



## Use of human neuroblastoma SH-SY5Y cells to evaluate glyphosate-induced effects on oxidative stress, neuronal development and cell death signaling pathways



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### ABSTRACT

Glyphosate-containing herbicides are the most used agrochemicals in the world. Their indiscriminate application raises some concerns regarding the possible health and environmental hazards. In this study, we investigated in human neuroblastoma cell line SH-SY5Y if oxidative stress, altered neurodevelopment and cell death pathways are involved in response to glyphosate and its metabolite aminomethylphosphonic acid (AMPA) exposures.

MTT and LDH assays were carried out to assess the glyphosate and AMPA cytotoxicity. Lipid peroxides measured as malondialdehyde (MDA), nitric oxide (NO) and reactive oxygen species (ROS) production, and caspase-Glo 3/7 activity were evaluated. The neuroprotective role of melatonin (MEL), Trolox, N-acetylcysteine (NAC) and Sylibin against glyphosate- and AMPA-induced oxidative stress was examined. Glyphosate and AMPA effects on neuronal development related gene transcriptions, and gene expression profiling of cell death pathways by Real-Time PCR array were also investigated. Glyphosate (5 mM) and AMPA (10 mM) induced a significant increase in MDA levels, NO and ROS production and caspase 3/7 activity. Glyphosate exposure induced

**Abbreviations:** ABL1, C-abl oncogene 1, non-receptor tyrosine kinase; AKT1, V-akt murine thymoma viral oncogene homolog 1; AMPA, Aminomethylphosphonic acid; APAF1, Apoptotic peptidase activating factor 1; APP, Amyloid beta (A4) precursor protein; ATG12, ATG12 autophagy related 12 homolog (*S. cerevisiae*); ATG3, ATG3 autophagy related 3 homolog (*S. cerevisiae*); ATG5, ATG5 autophagy related 5 homolog (*S. cerevisiae*); BAX, BCL2-associated X protein; BCL2, B-cell CLL/lymphoma 2; BCL2A1, BCL2-related protein A1; BCL2L11, BCL2-like 11 (apoptosis facilitator); BIRC2, Baculoviral IAP repeat containing 2; CAMK2A, Calcium/calmodulin-dependent protein kinase II alpha; CAMK2B, Calcium/calmodulin dependent protein kinase II beta; CASP1, Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase); CASP3, Caspase 3, apoptosis-related cysteine peptidase; CASP6, Caspase 6, apoptosis-related cysteine peptidase; CASP7, Caspase 7, apoptosis-related cysteine peptidase; CASP9, Caspase 9, apoptosis-related cysteine peptidase; CD40, CD40 molecule, TNF receptor superfamily member 5; CD40LG, CD40 ligand; cDNA, Complementary DNA; CFLAR, CASP8 and FADD-like apoptosis regulator; CNS, Central nervous system; Ct, Cycle threshold; CTSB, Cathepsin B; CYLD, Cylindromatosis (turban tumor syndrome); DAF-2 DA, 4,5-diaminofluorescein; DAF-2 DA, 4,5-diaminofluorescein diacetate; DAF-2T, Triazolofluorescein; DAF-FM, 4 amino-5-methylamino-2',7'-difluorescein; DAF-FM-DA, 4 amino-5-methylamino-2',7'-difluorescein diacetate; DCFH, 2,7-dichlorofluorescein; DCFH-DA, 2,7-dichlorofluorescein diacetate; DENND4A, DENN/MADD domain containing 4A; DMEM F-12, Dulbecco's modified eagle medium:F12; DMSO, Dimethyl sulfoxide; DPBS, Dulbecco's phosphate buffered saline; EIF5B, Eukaryotic translation initiation factor 5B; FAS, Fas (TNF receptor superfamily, member 6); FASLG, Fas ligand (TNF superfamily, member 6); FBS, Fetal bovine serum; FOXO1, Forkhead box O1; GADD45A, Growth arrest and DNA-damage-inducible, alpha; GALNT5, UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5); GAP43, Growth-associated protein 43; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HSPBAP1, HSPB (heat shock 27kDa) associated protein 1; IFNG, Interferon, gamma; IGF1, Insulin-like growth factor 1 (somatomedin C); IL6, Interleukin 6; INS, Insulin; IPA, Ingenuity Pathway Analysis Tool; IPKB, Ingenuity Pathway Knowledge Base; IRGM, Immunity-related GTPase family, M; KCNIP1, Potassium channel interacting protein 1; LD<sub>50</sub>, Lethal dose for 50% of test animals; LDH, Lactate dehydrogenase; MAPK8, Mitogen-activated protein kinase 8; MCL1, Myeloid cell leukemia sequence 1 (BCL2-related); MDA, Malondialdehyde tetrabutylammonium salt; MEL, Melatonin; mRNA, Messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NAC, N-acetylcysteine; NFκB1, Nuclear transcription factor kappa B subunit 1; NO, Nitric oxide; NOL3, Nucleolar protein 3 (apoptosis repressor with CARD domain); OR10J3, Olfactory receptor, family 10, subfamily J, member 3; PARP2, Poly (ADP-ribose) polymerase 2; PCR, Polymerase chain reaction; PIK3C3, Phosphoinositide-3-kinase, class 3; RAB25, RAB25, member RAS oncogene family; ROS, Reactive oxygen species; RPS6KB1, Ribosomal protein S6 kinase, 70kDa, polypeptide 1; S100A7A, S100 calcium binding protein A7A; SEM, Standard error of the mean; SH-SY5Y, Human neuroblastoma cell line; SNCA, Synuclein, alpha (non A4 component of amyloid precursor); SQSTM1, Sequestosome 1; SYCP2, Synaptonemal complex protein 2; TBA, Thiobarbituric acid; TBARS, Thiobarbituric acid reactive substance; TMEM57, Transmembrane protein 57; TNF, Tumor necrosis factor; TNFRSF11B, Tumor necrosis factor receptor superfamily, member 11b; TNFα, Tumor necrosis factor-α; TP53, Tumor protein p53; TUBB3, Tubulin beta 3 class III; Wnt3a, Wnt family member 3A; Wnt5a, Wnt family member 5A; Wnt7a, Wnt family member 7A; XIAP, X-linked inhibitor of apoptosis

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up-regulation of Wnt3a, Wnt5a, Wnt7a, CAMK2A, CAMK2B and down-regulation of GAP43 and TUBB3 mRNA expression involved in normal neural cell development. In relation to gene expression profiling of cell death pathways, of the 84 genes examined in cells a greater than 2-fold change was observed for APAF1, BAX, BCL2, CASP3, CASP7, CASP9, SYCP2, TNF, TP53, CTSB, NFκB1, PIK3C3, SNCA, SQSTM1, HSPBAP1 and KCNIP1 mRNA expression for glyphosate and AMPA exposures. These gene expression data can help to define neurotoxic mechanisms of glyphosate and AMPA.

Our results demonstrated that glyphosate and AMPA induced cytotoxic effects on neuronal development, oxidative stress and cell death via apoptotic, autophagy and necrotic pathways and confirmed that glyphosate environmental exposure becomes a concern.

This study demonstrates that SH-SY5Y cell line could be considered an *in vitro* system for pesticide screening.

## 1. Introduction

Glyphosate (*N*-[phosphonomethyl] glycine) is a nonselective post-emergent herbicide that inhibits the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate synthase, functionality of which is absolutely required for the survival of plants (Funke et al., 2006). The main breakdown product of glyphosate in mammals, plants, soil, and water is AMPA (Fig. 1) (Anadón et al., 2009; USEPA, 2009). Glyphosate is already one of the most used herbicides in modern agriculture. This herbicide is spread on most eaten transgenic plants, modified to tolerate high levels of this compound in their cells. Glyphosate may also be used for weed killing in non-agricultural areas such as water systems, including irrigation and temporarily drained waters, parks, road verges and gardens (WSSA, 1983). Its indiscriminate application arises some concerns regarding the possible health and environmental hazards. Glyphosate contamination may come from air (dermal or pulmonary during spraying), soil, water, feed and food. At present, few studies have been conducted to examine the possible bioaccumulation. It has been shown in rats after oral ingestion of 400 mg/kg of the herbicide, a maximum glyphosate plasma concentration of 5 µg/mL at 5 h after dosing; glyphosate diffuses in mammalian tissues, with half-lives of distribution and elimination of 4 h and 15 h, respectively (Anadón et al., 2009). Half-lives of glyphosate and AMPA in soil range from 2 to 197 days and 76 to 240 days, respectively (Battaglin et al., 2005). They can persist in the environment as residues in soils and crops for up to 3 years (Vereecken, 2005). Although humans are not a direct target, they could be in contact with glyphosate due to occupational exposure (Acquavella et al., 2004; Paz-y-Miño et al., 2007) and/or through dietary exposure (McQueen et al., 2012). Based on available information and taking into account that glyphosate has low oral acute mammalian toxicity (rat LD<sub>50</sub> oral > 2000 mg/kg bw) it is categorized as “unlikely to present acute hazard in normal use” (Solomon et al., 2007; EFSA, 2015), but the ingestion of large amounts of glyphosate results in grave clinical outcomes (Palli et al., 2011; Lee et al., 2008). Patients who ingest large volumes of glyphosate herbicides are at risk of developing hypotension, mental deterioration, respiratory failure, and arrhythmia (Seok et al., 2011). In the other hand, the International Agency for Research on Cancer published the Glyphosate Monograph of Volume 112 (IARC, 2015) which concluded that there was strong evidence supporting that “glyphosate can operate through two key characteristics of known human carcinogens” including genotoxicity and induction of oxidative stress. This was viewed as providing strong support for IARC classifying glyphosate as probably carcinogenic to humans, Group 2A. However, European Food Safety Authority peer review concluded that glyphosate is unlikely to pose a carcinogenic hazard to humans (EFSA, 2015), and also a Joint FAO/WHO Meeting on Pesticide Residues (JMPR, 2016) resolved that glyphosate is unlikely to be genotoxic at anticipated dietary exposures and unlikely to cause a carcinogenic risk to humans from dietary exposure; AMPA presents a similar toxicological profile and the glyphosate reference values are applied to its metabolite AMPA.

Despite the apparent well-established safety of glyphosate for humans by regulatory agencies, it has been suggested that long-term low

level exposure might lead to human neurodegenerative disorders. Pesticides have been recognized as the main environmental risk factor associated with human neurodegenerative disorders. Several factors, including mitochondrial dysfunction, oxidative stress, altered protein handling and inflammatory change, are involved in the degenerative process through apoptosis and/or autophagy pathways (Bredesen et al., 2006). Occupational pesticide exposure leads to oxidative damage, increases the risk of incidence of Parkinson's and Alzheimer's disease, and also might accelerate age-related neurodegeneration (Peng et al., 2010; Hayden et al., 2010). In this context, it has been suggested that acute and chronic exposure to glyphosate might be linked to Parkinsonism (Barbosa et al., 2001; Wang et al., 2011). It has been reported that exposure to glyphosate-containing pesticide leads to degeneration of γ-aminobutyric acid and dopamine neurons in *Caenorhabditis elegans* (Negga et al., 2012). Recently, in our laboratory, it has been also demonstrated that in rats, the oral glyphosate exposure leads to loss of serotonin, dopamine and norepinephrine levels in central nervous system (CNS) mainly in the striatum, prefrontal cortex, hypothalamus and hippocampus tissues (Martínez et al., 2018) which play an important role in brain functions. Both prefrontal cortex and hippocampal areas are essential brain structures for learning, memory and attention processes in a variety of rat models (Broersen, 2000; Wang and Cai, 2006). Therefore, glyphosate neurotoxicity and its mechanism of action on the nervous system need to be primarily elucidated. Glyphosate formulations contain surfactants have been also associated with induction of apoptosis and necrosis in human umbilical, embryonic, and placental cells evaluating three enzymatic biomarkers (adenylate kinase, succinate dehydrogenase and caspase 3/7 activities) (Benachour and Seralini, 2009), as well as with induction of oxidative and inflammatory processes evaluating serum NO and tumor necrosis factor-α (TNF-α) levels from rats treated intraperitoneally (El-Shenawy, 2009). It has also been reported that glyphosate exposure produced high BCL2-associated X protein (BAX) and low B-cell CLL/lymphoma 2 (BCL2) protein expression in neuronal differentiated PC12 cells (Gui et al., 2012) suggesting that two important cell death pathway, apoptosis and

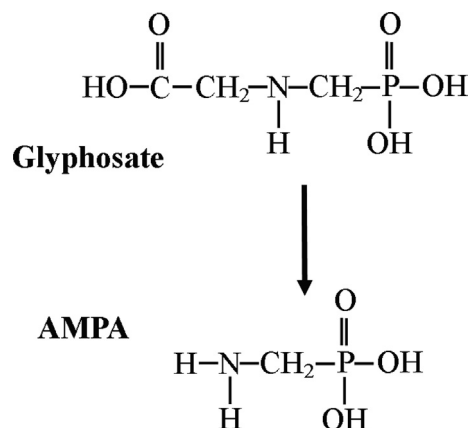


Fig. 1. Chemical structures of glyphosate and its metabolite AMPA.

autophagy are involved in the glyphosate-induced toxicity mechanism. Cattani et al. (2014) demonstrated that a glyphosate-based herbicide might lead to excessive extracellular glutamate levels and consequently to glutamate excitotoxicity and oxidative stress in hippocampus from immature rats following acute and chronic (pregnancy and lactation) pesticide exposure.

