



Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: Involvement of glutamate excitotoxicity



Daiane Cattani, Vera Lúcia de Liz Oliveira Cavalli, Carla Elise Heinz Rieg, Juliana Tonietto Domingues, Tharine Dal-Cim, Carla Inês Tasca, Fátima Regina Mena Barreto Silva, Ariane Zamoner*

Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil

ARTICLE INFO

Article history:

Received 23 August 2013

Received in revised form 12 February 2014

Accepted 6 March 2014

Available online 15 March 2014

Keywords:

Glyphosate

Calcium

Glutamatergic excitotoxicity

Oxidative stress

Kinase pathways

ABSTRACT

Previous studies demonstrate that glyphosate exposure is associated with oxidative damage and neurotoxicity. Therefore, the mechanism of glyphosate-induced neurotoxic effects needs to be determined. The aim of this study was to investigate whether Roundup® (a glyphosate-based herbicide) leads to neurotoxicity in hippocampus of immature rats following acute (30 min) and chronic (pregnancy and lactation) pesticide exposure. Maternal exposure to pesticide was undertaken by treating dams orally with 1% Roundup® (0.38% glyphosate) during pregnancy and lactation (till 15-day-old). Hippocampal slices from 15 day old rats were acutely exposed to Roundup® (0.0005–0.1%) during 30 min and experiments were carried out to determine whether glyphosate affects $^{45}\text{Ca}^{2+}$ influx and cell viability. Moreover, we investigated the pesticide effects on oxidative stress parameters, ^{14}C - α -methyl-amino-isobutyric acid (^{14}C -MeAIB) accumulation, as well as glutamate uptake, release and metabolism. Results showed that acute exposure to Roundup® (30 min) increases $^{45}\text{Ca}^{2+}$ influx by activating NMDA receptors and voltage-dependent Ca^{2+} channels, leading to oxidative stress and neural cell death. The mechanisms underlying Roundup®-induced neurotoxicity also involve the activation of CaMKII and ERK. Moreover, acute exposure to Roundup® increased ^3H -glutamate released into the synaptic cleft, decreased GSH content and increased the lipoperoxidation, characterizing excitotoxicity and oxidative damage. We also observed that both acute and chronic exposure to Roundup® decreased ^3H -glutamate uptake and metabolism, while induced $^{45}\text{Ca}^{2+}$ uptake and ^{14}C -MeAIB accumulation in immature rat hippocampus. Taken together, these results demonstrated that Roundup® might lead to excessive extracellular glutamate levels and consequently to glutamate excitotoxicity and oxidative stress in rat hippocampus.

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1. Introduction

The annual consumption of pesticides in Brazil has increased alarmingly in recent years. The justification for the use of these substances is based on the improvement of agricultural productivity, which places the country as one of the world's largest producers of food. However, the risks to the environment and health are neglected. According to the Brazilian National Trade Union for the

Agricultural Defense Products (Sindicato Nacional da Indústria de Produtos para Defesa Agrícola – SINDAG), in 2008 Brazil became the largest consumer of pesticides in the world. The increase in pesticide consumption leads to high rates of intoxication among farmers. Moreover, it has been suggested important associations between the bulk sale of pesticides and the increased rates of several types of cancer, endocrine disorders and a high prevalence of neurodegenerative diseases in agricultural workers (Londres, 2011), reinforcing the need for mechanistic investigations.

Glyphosate-based herbicides lead the pesticide world market. Moreover, glyphosate is the primary active ingredient present in Roundup® (Monsanto Company, St. Louis, MO). It has been suggested that the toxicity of Roundup® is probably due to synergistic effects between glyphosate and other formulation products (Marc et al., 2002; El-Shenawy, 2009), such as the surfactant

* Corresponding author at: Departamento de Bioquímica, Centro de Ciências Biológicas, UFSC, Campus Universitário, Bairro Trindade, CEP: 88040-970, Florianópolis, Santa Catarina, Brazil. Tel.: +55 48 3721 4747; fax: +55 48 3721 9672.

