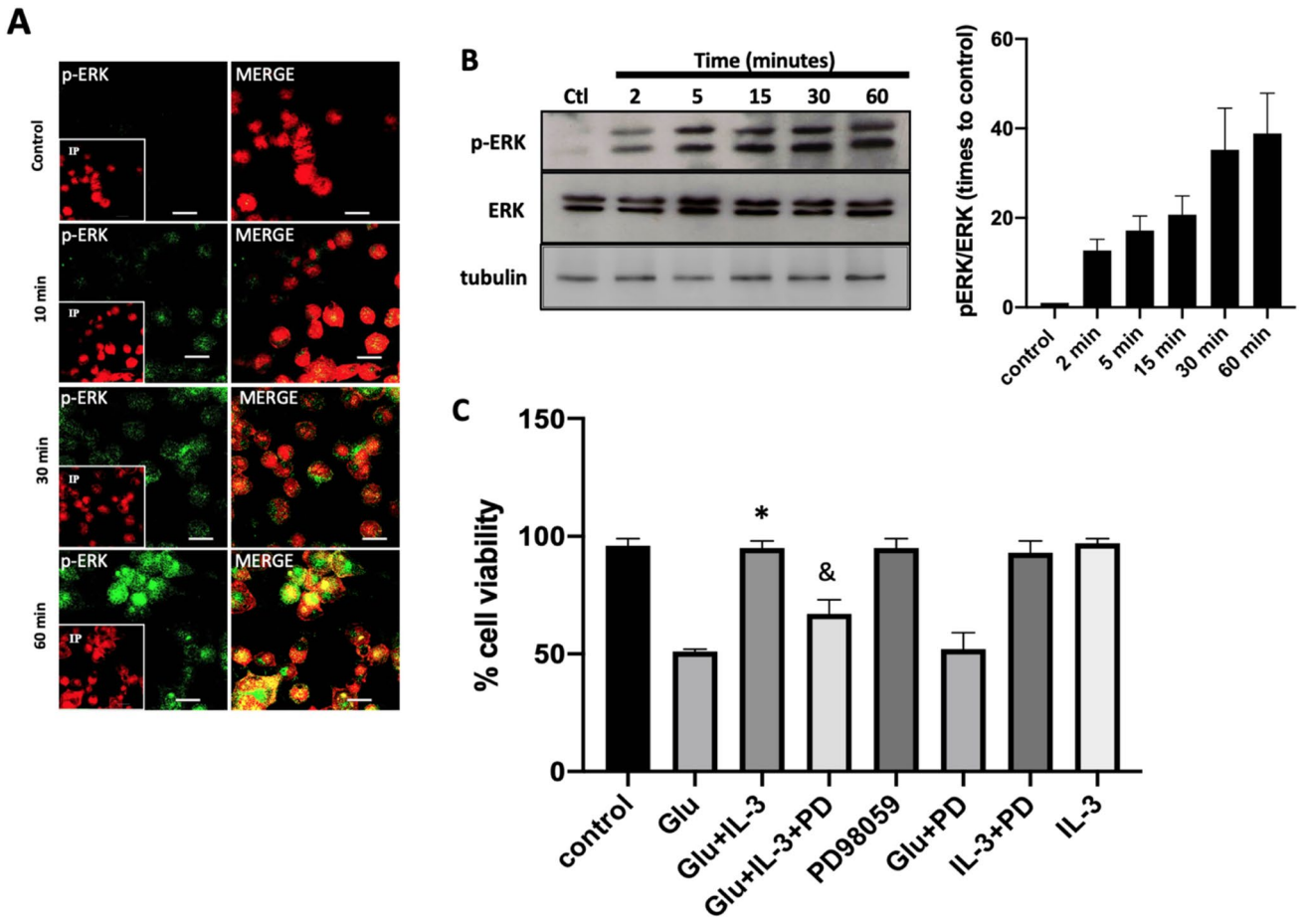


Fig. 6 Role of ERK signaling pathway. **A** Indirect immunofluorescence of N2a cells treated with 3 nM IL-3 for different time (from 0 to 60 min), using anti-p-ERK (anti-p-p44/42 MAPK, green). The nuclei were stained with propidium iodide (IP; red). Scale bar, 20 μM. **B** Western blot analysis of total proteins from cells treated with 3 nM IL-3 during different time periods, using specific antibodies anti-p-p44/42 MAPK (ERK1 and ERK2) and anti-total ERK. Antibody against tubulin was used as a loading control. As a negative control the cells were treated with vehicle. Graph represents the quantitative analysis of p-ERK normalized to total ERK. **C** Cells were pre-incubated 30 min with 20 μM PD98059, then treated with 3 nM IL-3 and 7 mM glutamate for 24 h. Cell viability was measured using MTT assay. Results are representative of three independent experiments. Data are presented as mean± S.E. (*$p<0.001$ comparing to Glutamate treatment, and & $p<0.001$ comparing to glutamate with IL-3 treatment)

result suggests that this pathway has an essential part of the protective effect observed for IL-3 in this model.