Currently, oxidative stress is among the most important subjects in pesticide toxicology (Mansour and Mossa, 2010). Oxidative stress in cells due either to excessive production of reactive oxygen species (ROS) or insufficient antioxidant defense (particularly in the elderly) can damage cellular proteins, lipids and DNA and activate apoptotic pathways (Mattson, 2006). Cell death, the result of progressive cell injury, is one of the most critical events in the evolution of disease in any tissue or organ. There are two principle types of cell death, necrosis and apoptosis, which differ in their morphology, mechanism, and role in physiology and disease (Edinger and Thompson, 2004). In addition, cell death during autophagy may have many of the biochemical characteristics of apoptosis. Environmental stressors including pesticides contribute to neurological conditions through mechanisms involving inflammation, oxidative stress and further apoptotic cell death.

On the other hand, the growth and morphological differentiation of neurons are critical events in the establishment of proper neuronal connectivity and functioning. The developing nervous system is highly susceptible to damage caused by exposure to environmental contaminants. In the field of developmental neurotoxicity, efforts have been directed toward the development of alternative models utilizing mammalian cells *in vitro*, which could serve as tools for neurotoxicity and developmental neurotoxicity testing, particularly for screening purposes. Novel approaches, part of the “omics” technologies, may also find a role in such endeavor.

Considering that major information regarding glyphosate neurotoxic effects and underlying mechanisms involved after mammalian exposures are needed for risk evaluation, in this study, we investigated the neurotoxicity of glyphosate in human neuroblastoma SH-SY5Y cells evaluating oxidative stress and neurodevelopment markers and further we explored whether cell death pathways were involved in the glyphosate-induced toxicity mechanisms. This *in vitro* study was undertaken (i) to characterize the concentration-dependent cytotoxicity of glyphosate and AMPA using cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays and to determine the protective role of selected antioxidant substances (MEL, Trolox, NAC and Sylibin) on MTT reduction, lipid peroxidation, NO and ROS production, and caspase 3/7 activity, (ii) to evaluate gene expressions of neuronal development: growth-associated protein 43 (GAP43), calcium/calmodulin-dependent protein kinase II alpha/protein kinase II beta (CAMK2A/CAMK2B), tubulin beta 3 class III (TUBB3), Wnt proteins (Wnt3a, Wnt5a and Wnt7a) and proinflammatory [interleukin 6 (IL6) and TNF $\alpha$ ] mediators and (iii) to examine by Real-Time polymerase chain reaction (PCR) array the expression of key genes related to cell death (apoptosis, autophagy and necrosis genes) after glyphosate and AMPA exposures. In the present study, the dopaminergic human neuroblastoma cell line SH-SY5Y was used as an *in vitro* model to determine cytotoxic mechanisms of glyphosate and AMPA. SH-SY5Y cells have been extensively used as an *in vitro* model for research in neuroscience (Pahlman et al., 1990; Krishna et al., 2014).

## 2. Materials and methods

### 2.1. Chemicals and reagents

The compounds [N-(phosphonomethyl) glycine] glyphosate, molecular formula  $C_3H_8NO_5P$ , CAS No 107-83-6, purity  $\geq 98\%$  (Fig. 1), [(aminomethyl)phosphonic acid] AMPA, molecular formula  $CH_6NO_3P$ , CAS 1066-51-9, purity  $> 99\%$  (Fig. 1), 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), melatonin (N-acetyl-5-

methoxytryptamine) (MEL), N-acetyl-cysteine (NAC), Trolox [( $\pm$ )-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid], Sylibin (Silibinin), menadiolone, dicoumarol, malondialdehyde tetrabutylammonium salt (MDA), dimethyl sulfoxide (DMSO), Dulbecco's phosphate buffered saline (DPBS, D8537), fetal bovine serum (FBS) and 2-thiobarbituric acid (TBA) were purchased from Sigma-Aldrich, St Louis, MO 63103, USA. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F-12) was obtained from Biowhitaker Lonza (Walkersville, MD, USA). Penicillin and streptomycin were obtained from Invitrogen (Madrid, Spain). The fluorescent probes 2',7'-dichlorofluorescein diacetate (DCFH-DA) and 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM-DA) were from Sigma-Aldrich and Invitrogen, respectively. The kit Caspase-Glo<sup>®</sup> 3/7 assay was purchased from Promega Corporation, Madison, WI 53711-5399, USA. All other chemicals were of the highest quality grade and obtained from commercial sources.

### 2.2. Culture of SH-SY5Y cells

The human dopaminergic neuroblastoma SH-SY5Y cell line was obtained from European Collection of Authenticated Cell Cultures (ECACC 94030304), Sigma-Aldrich. SH-SY5Y cells were maintained in DMEM-F-12 medium supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin. Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. For assays, SH-SY5Y cells were subcultured in 96-well plates at a seeding density of  $5 \times 10^4$  cells per well. Cells were treated with the drugs before confluence in DMEM-F12 with 1% FBS. Glyphosate and AMPA were dissolved in DMEM-F12 medium supplemented with 1% FBS. All SH-SY5Y cells used in this study were used at a low passage number ( $< 13$ ).

### 2.3. MTT assay and cell viability

Cell viability, virtually the mitochondrial activity of living cells, of glyphosate and its main metabolite AMPA were measured by quantitative colorimetric assay with MTT, as described previously (Denizot and Lang, 1986). Briefly, 50  $\mu$ L of the MTT labeling reagent, at a final concentration of 0.5 mg/mL, was added to each well at the end of the incubation period and the plate was placed in a humidified incubator at 37 °C with 5% CO<sub>2</sub> and 95% air (v/v) for an additional 2 h period. Metabolically active cells convert the yellow MTT tetrazolium compound to a purple formazan product. The insoluble formazan was dissolved with DMSO; colorimetric determination of MTT reduction was measured at 540 nm. Control cells treated with DMEM-F12 were taken as 100% viability.

### 2.4. Lactate dehydrogenase (LDH) assay

The possible cytotoxic effects of glyphosate and its main metabolite AMPA exposures on SH-SY5Y cells were also evaluated by measuring LDH leakage into the extracellular fluid. LDH is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. After glyphosate and AMPA exposures (48 h) at several concentrations, samples were collected to estimate extracellular LDH as indication of cell death. LDH activity was spectrophotometrically measured using a Cytotoxicity Cell Death kit (Roche-Boehringer, Mannheim, Germany) according to the manufacturer's indications. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity; total LDH (intracellular plus extracellular) was normalized as 100%; then, the amount of LDH released to the extracellular medium was expressed as percentage of this total value. LDH activity was measured spectrophotometrically at 490–620 nm, using a microplate reader (Biochrom ASYS UVM 340, Cambridge, UK).

## 2.5. Determination of lipid peroxidation

Malondialdehyde (MDA) is a breakdown product of the oxidative degradation of cell membrane lipids and it is generally considered an indicator of lipid peroxidation. In this study, we evaluated lipid peroxidation induced by glyphosate, AMPA and antioxidants (MEL, Trolox, NAC and Sylibin) after 48 h incubation period. Intracellular MDA production was quantified using a thiobarbituric acid reactive substance (TBARS) assay kit (Cell Biolabs Inc., San Diego, CA). Briefly,  $1 \times 10^6$  cells per well were seeded in a six-well plate, then collected in 200  $\mu$ L of culture medium and sonicated for  $3 \times 5$  s intervals at 40 V over ice. SDS Lysis solution (100  $\mu$ L) was added to the sample solution and the MDA standards in a microcentrifuge tube and mix thoroughly. Then, 250  $\mu$ L of TBA reagent was added to each sample and standard to be tested, and incubate at 95  $^{\circ}$ C for 45–60 min. Each sample and standard (200  $\mu$ L) were loaded (in duplicate) into a clear 96-well plate and the absorbance at 532 nm was recorded using a microplate reader (Biochrom ASYS UVM 340, Cambridge, UK). The content of MDA was calculated for each sample from a standard curve.

## 2.6. Nitric oxide (NO) production

4,5-Diaminofluorescein diacetate (DAF-2 DA) has been widely used for the measurement of nitric oxide (NO) in cells and tissues. The diacetate form of DAF-FM (DAF-2-DA) cross the cell membrane where is cleaved by intracellular esterases to form the negatively charged parent compound 4,5-diaminofluorescein (DAF-2) which cannot cross the cell membrane and thus accumulates inside the cells. In the presence of NO, DAF-2 is transformed in a highly fluorescent triazole triazolofluorescein (DAF-2 T). NO production was detected by spectrofluorometric following the optimized method for the detection of NO in the low-nM range developed for Leikert et al. (2001). Briefly, 1  $\mu$ M DAF-2 was added to the wells ( $8 \times 10^4$  cells per well) and incubated for 30 min at 37  $^{\circ}$ C. Then, cells were washed twice with DPBS and serum-free medium or serum-free medium with glyphosate, AMPA and antioxidants (MEL, Trolox, NAC and Sylibin) were added per well. NO production was evaluated in a fluorescent microplate reader at an excitation wavelength of 495 nm and an emission wavelength of 515 nm (FLx800 Fluorimeter, BioTek, Winooski, VT, USA).

## 2.7. Determination of reactive oxygen species (ROS) production

Cellular ROS were quantified by the 2,7-dichlorofluorescein (DCFH) assay (Wang and Joseph, 1999) using a microplate reader. After being oxidized by intracellular oxidants, DCFH becomes dichlorofluorescein and emits fluorescence. By quantifying fluorescence, a fair estimation of the overall oxygen species generated under the different conditions was obtained. Briefly, 10  $\mu$ M DCFH was added to the wells ( $8 \times 10^4$  cells per well) and incubated for 30 min at 37  $^{\circ}$ C. Then, cells were washed twice with DPBS and serum-free medium or serum-free medium with glyphosate, AMPA and antioxidants (MEL, Trolox, NAC and Sylibin) were added per well. ROS generation was evaluated in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (FLx800 Fluorimeter, BioTek, Winooski, VT, USA).

## 2.8. Caspase-Glo 3/7 assay

The caspase-Glo 3/7 assay is a homogenous, luminescent assay that measures caspase-3 and -7 activities, key-caspases of apoptosis. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD (aspartic acid-glutamic acid-valine-aspartic acid), in a reagent optimized for caspase activity, luciferase activity, and cell lysis. The addition of the Caspase-Glo reagent results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal produced by luciferase. Luminescence is

proportional to the amount of caspase present.

The kit Caspase-Glo<sup>®</sup> 3/7 assay (Promega) provides the Caspase-Glo 3/7 buffer and lyophilized Caspase-Glo 3/7 substrate. These were equilibrated at room temperature prior to use. The contents of the buffer solution were transferred into the bottle containing the substrate. The content was dissolved by mixing to form the Caspase-Glo 3/7 reagent. This reagent can be stored at 4  $^{\circ}$ C for up to 1 week. The reagent can be frozen at  $-20$   $^{\circ}$ C for longer storage.

SH-SY5Y cells ( $15 \times 10^3$  cells per well) were grown in a 96-well white plate and exposed to glyphosate (5 mM) and AMPA (10 mM) for 48 h. After treatment, the Caspase-Glo<sup>®</sup> 3/7 was prepared according to manufacturer's guidelines. After 30 min at room temperature, 50  $\mu$ L of Caspase-Glo 3/7 reagent was added to 50  $\mu$ L of culture medium containing the cells previously treated in each well. After shaking the plate at 350 rpm during 30 s, an incubation period of 60 min at room temperature in the dark was needed to stabilize the signal before luminescence measurement with the luminometer. Luminescence was measured using the plate reader (FLx800, BioTek, Winooski, VT, USA).

## 2.9. RNA isolation and cDNA synthesis

Neuroblastoma SH-SY5Y cells were co-incubated with glyphosate or with AMPA for 48 h. Total RNA was extracted using the Trizol Reagent method (Invitrogen) and purified using RNeasy MinElute Cleanup Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany) according to the manufacturer protocol. The final RNA concentration and purity was determined using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Madrid, Spain), obtaining A260/A280 ratios between 1.9 and 2.1 in all the samples. First-strand complementary DNA (cDNA) was synthesized from 5  $\mu$ g total RNA by reverse transcription using RT2 First Strand kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, starting with a genomic DNA elimination step. At the end, cDNA was diluted 1:10 in nuclease-free water and stored at  $-80$   $^{\circ}$ C for further analysis.