E-mail addresses: ariane.zamoner@ufsc.br, arianezps@gmail.com, arianezamoner@yahoo.com.br (A. Zamoner).

polyethoxylated tallowamine (POEA) which might facilitate glyphosate penetration through plasma membranes potentiating its toxicity (Williams et al., 2000; Tsui and Chu, 2003; Richard et al., 2005; Benachour and Séralini, 2009).

The planting of transgenic soybeans resistant to glyphosate-Roundup® (Glyphosate resistant soybeans, GRS) has greatly enhanced the consumption of this herbicide in Brazilian crops. In this context, it was recently reported a teratogenic action of herbicides based on glyphosate to vertebrates (Paganelli et al., 2010), warning of the need to evaluate the use of the product in agriculture. Moreover, Benachour and Séralini (2009) recently demonstrated that glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. This herbicide was also associated with induction of oxidative stress and neuroinflammation (El-Shenawy, 2009; Astiz et al., 2012). Moreover, glyphosate was able to provoke oxidative stress in specific brain regions: substantia nigra, cerebral cortex and hippocampus. However, most of these studies were carried out by using the commercial formulations, and not pure glyphosate, suggesting that further studies are necessary to examine whether the pesticide formulation may lead to neurotoxicity.

It has been suggested that pesticide exposure could be a risk factor for neurodegenerative disorders (Le Couteur et al., 1999; Kirby et al., 2001; Barlow et al., 2005; Patel et al., 2006). In this context, 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 (Wang et al., 2006; Peng et al., 2007; Hayden et al., 2010); however, it is unclear as to which pesticides and what mechanisms of action may contribute to the neurodegenerative condition. In this context, it has been reported that acute and chronic exposure to glyphosate might cause parkinsonism, a condition similar to Parkinson's disease (Barbosa et al., 2001; Wang et al., 2011). Barbosa and colleagues (2001) reported a case of a 54-year-old man who accidentally sprayed himself with the chemical agent glyphosate and one month later he developed parkinsonism. One year later, the patient presented a slow resting tremor in the left hand and arm, accompanied by impairment of short-term memory. Further, Wang et al. (2011) reported a case of parkinsonism following chronic exposure to glyphosate in a previously healthy 44-year-old woman who worked for 3 years in a chemical factory, exclusively in the glyphosate production division. The glyphosate neurotoxicity was associated with rigidity, slowness and resting tremor in all four limbs with no impairment of short-term memory.

It has been recently demonstrated that glyphosate induced both apoptotic and autophagic cell death in neuronal differentiated PC12 cells, providing a link between glyphosate and Parkinson's disease (Gui et al., 2012). Moreover, Chorfa et al. (2013) shown that glyphosate causes a significant cytotoxicity to SH-SY5Y neuronal cell line. The cytotoxicity was estimated by MTT assay and the half-maximal (50%) inhibitory concentration (IC50) for glyphosate was 9 µM. In addition, Negga and colleagues (2011) demonstrated that *Caenorhabditis elegans* were susceptible to glyphosate concentrations within environmentally relevant ranges. Further, the same group demonstrated that exposure to glyphosate-containing pesticide leads to degeneration of γ -aminobutyric acid and dopamine neurons in *C. elegans* (Negga et al., 2012a,b). Taken together, these studies suggest that glyphosate might affect neural cells leading to oxidative damage, neuronal cell death and neurodegenerative conditions.

The neurodegenerative conditions are frequently associated with glutamatergic excitotoxicity and oxidative stress (Pessoa-Pureur and Wajner, 2007). The central nervous system presents high vulnerability to free radical damage due to its elevated oxidative metabolic rate and enriched content of unsaturated lipids, as well as to its elevated rate of free radical generation derived from

neurotransmitters metabolism, and poor radical scavenging mechanisms (Chong et al., 2005).