IL-3 Regulated Bax and Bcl-2 Expression

The Bcl-2 family of proteins includes apoptosis-inhibiting gene products (Bcl-2, Bcl-X L, Mcl-1, among others) and apoptosis-promoting gene products (Bax, Bak, Bim, among others) [34]. In several works, it has been demonstrated that glutamate decreases the protein levels and transcription of Bcl-2, while the increase in the Bcl-2 expression rescues cells from glutamate-induced apoptosis [35, 36]. Here we analyzed the expression of Bcl-2 in N2a cells treated with glutamate in the presence or absence of IL-3, and the role of different signaling pathways in the expression of these genes. We observed that glutamate affects the expression of Bcl-2 (Fig. 7A), inducing a significant decrease in its expression. The treatment with IL-3 prevents the decrease in the expression of this antiapoptotic protein; however, the pre-treatment with different inhibitors, such as LY294002, AKT4, and AG490 and PD98059 inhibited the rescue on Bcl-2 gene expression induced by IL-3 treatment. We also analyzed Bax gene expression, a proapoptotic gene, in cells treated with glutamate in the presence or absence of IL-3. We observed that glutamate affects the expression of Bax, increasing its expression several times. The treatment with IL-3 reduced the expression of Bax induced by glutamate. Analyzing the effect of specific inhibitors such as LY294002, AKT4, and AG490 the results show that these inhibitors can keep the high levels of Bax expression induced by glutamate, suggesting that the downregulation of Bax gene expression could be involved in the survival

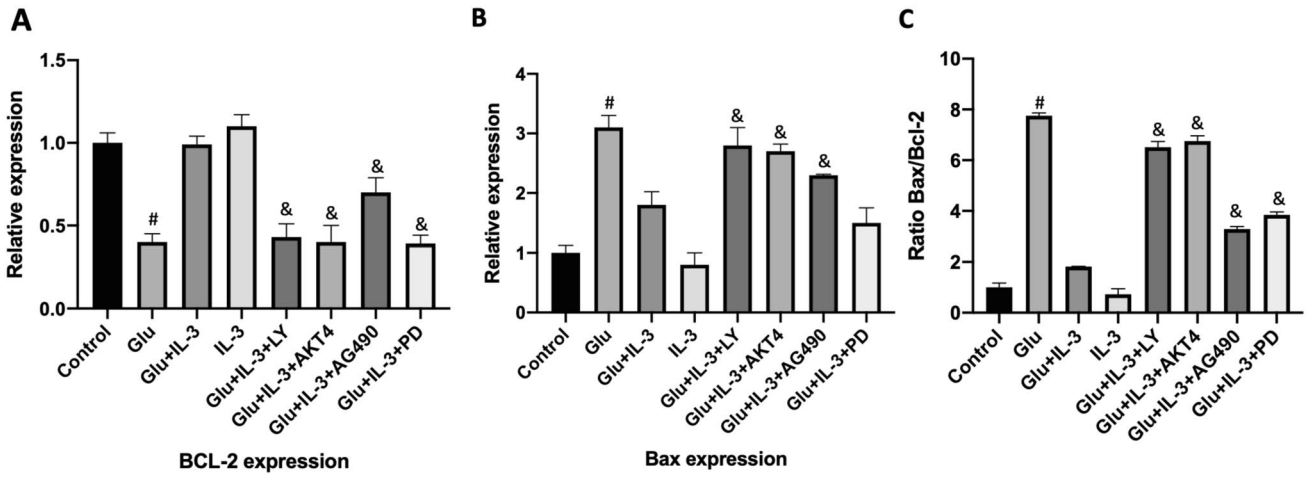


Fig. 7 Effect of glutamate and IL-3 on Bcl2 and Bax gene expression. Cells were previously incubated with 3 nM IL-3 for 30 min in the presence or absence of specific signaling pathway inhibitor (50 µM LY294002, 10 µM AKT4 inhibitor, 10 µM AG490), then treated with 7 mM glutamate for 24 h. **A** Analysis of gene expression of *Bcl-2* under different treatments. **B** Analysis of gene expression of *Bax* under different treatment. Data presented as mean ± S.E. β-actin was used as a housekeeping gene. **C** Ratio between Bax and Bcl-2 gene expression. P-values determined by Mann-Whitney test (nonparametric t-test; #$p < 0.05$ comparing to control, and & $p < 0.001$ comparing to glutamate with IL-3 treatment)