## 2.10. Gene expressions linked to neuronal development and proinflammation by Real-Time PCR

Quantitative Real-Time PCR assays for GAP43, CAMK2A, CAMK2B, TUBB3, Wnt3a, Wnt5a, Wnt7a, IL6 and TNF $\alpha$  were performed to analyze messenger RNA (mRNA) gene expressions (Table 1). Reactions were run on a Real-Time PCR system, BioRad CFX96, using RT<sup>2</sup> SYBR Green qPCR master mix (Qiagen, Hilden, Germany) according to manufacturer's protocol. Concentration of each primer was 400 nM and thermal protocol was as follows: 95  $^{\circ}$ C for 10 min, followed by 40 cycles composed of 15 s at 95  $^{\circ}$ C and 1 min at 60  $^{\circ}$ C. Sequences of primers are presented in Table 1. Relative changes in gene expression were

**Table 1**

Sequences of forward and reverse primers for proinflammation and neurodevelopment related genes.

Genes	Primer forward sequence	Primer reverse sequence
<b>Housekeeping gene</b>		
GAPDH	GAGAAGGCTGGGGCTCATT	AGTGATGGCATGGACTGTGG
<b>Proinflammation related genes</b>		
IL6	CCAGTACCCCGAGGAGAAGA	CAGCTCTGGCTTGTCTCTCA
TNF $\alpha$	CTGGAAAGGACACCATGAGCA	TCTCTCAGCTCCACGCCATT
<b>Neuronal development related genes</b>		
GAP43	AGGGAGAAGGCACCACTACT	GGAGGACGGCGAGTATACAG
CAMK2A	CATGGTTTGGGTTTGCAGGG	CCGGCTTTGATCTGCTGGTA
CAMK2B	GAGGACGGAGCGAGCAGAT	GACGCACGATGTTGGAATGC
TUBB3	CCGAAGCCAGCAGTGTCTAA	AGGCCTGGAGCTGCAATAAG
Wnt3a	TCTACGACGTGCACACCTG	CCTGCCTTCAGGTAGGAGTT
Wnt5a	AGCAGACGTTTCGGCTACAG	TGCCCCAGTTCATTACAC
Wnt7a	GCGTCTCGCACACTTGAC	CCGGCTTTCCGGTTTCATAG



calculated according to Pfaffl (2001), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene (with tested no differences between groups) and extracting efficiencies from raw data using LinRegPCR free software (Ramakers et al., 2003).

### 2.11. Gene expressions involved in cell death by Real-Time PCR array

The Human Cell Death Pathway Finder PCR Array (PAHS-212Z) was used to analyze mRNA levels of 84 key genes involved in cell death, in a 96-well format, according to the manufacturer's instructions (SABiosciences Inc.). Reactions were run (9 arrays in total) on a real-time PCR system, Bio-Rad CFX96, using RT<sup>2</sup> SYBR Green PCR master mix (Qiagen, Hilden, Germany). The thermocycler parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative changes in gene expression as well as cluster analysis were performed via RT<sup>2</sup> Profiler PCR Array Data Analysis Software version 3.5 (Qiagen, Hilden, Germany), using the Ct (cycle threshold) method with normalization of the raw data to several housekeeping genes. The expression data are presented as real change multiples. For cluster analysis of the differentially expressed genes, average-link clustering was chosen. Genes with altered expression profile compared to control with fold change value  $\geq \pm 1.5$  and  $P < 0.05$  were presented.

### 2.12. Ingenuity pathway analysis (IPA)

Datasets representing genes with altered expression profile derived

from Real-Time PCR array analyses were imported into the Ingenuity Pathway Analysis Tool (IPA Tool; Ingenuity H Systems, Redwood City, CA, USA; <http://www.ingenuity.com>). In IPA, differentially expressed genes are mapped to genetic networks available in the Ingenuity database and then ranked by score. The basis of the IPA program consists of the Ingenuity Pathway Knowledge Base (IPKB) which is derived from known functions and interactions of genes published in the literature. Thus, the IPA Tool allows the identification of biological networks, global functions and functional pathways of a particular dataset.

Datasets derived from real-time PCR array analyses were uploaded into the IPA Tool. Canonical pathways and network analysis in IPA were used to identify the pathways that were altered by glyphosate and AMPA treatments.

### 2.13. Statistical analysis

Six replicates for each experimental condition were performed. Data are represented as mean values  $\pm$  standard error of the mean (SEM). Comparisons between experimental and control among groups were performed by one-way ANOVA followed by the Tukey's *post-hoc* test, using GraphPad Prism 6. Statistical difference was accepted when  $P < 0.05$ .

For MTT and LDH assays, cytotoxic concentrations (IC<sub>50</sub> concentrations) were calculated by concentration-response (Sigmoidal fitting) with Origin-Pro 9 software.

For quantitative Real-Time PCR assays, data are represented as mean value  $\pm$  SEM. Statistical analysis was carried out using the

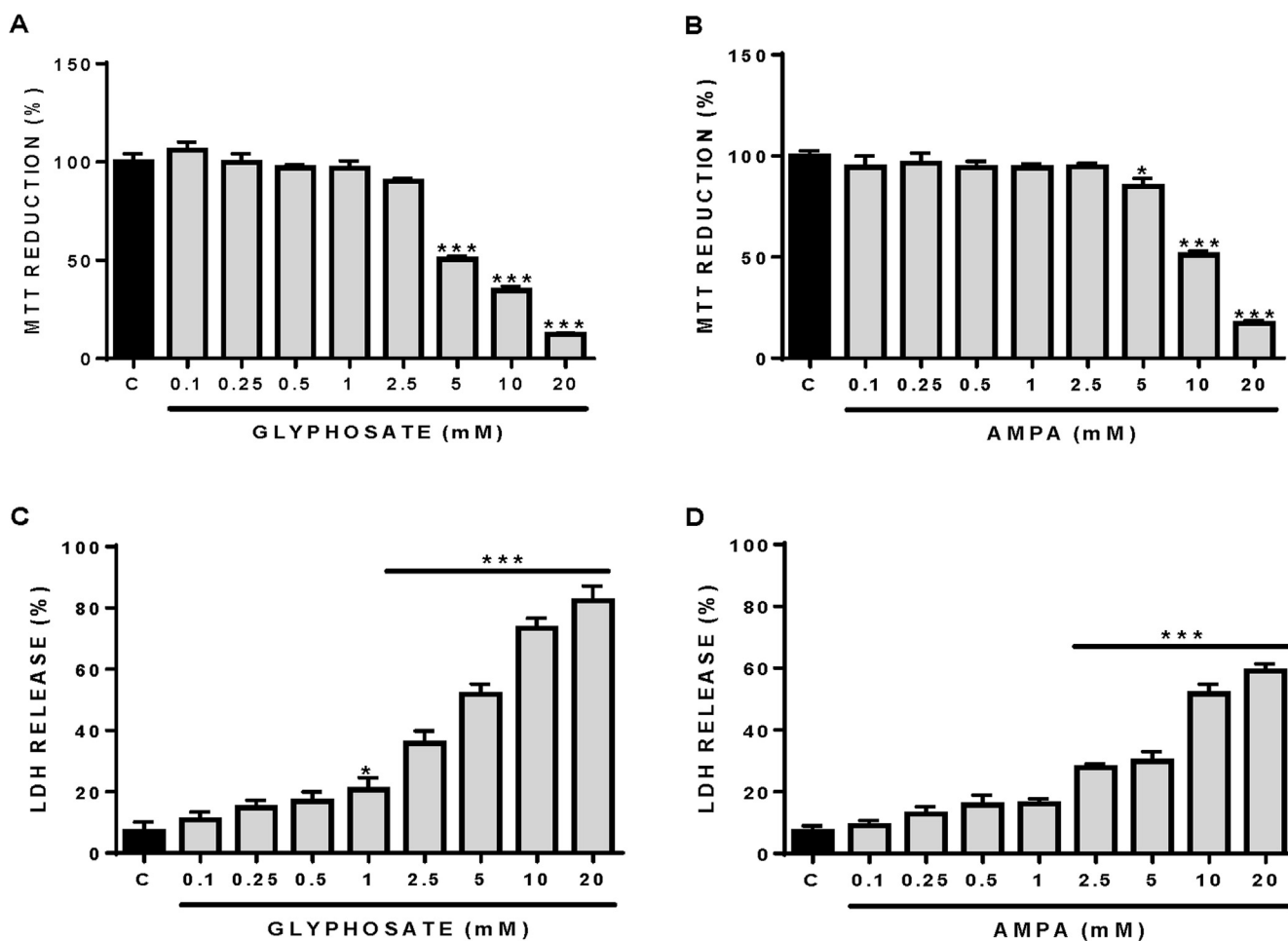


Fig. 2. Cytotoxicity induced by glyphosate and AMPA on SH-SY5Y cell viability after 48-h incubation period. Cell viability was measured as MTT reduction (A and B) or as LDH release (C and D). Data were normalized as % control (black column). Data represent the mean  $\pm$  SEM of six independent experiments in triplicate. \* $P < 0.05$ , \*\*\* $P < 0.001$  compared to control.

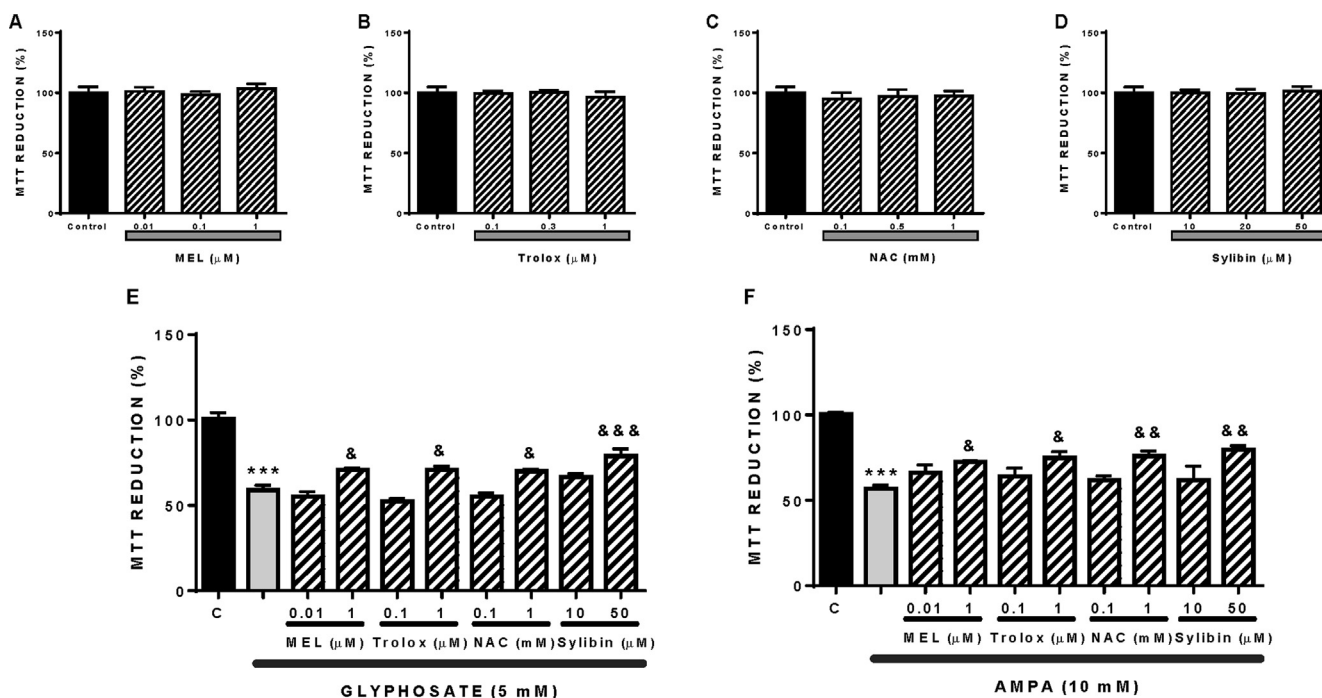
Student's *t*-test. Statistical significance was defined at  $P < 0.05$ .

### 3. Results

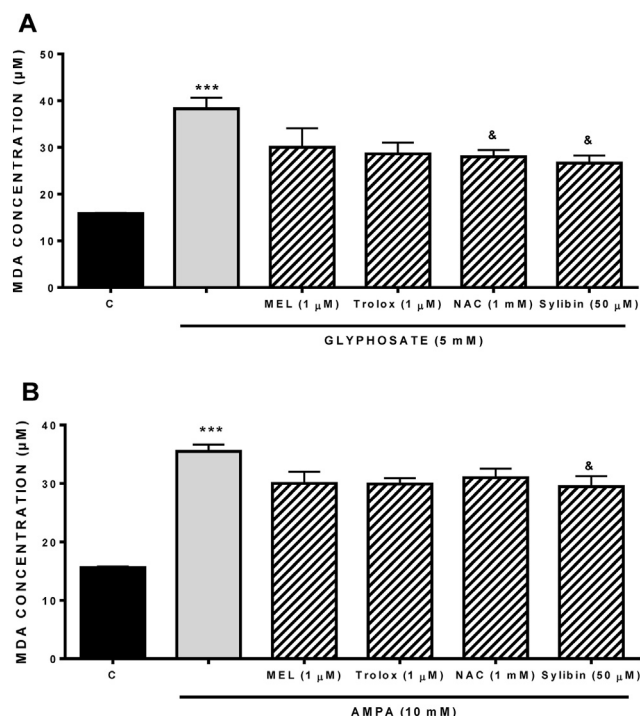
#### 3.1. Effects of glyphosate and AMPA on SH-SY5Y cell viability MTT and LDH assays

In order to evaluate cell survival we used MTT assay. A 48 h incubation period with glyphosate or AMPA at increasing concentrations (0.1–20 mM) reduced cell viability in a concentration-dependent manner compared with control group (Fig. 2A, B). Results showed that the lower concentrations, 0.1, 0.25, 0.5, 1, and 2.5 mM of glyphosate and AMPA, did not affect neuronal survival. The  $IC_{50}$  value was calculated to be  $5.36 \pm 1.12$  mM for glyphosate and  $9.93 \pm 0.43$  mM for AMPA. We also examined whether exposure to glyphosate or AMPA induced cell death by assessing LDH leakage. SH-SY5Y cells incubated for a 48 h period with glyphosate or AMPA (0.1–20 mM) produced in concentration-dependent manner significant elevations in LDH leakage compared with control group (Fig. 2C, D). The  $IC_{50}$  value was calculated to be  $4.93 \pm 0.62$  mM for glyphosate and  $9.70 \pm 2.77$  mM for AMPA. These observations allowed us to choose a glyphosate concentration of 5 mM and an AMPA concentration of 10 mM in order to perform the subsequent experiments.

We examined whether exposure to compounds commonly used as antioxidants such as MEL, Trolox, NAC and Sylibin induced cytotoxicity determining dose-response curves for cell viability MTT assay (Fig. 3A, B, C, D). Since the doses of MEL (0.01, 0.1 and 1  $\mu$ M), Trolox (0.1, 0.3 and 1  $\mu$ M), NAC (0.1, 0.5 and 1 mM) and Sylibin (10, 20 and 50  $\mu$ M) did not produced significant difference between data of antioxidant treated cells and control cells, this dose range was used to determine the potential of these antioxidants to prevent cell viability affected by glyphosate (5 mM) and AMPA (10 mM) (Fig. 3E, F). MEL (1  $\mu$ M), Trolox (1  $\mu$ M), NAC (1 mM) and Sylibin (50  $\mu$ M) showed effective protection against the glyphosate and AMPA cytotoxicity measured as MTT reduction (Fig. 3E, F).



**Fig. 3.** Cytotoxicity induced by MEL (A), Trolox (B), NAC (C) and Sylibin (D) after 48-h incubation period on SH-SY5Y cell viability. Cytoprotection of MEL, Trolox, NAC and Sylibin after 48-h co-incubation period against cell death elicited by glyphosate (5 mM) (E) and AMPA (10 mM) (F). Cell viability was measured as MTT reduction (A - F) and data were normalized as % control (black column). Data represent the mean  $\pm$  SEM of six independent experiments in triplicate.  $***P < 0.001$  compared to control.  $^*P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  compared to glyphosate or compared to AMPA in the absence of antioxidants.



**Fig. 4.** MDA production induced by glyphosate (5 mM) (A) and AMPA (10 mM) (B) after 48-h incubation period on SH-SY5Y cells. Effect of antioxidants MEL (1  $\mu$ M), Trolox (1  $\mu$ M), NAC (1 mM) and Sylibin (50  $\mu$ M) on MDA levels induced by glyphosate (5 mM) (A) and AMPA (10 mM) (B) after 48-h co-incubation period. Data represent the mean  $\pm$  SEM of six independent experiments in triplicate.  $***P < 0.001$  compared to control.  $^*P < 0.05$  compared to glyphosate or AMPA, in the absence of antioxidants.

### 3.2. Effects of glyphosate and AMPA on oxidative stress markers: Induction of oxidative stress

Effects of glyphosate or AMPA exposures on MDA levels, NO and ROS production and caspase 3/7 activity in human neuroblastoma SH-SY5Y cells are depicted in Figs. 4–7, respectively.

MDA is one of the most important intermediates produced during lipid peroxidation. Fig. 4A shows the lipid peroxidation induced by glyphosate (5 mM) after 48 h incubation period. Incubation with glyphosate (5 mM) induced a significant increase in MDA levels (142%,  $P < 0.001$  compared to control). Also Fig. 4A shows the effect of glyphosate (5 mM) on the lipid peroxidation after 48 h incubation with the antioxidants MEL, Trolox, NAC and Sylibin. Compared to other antioxidants, NAC (1 mM) and Sylibin (50  $\mu$ M) provided a significant decrease (27%,  $P < 0.05$ , and 30%,  $P < 0.05$ , compared to glyphosate) on MDA levels induced by glyphosate, but not MEL and Trolox (Fig. 4A).

Fig. 4B shows the lipid peroxidation induced by AMPA (10 mM) after 48 h incubation period. Incubation with AMPA (10 mM) induced a significant increase in MDA levels (127%,  $P < 0.001$  compared to control). Also Fig. 4B shows the effect of AMPA (10 mM) on the lipid peroxidation after 48 h incubation with the antioxidants MEL, Trolox, NAC and Sylibin. Compared to other antioxidants, Sylibin provided a significant decrease (17%,  $P < 0.05$  compared to AMPA) on MDA

levels induced by AMPA, but not MEL, Trolox and NAC.

A marked increase in NO is detrimental of many pathological conditions; furthermore excessive NO can trigger a neurotoxic cascade. Fig. 5A shows the NO production induced by glyphosate (5 mM) after 48 h incubation period. Incubation with glyphosate (5 mM) induced a significant increase on NO production (23%,  $P < 0.001$ ). Also Fig. 5A shows the effect of glyphosate (5 mM) on NO production after 48 h incubation with the antioxidants MEL (1  $\mu$ M), Trolox (1  $\mu$ M), NAC (1 mM) and Sylibin (50  $\mu$ M). MEL, NAC and Sylibin provided a significant decrease (–10%,  $P < 0.05$ ; –10%,  $P < 0.05$  and –12%,  $P < 0.01$ , respectively, compared to glyphosate), but not Trolox.

Fig. 5B shows the NO production induced by AMPA (10 mM) after 48 h incubation period. Incubation with AMPA (10 mM) induced a significant increase in NO levels (23%,  $P < 0.001$  compared to control). Also Fig. 5B shows the effect of AMPA (10 mM) on the NO production after 48 h incubation with the antioxidants MEL (1  $\mu$ M), Trolox (1  $\mu$ M), NAC (1 mM) and Sylibin (50  $\mu$ M). Trolox, NAC and Sylibin provided a significant decrease (–10%,  $P < 0.05$ ; –16%,  $P < 0.01$  and –19%,  $P < 0.001$ , respectively, compared to AMPA), but not MEL.

In order to determine the presence of oxidative imbalance caused by glyphosate or AMPA as well as the efficacy of antioxidants (MEL, Trolox, NAC and Sylibin) against oxidative stress in the cells, cellular ROS formation was quantified by the DCFH assay. Glyphosate (5 mM)

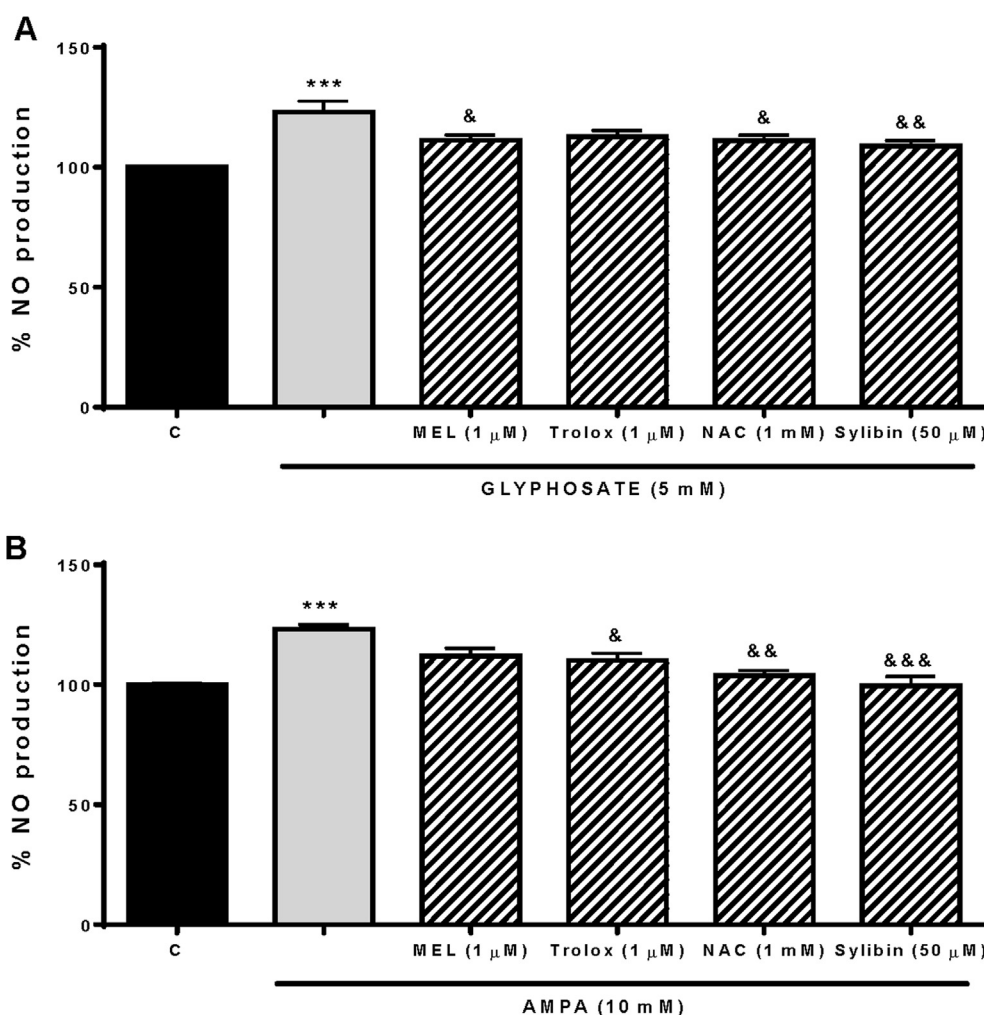
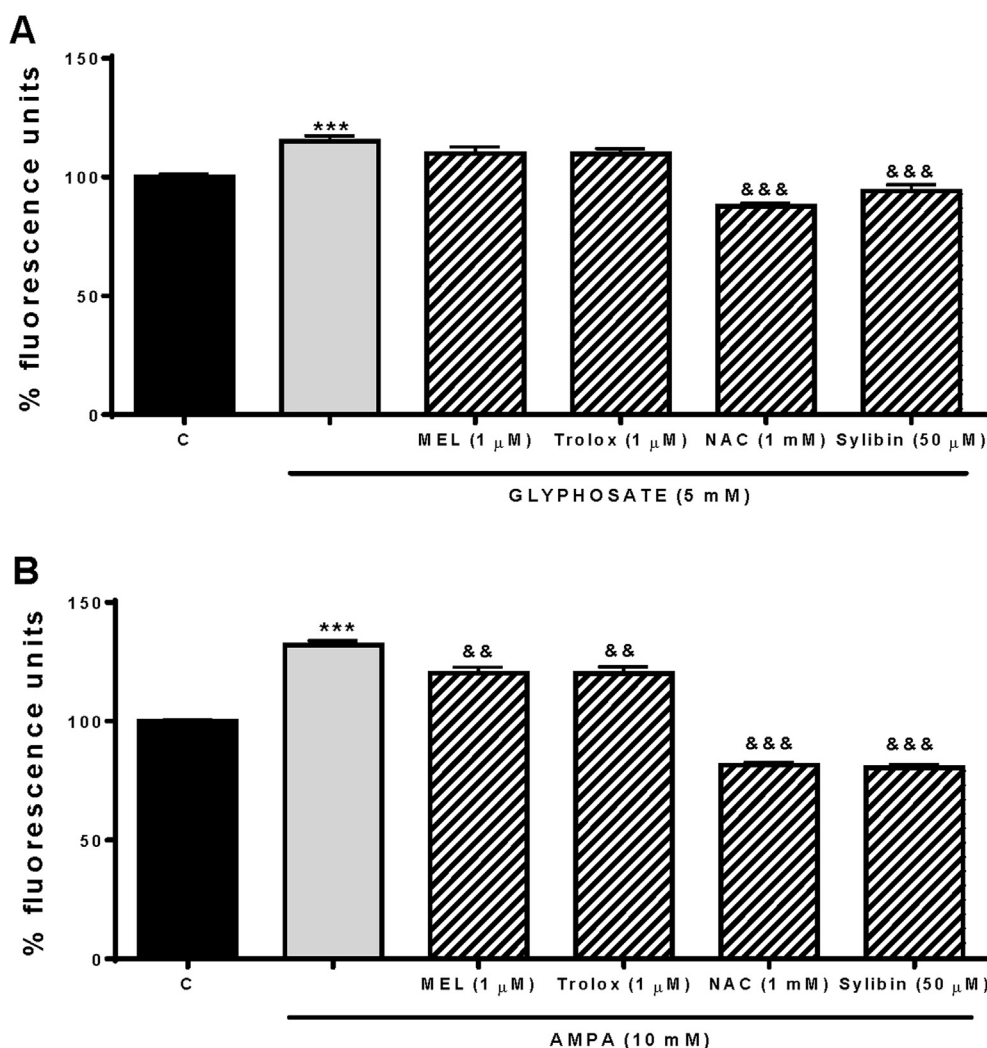