effect of IL-3 through the activation of Jak2, and PI3 K/Akt signaling pathways. However, using the inhibitor PD98059, the levels of Bax are still down, suggesting that MAPK signaling pathway can regulate Bcl-2 expression but has no role in Bax expression. Also, we analyze the ratio between Bax and Bcl-2 expression, which better represents the relation of these proteins associated with cell death. In Fig. 7C, we can see a clear increase in this ratio using both inhibitors to the Akt signaling pathway, LY294002 and Akt4 inhibitor, suggesting that this signaling is crucial to keep this ratio low in response to IL-3 treatment. At the same time, we can see that the inhibitors AG490 and PD98059 also have a significant role in maintaining the expression of these proteins, but their role is minor compared to the Akt signaling pathway. All these results suggest that these signaling pathways are involved in the survival effect of IL-3 by activating several signaling pathways.

Discussion

Glutamate is one of the most important neurotransmitters in the central nervous system; however, high glutamate concentrations produce several deleterious effects on neuronal metabolism, survival, and neurogenesis. Indeed, glutamate is considered a potent neurotoxin capable of destroying neurons by apoptosis [37]. In this work, we demonstrated that IL-3 reduces the glutamate-toxicity in the N2a cell line. We examined the effect of specific signaling pathways inhibitors on IL-3-mediated protection as a first attempt to elucidate the molecular pathways involved in IL-3-mediated induction reduction of glutamate-toxicity in N2A cells. The results show that activation of JAK/STAT, Ras/MAPK, and PI3 K signaling pathways are required for IL-3 to display its protective effects on glutamate toxicity. These data are the first experimental evidence that IL-3 exerts a protective role on glutamate-toxicity in cells of neuronal origin, and they may be of particular interest in the context that many neurodegenerative diseases have elements related to glutamate toxicity in their pathogenesis.

We detected the expression of IL-3 receptors in neuroblastoma N2a cells (Fig. 1). Different types of cells in the CNS produce IL-3 [38, 39], and different cells in the CNS show effects caused by IL-3, including neurons like isolated rat retinal ganglion cells [27, 40] and hippocampal neurons [41]. In the present work, pre-incubation with IL-3 reduces cell death induced by glutamate and reduces the amount of DNA fragmentation in cells treated with high glutamate concentration. In some in vivo models of lethal ischemic damage, IL-3 seems to have similar results because IL-3 infusion caused a reduction of CA1 neurons with DNA fragmentation after ischemia, and IL-3 prevented the decrease in Bcl-xL protein expression, which is associated with a reduction in apoptosis cell death [41]. It is clear that the function of IL-3 in neurons is still under research, but IL-3 is strongly associated with brain volume variation in human populations [25, 26] by playing a role in the regulation of proliferation and survival of neural progenitors [25]. IL-3 protects against the loss of neurons in isolated rat retinal ganglion cells [27], stimulates sympathetic nerve growth [28], increases sensory neuron survival, and stimulates the formation of the neural

network in vitro, and promotes the process extension of cultured cholinergic neurons [42–44].

Recently, it has been demonstrated that astrocytes produce IL-3, which induces transcriptional and functional programming of microglia, which enhances the capacity to cluster and clear aggregates of Aβ and tau [45]. At the same time, an essential decrease in IL-3Rα levels was observed in the brains of scrapie-infected rodents, and in the prion-infected cell model, the downregulation included some downstream components of IL-3R such as JAK2-STAT5 and PI3 K/Akt/mTOR pathways, blocking the neuroregulatory and neuroprotective activities of IL-3 [46]. Jak2 is an upstream kinase of Akt and ERK signaling, so it stands to reason that its inhibition may lead to a decrease in the activation of these signaling pathways. At the same time, there is much evidence of the possible crosstalk between the Akt and ERK signaling, mainly associated with cell viability processes. The PI3K/AKT and RAF/MEK/ERK signaling cascades are compensatory pathways that mediate cell survival through co-regulated proteins. Indeed, it is well known that signaling by the PI3K-AKT and MEK1/2-ERK1/2 pathways can collaborate to maintain cell viability. Signaling pathways can also crosstalk with each other, wherein one pathway can signal to either enhance or suppress signaling by another [47, 48].