Fig. 5. NO production induced by glyphosate (5 mM) (A) and AMPA (10 mM) (B) after 48-h incubation period on SH-SY5Y cells. Effect of antioxidants MEL (1  $\mu$ M), Trolox (1  $\mu$ M), NAC (1 mM) and Sylibin (50  $\mu$ M) on NO production induced by glyphosate (5 mM) (A) and AMPA (10 mM) (B) after 48-h co-incubation period. Data were normalized as % control (black column). Data represent the mean  $\pm$  SEM of six independent experiments in triplicate. \*\*\* $P < 0.001$  compared to control. &  $P < 0.05$ , && $P < 0.01$ , &&& $P < 0.001$  compared to glyphosate or AMPA, in the absence of antioxidants.



**Fig. 6.** ROS production induced by glyphosate (5 mM) (A) and AMPA (10 mM) (B) after 48-h incubation period on SH-SY5Y cells. Effect of antioxidants MEL (1 μM), Trolox (1 μM), NAC (1 mM) and Sylibin (50 μM) on ROS production induced by glyphosate (5 mM) (A) and AMPA (10 mM) (B) after 48-h co-incubation period. Data were normalized as % control (black column). Data represent the mean  $\pm$  SEM of six independent experiments in triplicate. \*\*\* $P < 0.001$  compared to control. &&&  $P < 0.01$ , &&& $P < 0.001$  compared to glyphosate or AMPA in the absence of antioxidants.

increased significantly ROS production (72%,  $P < 0.001$ ) reflecting acted as a pro-oxidant (Fig. 6A). The increase on ROS production by glyphosate (5 mM) was significantly reduced by NAC (1 mM) and Sylibin (50 μM) (24%,  $P < 0.001$ ; 18%,  $P < 0.001$ , respectively), but not MEL and Trolox (Fig. 6A).

Fig. 6B shows the ROS production induced by AMPA (10 mM) after 48 h incubation period. Incubation with AMPA (10 mM) increased significantly ROS production (53%,  $P < 0.001$  compared to control). The increase on ROS production by AMPA was significantly reduced by the four antioxidants; NAC (1 mM) and Sylibin (50 μM) showed the most effective protection (38%,  $P < 0.001$  and 39%,  $P < 0.001$ , respectively) (Fig. 6B).

Fig. 7 shows the caspase-3/7 activity of SH-SY5Y cells treated for 48 h with glyphosate (5 mM) (Fig. 7A) and AMPA (10 mM) (Fig. 7B). Glyphosate and AMPA significantly increased caspase-3/7 activity (71%,  $P < 0.001$ ; 78%,  $P < 0.001$ , respectively). These remarkable increases in caspase-3/7 activity were reduced by Sylibin (50 μM) (28%,  $P < 0.01$  and 18%,  $P < 0.05$ , respectively) but not by MEL, Trolox, and NAC.

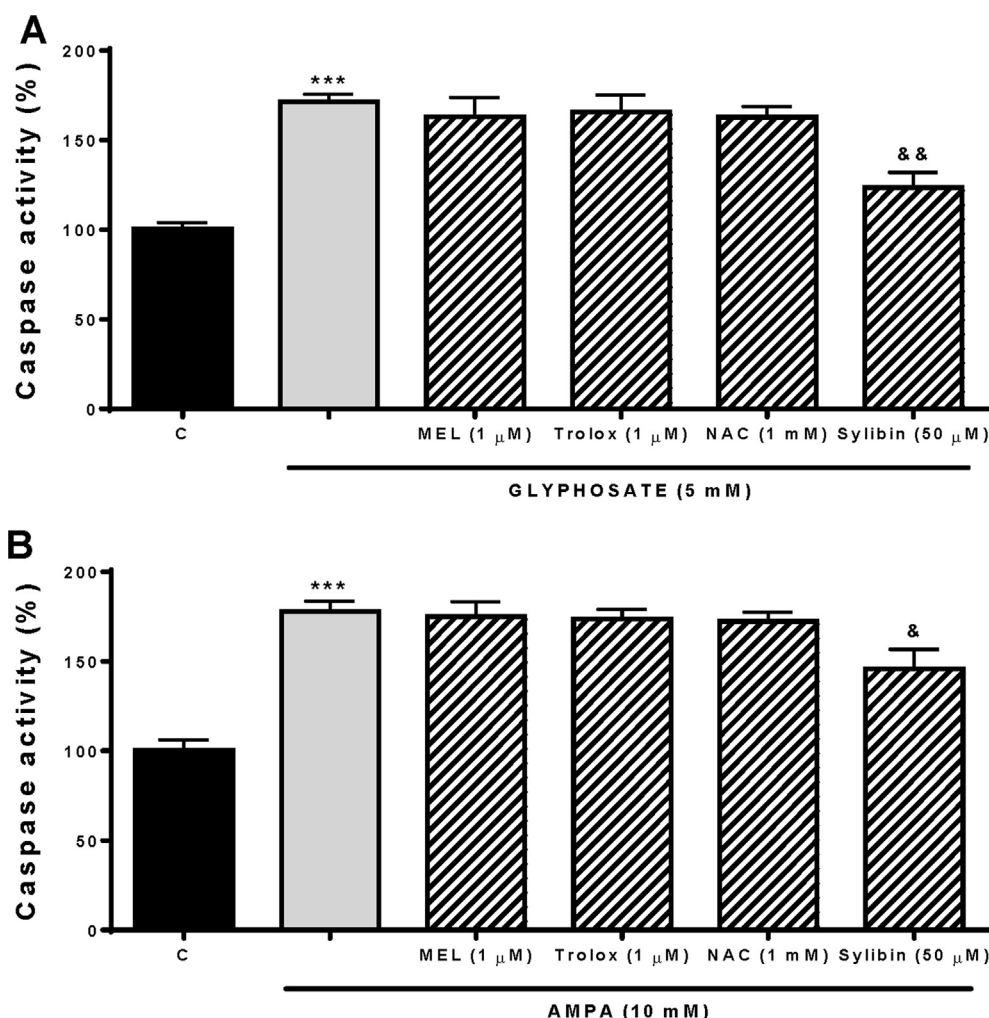
### 3.3. Effects of glyphosate and AMPA on neuronal development and proinflammation related gene transcriptions in SH-SY5Y cells

We evaluated whether glyphosate and AMPA might affect neuronal development (GAP43, CAMK2A, CAMK2B, TUBB3 and WNTs) and proinflammation (IL6 and TNF $\alpha$ ) signaling pathways by RT-PCR assays. Glyphosate (5 mM) reduced the gene expressions of GAP43 (-23%,  $P < 0.01$ ) and TUBB3 (-18%,  $P < 0.05$ ) compared to control group (Fig. 8A) and increased the gene expressions of CAMK2A, CAMK2B, Wnt3a, Wnt5a and Wnt7a (35%,  $P < 0.05$ ; 28%  $P < 0.05$ ; 27%,  $P < 0.05$ ; 26%,  $P < 0.05$  and 61%,  $P < 0.05$ , respectively) as well as increased the gene expressions of IL6 and TNF $\alpha$  (131%,  $P < 0.05$  and 66%,  $P < 0.05$ , respectively) compared to control group (Fig. 8A). The AMPA metabolite (10 mM) reduced the gene expressions of GAP43, CAMK2A, CAMK2B, TUBB3 and Wnt3a (-50%,  $P < 0.001$ ; -57%,  $P < 0.001$ ; -42%,  $P < 0.05$ ; -76%,  $P < 0.001$  and -64%,  $P < 0.01$ , respectively) and increased the gene expression of TNF $\alpha$  (103%,  $P < 0.05$ ) compared to control group (Fig. 8B).

### 3.4. Effects of glyphosate and AMPA on gene expression profiling of cell death pathways by Real-Time PCR array

Gene expression profiling of cell death pathways of glyphosate





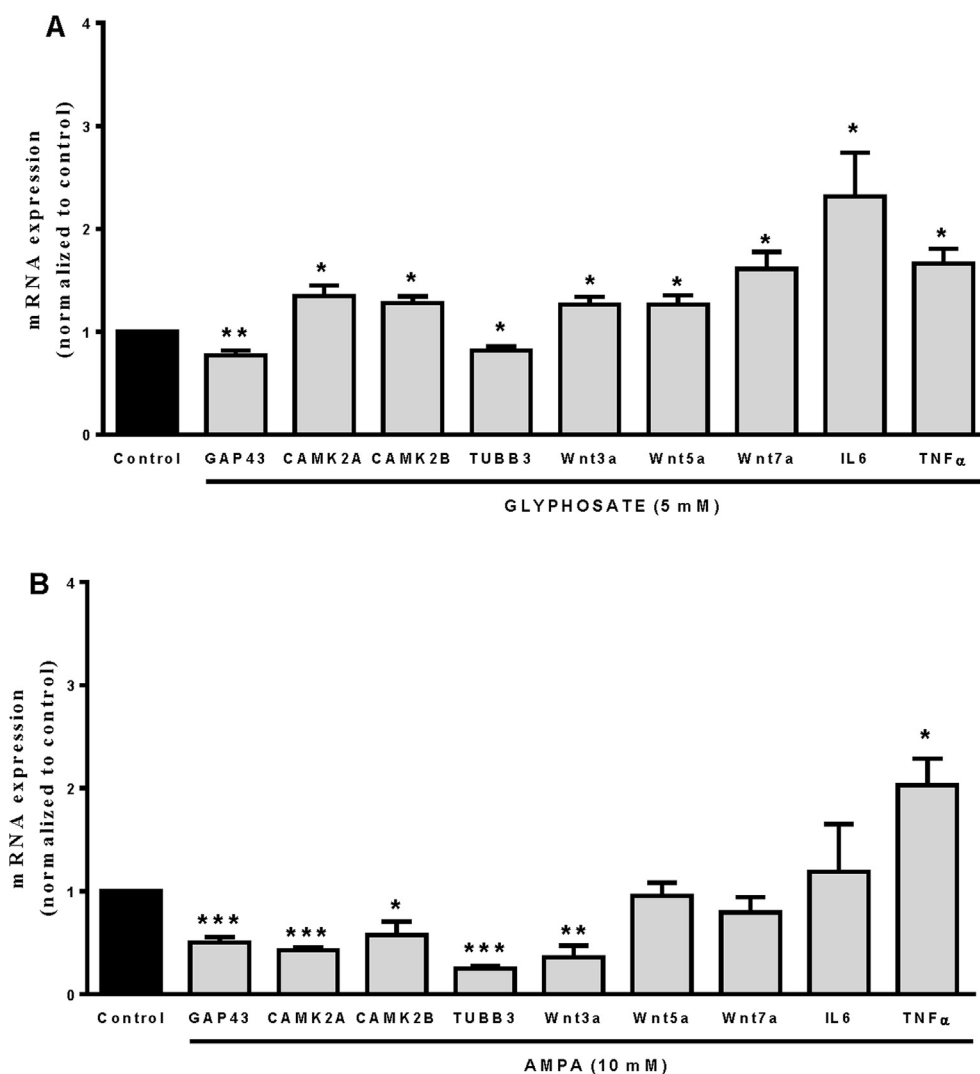
**Fig. 7.** Caspase-3/7 activity induced by glyphosate (5 mM) (A) and AMPA (10 mM) (B) after 48-h incubation period on SH-SY5Y cells. Effect of antioxidants MEL (1  $\mu$ M), Trolox (1  $\mu$ M), NAC (1 mM) and Sylibin (50  $\mu$ M) on caspase-3/7 activity induced by glyphosate (5 mM) (A) and AMPA (10 mM) (B) after 48-h co-incubation period. Data were normalized as % control (black column). Data represent the mean  $\pm$  SEM of six independent experiments in triplicate. \*\*\* $P$  < 0.001 compared to control. \* $P$  < 0.05, \*\* $P$  < 0.01 compared to glyphosate or AMPA in the absence of antioxidants.

(5 mM), AMPA (10 mM) and control samples were performed using Real-Time PCR array. The gene expression profiles of glyphosate and AMPA were significantly different from control, thus set of genes were successfully clustered (Fig. 9). Although there were some differences in intensities in gene expression within the major sub-branches, overall the pattern was similar within each cluster throughout the sub-branches. A global intensity map of the differentially expressed genes is shown in Fig. 9.

Genes were considered up- or down-regulated if the average fold change in expression was 1.5 or above compared to control group ( $P$  < 0.05). Of the 84 genes examined in the cells exposed to glyphosate (5 mM), changes in mRNA levels were detected in 49 genes: 47 were up-regulated and 2 were down-regulated. The differentially expressed genes are listed in Table 2. Of these genes, a greater fold change than 2-fold was observed on the up-regulated apoptotic peptidase activating factor 1 (APAF1) (2.77-fold), BAX (3.03-fold), caspase 3, apoptosis-related cysteine peptidase (CASP3) (2.84-fold), caspase 7, apoptosis-related cysteine peptidase (CASP7) (3.37-fold), caspase 9, apoptosis-related cysteine peptidase (CASP9) (2.13-fold), CD40 ligand (CD40LG) (2.04-fold), myeloid cell leukemia sequence 1 (MCL1) (2.07-fold), nucleolar protein 3 (NOL3) (2.03-fold), synaptonemal complex protein 2 (2SYCP2) (2.86-fold), tumor necrosis factor (TNF) (4.37-fold), tumor protein p53 (TP53) (3.86-fold), cathepsin B (CTSB) (2.10-fold), nuclear transcription factor kappa B subunit 1 (NF $\kappa$ B1) (4.16-fold),

phosphoinositide-3-kinase class 3 (PIK3C3) (2.05-fold) and synuclein, alpha (SNCA) (2.81-fold) genes (Table 2). In addition, the expression levels of V-akt murine thymoma viral oncogene homolog 1 (AKT1) and B-cell CLL/lymphoma 2 (BCL2) genes were significantly down-regulated in cells exposed to glyphosate (5 mM) to levels that were 3.0 and 2.41 times lower, respectively, than those in control cultures ( $P$  < 0.05; Table 2).

Of the 84 genes examined in the cells exposed to AMPA (10 mM), changes in mRNA levels were detected in 27 genes: 25 were up-regulated and 2 were down-regulated. The differentially expressed genes are listed in Table 2. A greater than 2-fold change was observed on the up-regulated APAF1 (4.95-fold), BAX (2.19-fold), CASP3 (3.29-fold), CASP7 (2.36-fold), CASP9 (2.91-fold), fas-TNF receptor superfamily, member 6 (FAS) (3.29-fold), fas ligand (FASLG) (2.17-fold), synaptonemal complex protein 2 (SYCP2) (2.34-fold), TNF (2.57-fold), TP53 (2.42-fold), NF $\kappa$ B1 (2.66-fold), SNCA (2.09-fold), sequestosome 1 (SQSTM1) (2.81-fold), HSPB (heat shock 27 kDa) associated protein 1 (HSPBAP1) (3.23-fold) and potassium channel interacting protein 1 (KCNI1) (2.17-fold) genes (Table 2). In addition, the expression level of AKT1 gene was significantly down-regulated in the cells exposed to AMPA (10 mM) to levels that were 2.37 times lower than those in control cultures ( $P$  < 0.05; Table 2).



**Fig. 8.** Effect of glyphosate (5 mM) (A) and AMPA (10 mM) (B) after 48-h incubation period on gene expressions of neuronal development (GAP43, CAMK2A, CAMK2B, TUBB3, Wnt3a, Wnt5a and Wnt7a) and proinflammation (IL6 and TNF $\alpha$ ) mediators in SH-SY5Y cells. Data were normalized to control (black column). Data represent the mean  $\pm$  SEM of three independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 compared to control.

### 3.5. Ingenuity pathway analysis (IPA)

To investigate possible biological interactions of differently regulated genes, datasets representing genes with altered expression profile derived from real-time PCR array analyses were imported into the Ingenuity Pathway Analysis (IPA) tool.

The list of differentially expressed genes analyzed by IPA revealed 4 significant networks after glyphosate (5 mM) exposure. Fig. 10A represents the list of 4 network functions identified by IPA: (1) Cell death and survival, embryonic development, organismal injury and abnormalities, score of 17, (2) Cell cycle, humoral immune response, protein synthesis, score of 3, (3) Cellular compromise, neurological diseases, organismal injury and abnormalities, score of 1, and (4) Cancer, neurological diseases, organismal injury and abnormalities, score of 1 (Fig. 10A). The score is the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. A score of 3 indicates a 1/1000 chance that the focus genes are in a network not due to random chance. The IPA analysis also groups the differentially expressed genes into to molecular and cellular functions, with 17 focus molecules for Cell death and survival (Fig. 10B). Apoptosis signaling was the main signaling canonical pathway (Fig. 10C).

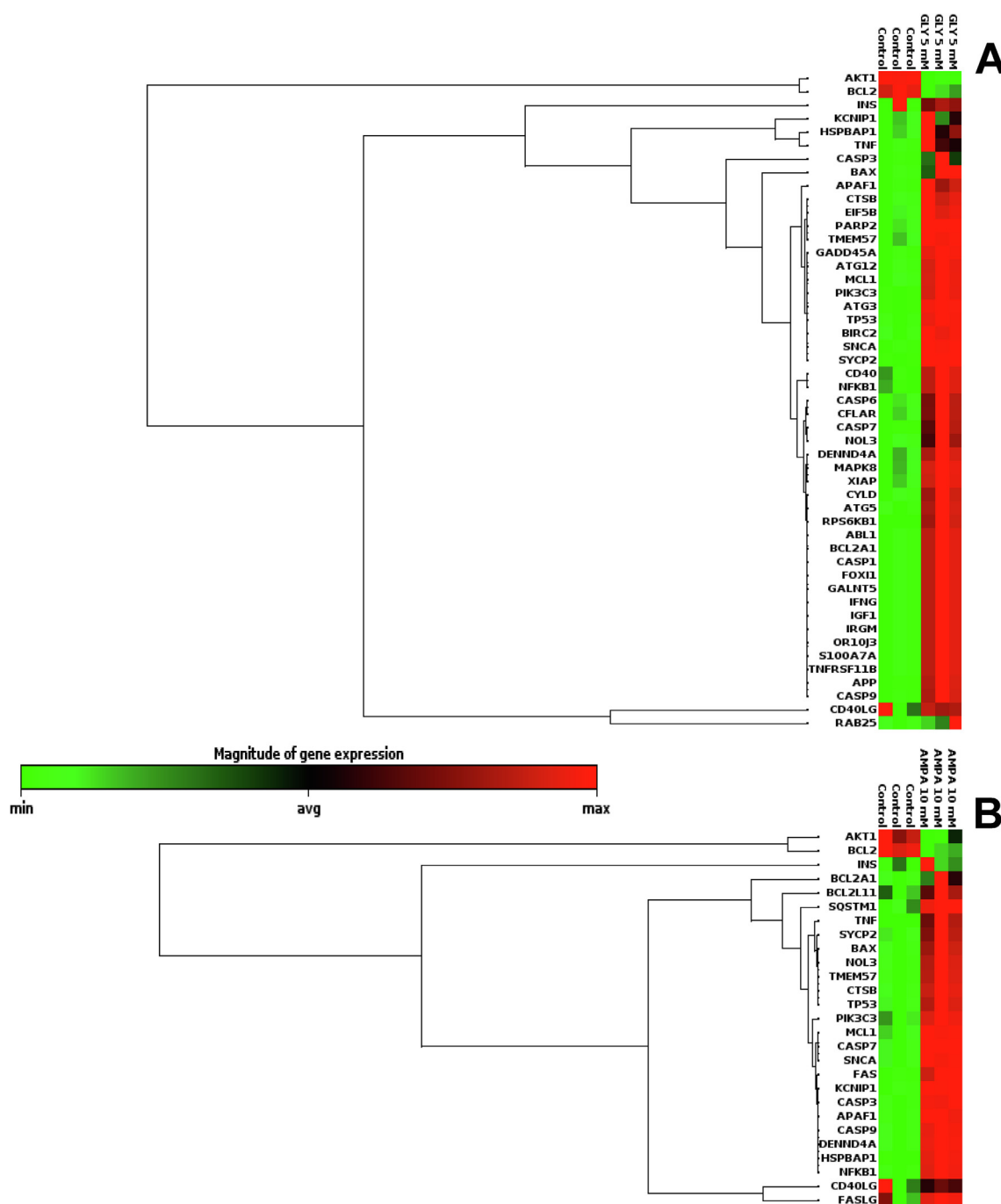
The list of differentially expressed genes analyzed by IPA revealed 3 significant networks after AMPA (10 mM) exposure. Fig. 10D represents

the list of 3 network functions identified by IPA: (1) Cell death and survival, organismal injury and abnormalities, score of 15, (2) Cellular compromise, neurological diseases, organismal injury and abnormalities, score of 1, and (3) Inflammatory response, hematological system development and function, lymphoid tissue structure, score of 1 (Fig. 10D). The IPA analysis also groups the differentially expressed genes into to molecular and cellular functions, with 12 focus molecules for Cell death and survival (Fig. 10E). Apoptosis signaling was the main signaling canonical pathway (Fig. 10F).

The genes up and down-regulated on apoptosis signaling canonical pathway and on the top network were represented for glyphosate (Fig. 11A, B) and AMPA (Fig. 11C, D). Top networks for glyphosate and AMPA summarize the highest number of connections among the larger number of differentially expressed genes which were mainly those related to the cell death process.

## 4. Discussion

Glyphosate-based herbicides are worldwide used indiscriminately with restricted control from environmental agencies. Pesticides have been recognized as the main environmental factor associated with human neurodegenerative disorders. Although there is growing evidence describing toxic effects of glyphosate, further studies are



**Fig. 9.** Hierarchical clustering of the relative expression of the 49 of 84 (glyphosate) and 24 of 84 (AMPA) cell death-related genes analyzed. **Green** represents genes whose transcription is down-regulated (the brightest green represents the smallest value), while **red** represents up-regulated genes (the brightest red represents the highest value) (fold change 1.5 or above in gene expression threshold) after glyphosate (5 mM) (A) or AMPA (10 mM) (B) exposure (color figure online). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

necessary to assess its neurotoxicity and the mechanisms leading to the potential damage on nervous system. Alternative *in vitro* testing strategies are required, in particular for screening, as part of a testing scheme (Anadón et al., 2013). Our study describes toxic effects of glyphosate and its metabolite AMPA using the SH-SY5Y cell line, a widely used *in vitro* model in studies related to neurotoxicity, oxidative stress and neurodegenerative diseases. This study demonstrates that glyphosate and AMPA can induce cell death promoting the involvement of oxidative stress, apoptosis, autophagy and necrosis mechanisms.

Our results show that glyphosate and its metabolite AMPA cause pronounced cytotoxic effects in human neuroblastoma SH-SY5Y cells.

Cell viability assays (MTT reduction) showed  $IC_{50}$  values of  $5.36 \pm 1.12$  mM for glyphosate and  $9.93 \pm 0.43$  mM for AMPA. In the present study, glyphosate cytotoxicity determined by MTT assay in SH-SY5Y cells was more sensitive than that reported in human fibroblasts cells ( $IC_{50}$  value > 75 mM) (Lueken et al., 2004), human liver HepG2 cells ( $IC_{50}$  value > 10 mM) (Gasnier et al., 2009) and PC12 cells ( $IC_{50}$  value > 40 mM) (Gui et al., 2012). The present study show that exposure to glyphosate (5 mM equivalent to 0.84 g/L) and AMPA (10 mM) induces significant oxidative stress in SH-SY5Y cells as was evident by the elevation of the MDA levels (142% and 127%, respectively) and ROS levels (72% and 53%, respectively). Also, caspase

**Table 2**

Results from Plus RT<sup>2</sup> Profiler PCR Array targeting select genes related to cell death (apoptosis, autophagy and necrosis genes). SH-SY5Y cells were treated with Glyphosate (5 mM), AMPA (10 mM) or DMSO (0.1%) as the carrier control.