Similar results have been demonstrated studying other related cytokines, such as G-CSF, which have a significant potential for neuronal regeneration and neuroprotection; much evidence has established G-CSF as a neuroprotective molecule in CNS disorders, including Parkinson's disease, and its role is mediated by increasing Bcl-2 expression levels and decreasing Bax expression levels in C57BL/6 mice [49, 50].

Analyzing the signaling pathways associated with IL-3 neuroprotection, our experiments in N2a cells showed that IL-3 stimulates the JAK/STAT pathway and induces the phosphorylation of ERK and Akt, thus activating these key signal transduction pathways associated with cell proliferation and survival [51, 52]. The neuroprotective effect of IL-3 on glutamate toxicity in N2a cells is blocked with pharmacological inhibitors for those signaling pathways, suggesting that all of them participate in the protective effect of IL-3. This triple requirement differs from the IL-3 neuroprotective role in neurons treated with Aβ fibril, where cell survival induced by IL-3 required PI3 K and JAK/STAT pathway activation but not MAP kinase [29]. Similar results have been demonstrated with Erythropoietin (Epo) administration which increases phosphorylated levels of JAK2 together with anti-apoptotic proteins Bcl-xL and Bcl- 2 as a part of its anti-apoptotic effect in the cerebral cortex [53]. At the same time, Epo mediates neurite outgrowth and anti-apoptosis of neurons and brain endothelial cells [54–56] and triggers anti-apoptosis, anti-excitotoxicity, and astrocyte formation [57, 58], through the activation of Akt and MAPK signaling pathways.

Glutamate induces DNA fragmentation and apoptotic cell death by regulating the ratio Bax/ Bcl-2 [35, 36]. Since it has been suggested that the ratio of Bax to Bcl-2 is an essential determinant of neuronal survival, the regulation of these Bcl-2 family proteins plays an important role in the neurotoxicity mediated by glutamate [35, 59]. In our cellular model, the treatment with IL-3 prevents the decrease in the expression of Bcl-2 produced by glutamate and induces a decrease in Bax expression triggered by glutamate. Interestingly, our findings in N2a cells are in the same direction as those from Choudhury et al. (2011) in a rat model of Parkinson's disease, where subcutaneous injection of a cytokine mixture containing granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-3 suppressed dopaminergic neurodegeneration and increased expression of the Bcl-2 family member Bcl-xL in these dopaminergic neurons [60].

Similarly, GM-CSF exerts its neuroprotective effect on neural injury by reducing the expression of pro-apoptotic proteins, including Bax, and inducing antiapoptotic proteins, such as Bcl-2 [61]. Another work suggests that GM-CSF induces the expression of Bcl-2 and Bcl-xl via Janus tyrosine kinase (JAK) [62]. PI3 K can also be involved in Bcl2 gene regulation, as described by Moon et al., 2009 were analyzing the role of Leukemia Inhibitory Factor (LIF), a neuropoietic cytokine, in the control of neuronal development, LIF induces the expression of the antiapoptotic molecule Bcl-2 dependent of PI3 K pathway [63]. It is essential to highlight that ERK can also regulate the expression of Bcl-2; indeed, the up-regulation of Bcl-2 through ERK phosphorylation is a common molecular mechanism in cell survival under many stimuli [64]. All these results suggest that a web of phosphorylation pathways acts at multiple levels to regulate Bcl-2 family members and cell death [65].

To summarize, IL-3 exerts a protective role on glutamate-toxicity in N2a cells. The molecular mechanism involves the activation of JAK/STAT, Ras/MAPK, and PI3 K signaling pathways and regulating the expression of Bcl-2 family members. The results demonstrate that IL-3 presents protective effects on glutamate toxicity. Protecting neuronal cells against glutamate-induced damage by IL-3 could be a promising strategy against some neurodegenerative processes; more research is necessary to test the therapeutic use in the future.

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Author Contributions VM, FJM, and AZ conceived and designed the experiments; VM, MCG, and AZ performed experiments and acquired the data; VM, MCG, JCF, FJM, and AZ analyzed and interpreted the data; FJM and AZ. drafted, edited, and revised manuscript. All authors read and approved the final manuscript.

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Data Availability The datasets generated during and/or analyzed during the current study are available by request; please contact the corresponding authors.

Declarations

Conflict of interest The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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