Gene symbol	GenBank accession no.	Fold change Glyphosate	Fold change AMPA	Gene symbol	GenBank accession no.	Fold change Glyphosate	Fold change AMPA	Gene symbol	GenBank accession no.	Fold change Glyphosate	Fold change AMPA
<b>Apoptosis</b>				<b>Autophagy</b>				<b>Necrosis</b>			
ABL1	NM_005157	1.94		APP	NM_000484	1.67		DENND4A	NM_005848	1.51	1.61
AKT1 <sup>a</sup>	NM_005163	-3.00	-2.37	ATG12	NM_004707	1.87		EIF5B	NM_015904	1.59	
APAF1	NM_001160	2.77	4.95	ATG3	NM_022488	1.97		FOXI1	NM_012188	1.94	
BAX	NM_004324	3.03	2.19	ATG5	NM_004849	1.71		GALNT5	NM_014568	1.94	
BCL2 <sup>a</sup>	NM_000633	-2.41	-1.96	CTSB	NM_001908	2.10	1.92	HSPBAP1	NM_024610	1.72	3.23
BCL2A1	NM_004049	1.94	1.93	IFNG	NM_000619	1.94		KCNIP1	NM_014592	1.67	2.17
BCL2L1	NM_006538		1.77	IGF1	NM_000618	1.94		OR10J3	NM_001004467	1.94	
BIRC2	NM_001166	1.72		INS	NM_000207	1.50	1.78	PARP2	NM_005484	1.61	
CASP1	NM_033292	1.94		IRGM	NM_001145805	1.94		RAB25	NM_020387	1.74	
CASP3 <sup>a</sup>	NM_004346	2.84	3.29	MAPK8	NM_002750	1.51		RPS6KB1	NM_003161	1.62	
CASP6	NM_032992	1.55		NFκB1	NM_003998	4.16	2.66	S100A7A	NM_176823	1.94	
CASP7	NM_001227	3.37	2.36	PIK3C3	NM_002647	2.05	1.64	TMEM57	NM_018202	1.68	1.79
CASP9	NM_001229	2.13	2.91	SNCA	NM_000345	2.81	2.09				
CD40	NM_001250	1.80		SQSTM1	NM_003900		2.81				
CD40LG	NM_000074		1.76								
CFLAR	NM_003879	1.60									
CYLD <sup>b</sup>	NM_015247	1.77									
GADD45A	NM_001924	1.79									
FAS <sup>a</sup>	NM_000043		3.29								
FASLG	NM_000639		2.17								
MCL1	NM_021960	2.07	1.53								
NOL3	NM_003946	2.03	1.74								
SYCP2 <sup>b</sup>	NM_014258	2.86	2.34								
TNF <sup>a</sup>	NM_000594	4.37	2.57								
TNFRSF11B	NM_002546	1.94									
TP53 <sup>a</sup>	NM_000546	3.86	2.42								
XIAP	NM_001167	1.55									

**Note:**

Data for gene expression represent fold change mean (> 1.5),  $P < 0.05$  compared to control of three independent experiments.

<sup>a</sup> Genes related to both apoptosis and autophagy.

<sup>b</sup> Genes related to both apoptosis and necrosis.

activity (caspases 3/7) increased when cells were exposed to glyphosate and AMPA (71% and 78%, respectively) pointing to the initiation of an apoptotic process, effect that was not observed previously for glyphosate in rat Leydig cells (Clair et al., 2012) but in agreement with other investigators who have demonstrated that glyphosate induces caspases activation in human umbilical, embryonic and placental cells using high concentrations (7–450 g/L) after 24 h exposure (Benachour and Seralini, 2009). To assay a possible protection against oxidative injury, antioxidant substances were evaluated including MEL, Trolox, NAC and Sylibin (Rodríguez et al., 2004; Cort et al., 1975; Li et al., 2007; Nencini et al., 2007). NAC (1 mM) and Sylibin (50 μM) showed the most effective protection.

Furthermore, because the detection of developmental neurotoxicity of chemicals has high relevance for protection of human health, we aimed to assess, in SH-SY5Y cells, whether glyphosate and AMPA might alter selected target genes. We determined alterations in the expression of genes linked to neuro-(developmental) toxicity including Wnt3a, Wnt5a, Wnt7a, GAP43, CAMK2A, CAMK2B and TUBB3. Wnt proteins are crucial for neuronal development and maturation. Wnts are involved in diverse cellular functions that include neuronal migration, neuronal polarization, axon guidance, dendrite development, and synapse formation (Inestrosa and Arenas, 2010; Rosso and Inestrosa, 2013), all of which are essential steps in the formation of functional neural connections. Wnt signaling pathway play a role not only during early neuronal connectivity but also in synaptic modulation in the adult (Salinas and Zou, 2008). Evidence shows the involvement of Wnt signaling in neurodevelopment as well as in neurodegenerative diseases (De Ferrari and Moon, 2006). Since Wnt signaling pathways play crucial roles on neuronal processes, we evaluated whether glyphosate and AMPA affect neuronal development through changes in Wnt signaling pathways. In this study we analyzed the mRNA levels of Wnt3a, Wnt5a,

and Wnt7a genes. Our results revealed that exposure of SH-SY5Y cells to glyphosate (5 mM), but not AMPA (10 mM) exposure, increased mRNA-Wnt3a, -Wnt5a and -Wnt7a levels as well as induced up-regulation of the pro-inflammatory cytokines, particularly IL6 and TNFα genes. In this context, previous studies in primary cultures of pyramidal cells also revealed that glyphosate (23 mM) exposure affects neuronal development through changes in the Wnt signaling pathways but particularly through a decrease in mRNA-Wnt5a level whereas no changes in others Wnt species were observed (Coullery et al., 2016). These observations highlight the critical role of Wnt proteins to mediate neuronal development. Our findings in SH-SY5Y cells suggest that glyphosate may promote neuronal morphological defects involving up-regulation of Wnts signaling pathways. Wnt signaling pathways enhanced by glyphosate could also exacerbate proinflammatory responses, possibly imposing enhanced neurotoxicity as long-term side effect. Recent studies demonstrated that Wnts do not only regulate synapse formation but also they promote the remodeling of mature terminals elicited by neurological disorders (Budnik and Salinas, 2011).

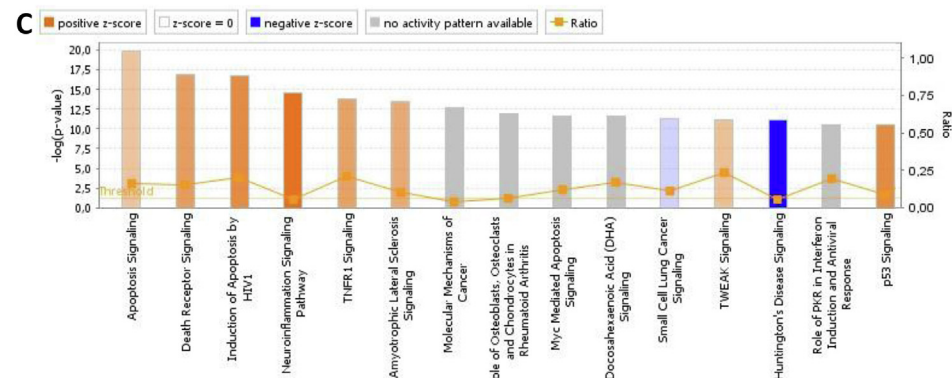
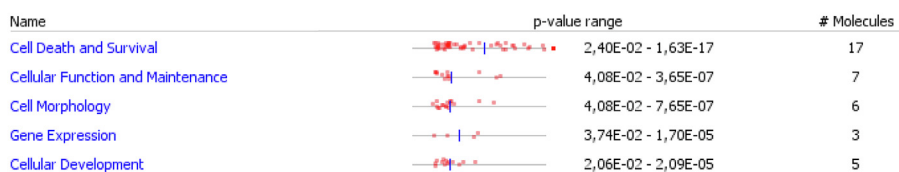
In relation to CAMK2A and CAMK2B genes that are abundantly expressed in neuronal tissues (Erondy and Kennedy, 1985) which play versatile roles in neuronal development and function, including synaptic plasticity and learning and memory (Saito et al., 2013), we observed that glyphosate increased the mRNA expression of the two CAMK2 isoforms in SH-SY5Y cells, which can lead to neuronal apoptosis (Chen et al., 2011). In contrast, AMPA decreased the expression of CAMK2A and CAMK2B mRNA, suggesting that the glyphosate metabolism partially prevents neuronal cell death. On the other hand, glyphosate and AMPA exposures induced a down-regulation of TUBB3 and GAP43 gene expressions. TUBB3 constitutes one of the earliest neuronal cytoskeletal proteins expressed in CNS development; TUBB3 has been strictly related to neurite outgrowth and is widely used as a neuronal



**A** Top Networks

ID	Associated Network Functions	Score
1	Cell Death and Survival, Embryonic Development, Organismal Injury and Abnormalities	17
2	Cell Cycle, Humoral Immune Response, Protein Synthesis	3
3	Cellular Compromise, Neurological Disease, Organismal Injury and Abnormalities	1
4	Cancer, Neurological Disease, Organismal Injury and Abnormalities	1

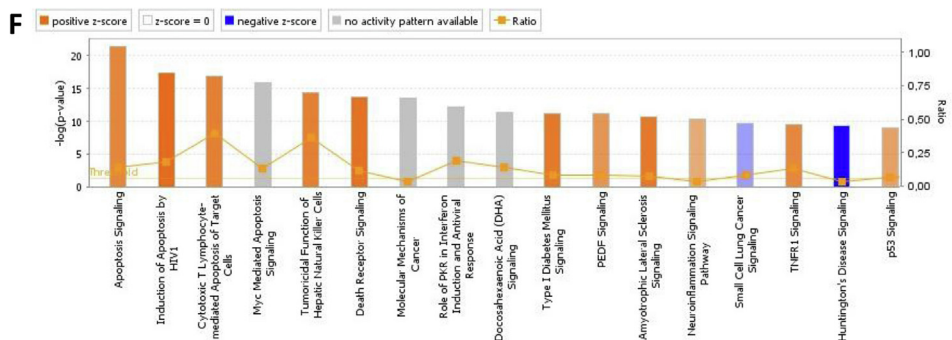
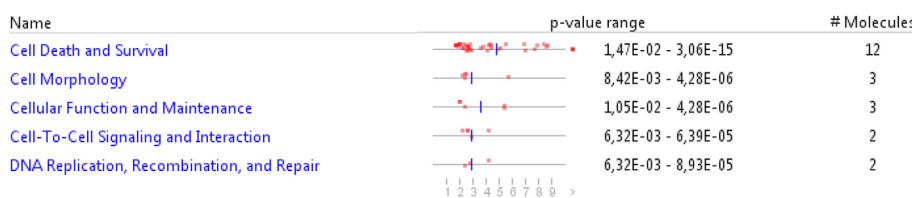
**B** Molecular and Cellular Functions



**D** Top Networks

ID	Associated Network Functions	Score
1	Cell Death and Survival, Organismal Injury and Abnormalities, Renal Necrosis/Cell Death	15
2	Cellular Compromise, Neurological Disease, Organismal Injury and Abnormalities	1
3	Inflammatory Response, Hematological System Development and Function, Lymphoid Tissue Structure	1

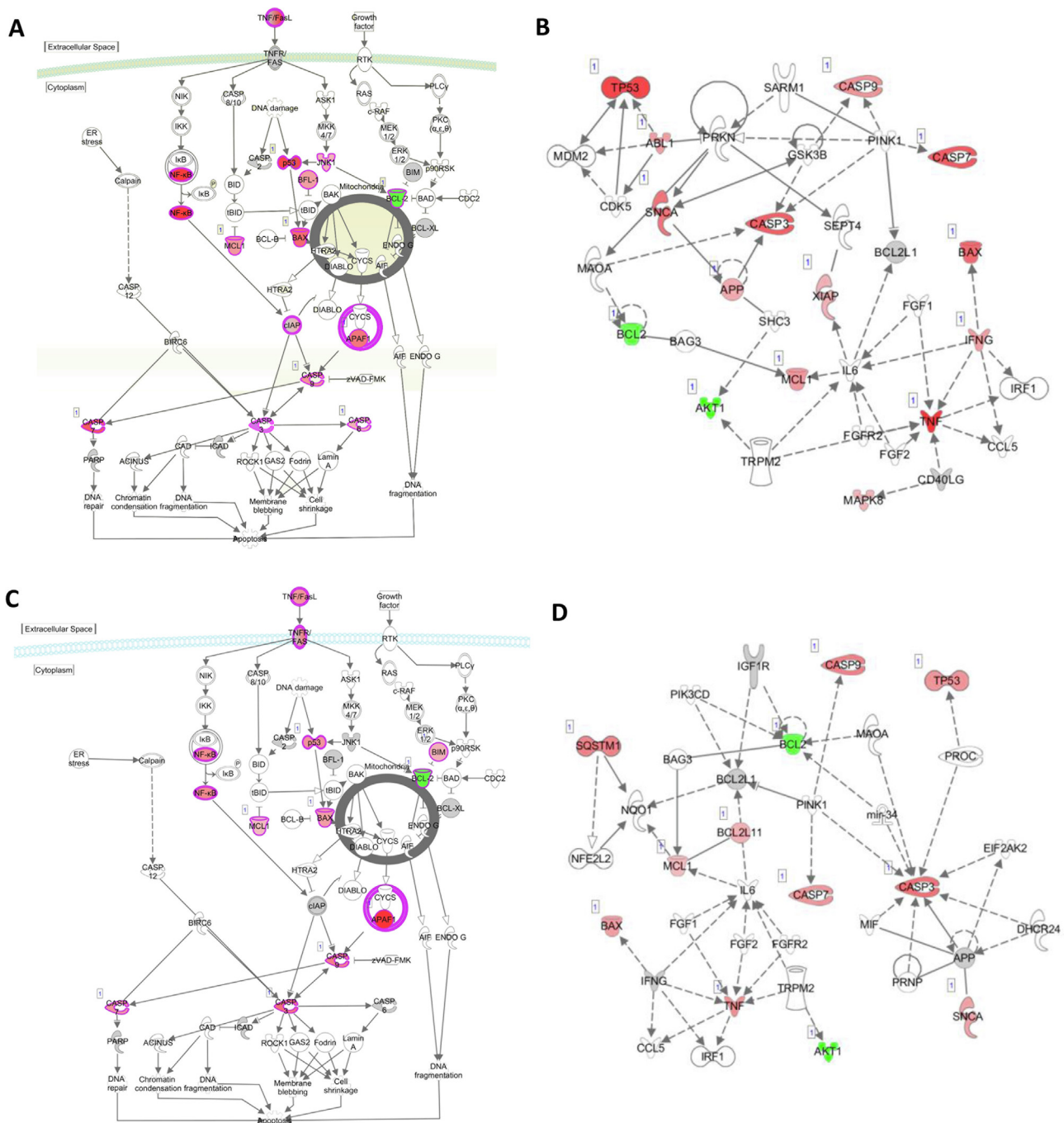
**E** Molecular and Cellular Functions



**Fig. 10.** Ingenuity Pathways Analysis (IPA) summary of glyphosate (5 mM) or AMPA (10 mM) exposure. To investigate possible interactions of differently regulated genes, datasets representing 84 genes with altered expression profile obtained from Real-Time PCR arrays were imported into the IPA tool and the following data are illustrated: (A, glyphosate and D, AMPA) The list of top network functions. (B, glyphosate and E, AMPA) The list of top molecular and cellular functions with their respective scores obtained from IPA. (C, glyphosate and F, AMPA) Toxicology pathway list in IPA analysis. Each histogram is a particular canonical pathway. The x-axis represents the top toxicology functions as calculated by IPA based on differentially expressed genes are highlighted, and the y-axis represents the ratio of number of genes from the dataset that map to the pathway and the number of all known genes ascribed to the pathway. The orange line represents the threshold of  $P < 0.05$  as calculated by Fischer's test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

marker in developmental studies (Katsetos et al., 2003). GAP43 may contribute to presynaptic membranes in the adult, leading to the phenomena of neurotransmitter release, endocytosis and synaptic vesicle recycling, long-term potentiation, spatial memory formation and

learning (Denny, 2006). In this context and taking into account that GAP43 is an intracellular growth-associated protein that is expressed during axonal remodeling and regeneration when neuronal axons are damaged both during the development in the CNS and during



**Fig. 11.** Specific elements of the canonical pathway for modulation of gene expression obtained in IPA for glyphosate (5 mM) or AMPA (10 mM). (A, glyphosate and C, AMPA) Toxicology pathway obtained in IPA. (B, glyphosate and D, AMPA) Most highly rated network in IPA analysis. The genes that are shaded were determined to be significant from the statistical analysis. A solid line represents a direct interaction between the two gene products and a dotted line means there is an indirect interaction. Green represents down-regulation, while red depicts up-regulation. White represents no change in expression (color figure online). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regeneration in the PNS (Grasselli and Strata, 2013), the decreased GAP43 mRNA levels by glyphosate and AMPA might contribute to adverse effects on axon outgrowth. Our results suggest that developmental nervous system seems to be highly vulnerable to glyphosate exposure. The observed changes in mRNA expression of the regulatory proteins (CAMK2A, CAMK2B, and GAP43) and the structural protein (TUBB3) are due to different mechanisms and these gene expressions could be proposed as potential biomarkers for detection and initial evaluation of developmental neurotoxicity in SH-SY5Y cells. Considering the limited data regarding glyphosate effect on the CNS, in this

work we demonstrate that glyphosate exerts neurotoxicity affecting neuronal development. Advances in this field may provide useful information to establish control on the use of glyphosate in order to protect human health.

This work also focuses on the cell death mechanisms induced by glyphosate and AMPA in human SH-SY5Y. We analyze by PCR array profiles the expression of key genes involved in three death processes: apoptosis, autophagy and necrosis. Cells that are exposed to a cytotoxic agent can react in different ways. Apoptosis has come to be used with the phrase ‘programmed cell death’ as it is a cell-intrinsic mechanism

for suicide that is regulated by a variety of cellular signaling pathways. The morphologic features of apoptosis result from the activation of caspases (cysteine proteases) by either death receptor ligation or the release of apoptotic mediators from the mitochondria. Dying by apoptosis requires energy in the form of ATP (Edinger and Thompson, 2004). In contrast to apoptosis, necrosis has been thought to be a passive form of cell death. Necrosis is the end result of a bioenergetics catastrophe resulting from ATP depletion and is characterized morphologically by vacuolation of the cytoplasm, breakdown of the plasma membrane and induction of inflammation around the dying cell by the release of cellular contents and proinflammatory molecules (Edinger and Thompson, 2004). Alternative, non-apoptotic forms of programmed cell death have been described and classified as programmed necrosis or autophagy cell death. Autophagy represents an alternative death pathway when apoptosis is disabled. Autophagy is an intracellular catabolic system that delivers cytoplasmic contents (e.g., proteins, lipids, and organelles) to lysosomes for degradation. When cells encounter environmental stresses, such as nutrient starvation, hypoxia, oxidative stress, pathogen infection, the level of autophagy can be dramatically augmented as a cytoprotective response, resulting in adaptation and survival; however, deregulated or excessive autophagy may lead to cell death. In certain contexts, autophagy and apoptosis are in a fine balance. Apoptosis and autophagy have extremely complex interrelationships and can overlap at the level of various signaling steps; morphologic features of both cell death mechanisms can be observed concurrently in the same cell (Maiuri et al., 2007). Our results obtained from microarray-based differential expression revealed the involvement of apoptosis, autophagy and necrosis mechanisms in glyphosate induced cell death (Table 2).

Our study showed that glyphosate and AMPA differentially altered expressions of 24 and 17 genes, respectively (fold change, see Table 2) related to apoptosis. The step of apoptosis regulation that is controlled by the BCL-2 family appears to be the most general final commitment step for the decision between cell life and death. Among the members of the BCL2 family, BCL2 protein acts to suppress cell death and BAX protein promotes cell death. BCL2/BAX index was confirmed as a significant independent prognostic factor for cell apoptosis (Gazzaniga et al., 1996). In the present study, we found in SH-SY5Y cell line, glyphosate (5 mM) and AMPA (10 mM) induced BCL2 down-expression (2.41-fold and 1.96-fold, respectively, Table 2) and BAX over-expression (3.03-fold and 2.19-fold, respectively, Table 2), being BCL2/BAX expression ratio of < 1. Thus expression level of BAX higher than expression level of BCL2 is consistent with our hypothesis that glyphosate and AMPA induced apoptosis in SH-SY5Y cells. This noting was previously supported in neuronal PC12 cells (Gui et al., 2012). Furthermore, transcriptional profiling indicated BCL2-related protein A1 (BCL2A1) overexpression (1.94-fold and 1.93-fold, respectively, Table 2); BCL2A1 overexpressed appears to be predominantly associated with advanced disease stages (Vogler, 2012). Exposure to glyphosate and AMPA also induced apoptotic cell death in SH-SY5Y cells by up-regulation of representative pro-apoptotic genes (Zimmermann et al., 2001) including APAF1 (2.77-fold and 4.95-fold, respectively), CASP3 (2.84-fold and 3.29-fold, respectively), CASP7 (3.77-fold and 2.36, respectively) and CASP9 (2.13-fold and 2.91, respectively) (Table 2). The maximal over-expression was exhibited after glyphosate or AMPA exposure in TP53 (3.86-fold and 2.42-fold, respectively), TNF (4.86-fold and 2.57-fold, respectively) and NFκB1 (4.16-fold and 2.66, respectively) genes (Table 2). TP53, a transcription factor that promotes apoptosis, is activated only when cells are stressed or damaged (Vogelstein et al., 2000). Up-regulation of TP53 gene expression observed in this study can be the cause of the down-regulation of AKT1 gene (3-fold and 2.37-fold, respectively, Table 2) and facilitate the apoptotic cell death in response to glyphosate and AMPA (Lee et al., 2011). TNF is a major mediator of apoptosis as well as inflammation and immunity, the up-regulation of TNF signaling observed in the SH-SY5Y cells exposed to glyphosate and AMPA (4.86-fold and 2.57-fold,

respectively, Table 2), directly may also promote autophagy and the up-regulation of NFκB1 gene expression (Dutta et al., 2006). The transcription factor NFκB is a critical regulator of many cellular processes including cell survival and inflammation. Nevertheless, depending on the cellular context, the duration and the intensity of NFκB activity, the redox-regulating function of NFκB can act either as an inhibitor or an activator of autophagy. Because autophagy is also induced in response to oxidative stress, interestingly, the ROS production induced by glyphosate and AMPA, in turn, can activate NFκB activity and promotes autophagy (Trocoli and Djavaheri-Mergny, 2011). Differentially altered expressions of 24 and 17 genes, respectively (fold change, see Table 2) related to apoptosis. We also demonstrated that glyphosate and AMPA caused up-regulation of other autophagy marker proteins (14 and 6 genes, respectively; fold change, see Table 2) including CTSB (2.10-fold and 1.92-fold, respectively) a key mediator in causing neuronal degeneration death in primary neurons (Gan et al., 2004), PIK3C3 (2.05-fold and 1.64-fold, respectively) protein widely accepted as a positive regulator of autophagy (Lindmo and Stenmark, 2006) and SNCA (2.81-fold and 2.09-fold, respectively) and SQSTM1 (2.81-fold, only by AMPA) both gene over-expressions related to neurodegenerative disorders (Dansithong et al., 2015; Homma et al., 2014). Finally, this study also demonstrated that mRNA levels of some key genes related to necrosis were significantly up-regulated by glyphosate and AMPA (12 and 4 genes, respectively; fold change, see Table 2). For this group of genes the major overexpression was observed for HSPBAP1 (1.72-fold and 3.23 fold, respectively) and KCNIP1 (1.67-fold and 2.17-fold, respectively); the overexpression of HSPBAP1 and KCNIP1 mRNAs might play roles in a neuroprotection under oxidative stress condition (Kirbach and Golenhofen, 2011) and in the GABAergic inhibitory system with participation in modulating inhibitory neuronal function (Xia et al., 2010).

In conclusion, these data from human neuroblastoma SH-SY5Y cells demonstrate that glyphosate and AMPA induce cytotoxicity and neurotoxicity via oxidative stress, neuronal development including neurite outgrowth, apoptotic, autophagy and necrotic signaling pathways. Our approach offers an efficient strategy for rapid identification of underlying molecular pathways leading to oxidative damage, neuronal cell death and neurodegeneration associated with glyphosate and AMPA exposure.

#### CRedit authorship contribution statement

**María-Aránzazu Martínez:** Conceptualization, Methodology, Investigation, Supervision. **José-Luis Rodríguez:** Investigation, Formal analysis. **Bernardo Lopez-Torres:** Investigation, Formal analysis, Resources. **Marta Martínez:** Investigation, Visualization, Writing - original draft, Writing - review & editing. **María-Rosa Martínez-Larrañaga:** Conceptualization, Methodology, Investigation. **Jorge-Enrique Maximiliano:** Investigation, Resources. **Arturo Anadón:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing. **Irma Ares:** Investigation, Validation, Supervision.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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