Taking into account the previous studies demonstrating that exposure to glyphosate might be associated with neurotoxicity and oxidative damage, the aim of this study was to determine whether Roundup® leads to neurotoxicity in hippocampus of immature rats following acute (30 min) and chronic (pregnancy and lactation) pesticide exposure. Then, we investigated the involvement of Ca²⁺, intracellular signaling pathways and oxidative damage on the mechanisms underlying Roundup®-induced neurotoxicity in rat hippocampus. Moreover, this study investigated whether acute or maternal exposure to a glyphosate-based herbicide alters glutamatergic system by interfering in the neurotransmitter uptake, release and/or metabolism within the hippocampal cells.

2. Material and methods

2.1. Radiochemical and compounds

L-[2,3-³H] glutamic acid (³H glutamate) (specific activity 49 Ci/mmol) was purchased from Amersham (Oakville, Ontario, Canada). ⁴⁵CaCl₂ (specific activity of 321 kBq/mg of Ca²⁺) and Optiphase Hisafe III biodegradable liquid scintillation were purchased from PerkinElmer (Waltham, MA). α -[¹⁴C] methylaminoisobutyric acid ([¹⁴C] MeAIB) (sp.act. 1.85 GBq/mmol) was purchased from Du Pont, NEN Products, MA, USA. The herbicide Roundup Original® (Homologation number 00898793) containing glyphosate 360 g/L is a commercial formulation registered in the Brazilian Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura, Pecuária e Abastecimento – MAPA). Nifedipine, N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H89), (Bisindolylmaleimidine IX, 2-{1-[3-(Amidinothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl)maleimide ethanesulfonate salt) Ro 31-8220, D(-)-2-amino-5-phosphonopentanoic acid (AP5), KN-93, (+)- α -methyl-4-carboxyphenylglycine (MCPG) and flunarizine were purchased from Sigma Chemical Company (St. Louis, MO, USA). The G6PD assay kit was kindly provided by INTERCIEN-TÍFICA (São José dos Campos, SP, Brazil). All other chemicals were of analytical grade.

2.2. Animals

Wistar rats were bred in animal house and maintained in an air-conditioned room (about 21 °C) with controlled lighting (12 h/12 h light/dark cycle). On the day of birth the litter size was culled to eight pups. Litters smaller than eight pups were not included. The suckling rats were kept with their mothers until euthanasia. Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All animals' procedures were carried out in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP00471).

2.3. In vivo exposure to Roundup®

Wistar rats were mated and the day of appearance of the vaginal plug was considered day 0 of fetal age. Maternal exposure to Roundup® was induced by adding 1% Roundup® (corresponding to 0.38% glyphosate) in the drinking water from gestation day 5 and continually up to lactation day 15. Control animals, receiving only water during the same period, were used as controls. The concentration chosen for the *in vivo* exposure was based on no observed adverse effect level (NOEL) for maternal toxicity which was 1000 mg/kg body weight/day dosage (Williams et al., 2000). The concentration we used was equivalent to 1/14 of the limits of NOEL. Each experiment was carried out by treating 4 different

dams during pregnancy and lactation, and the pups from their litters were used to investigate the neurotoxic effects of Roundup® on hippocampal cells. Therefore, each experimental group contained hippocampal slices from pups of different litters (different dams). All experiments were performed in triplicate. The body weight over pregnancy and water consumption by dams were measured daily. Also pup body weights were accompanied over the 15 days. Fifteen-day old rats were used in our experimental condition considering that in rats the period of maximum synaptogenesis occurs between postnatal day 11 and postnatal day 20, a period that is associated with increased synaptic plasticity in order to establish proper synaptic connections (Sutor and Luhmann, 1995).

2.4. Preparation of hippocampus slices

Rats were killed by decapitation and the hippocampus was dissected onto Petri dishes placed on ice and the parietal region was cut into 300 µm thick slices with a McIlwain chopper.

2.5. ⁴⁵Ca²⁺ uptake

Hippocampal slices from 15-day-old male rats were preincubated in Krebs Ringer-bicarbonate (KRb) buffer (122 mM NaCl; 3 mM KCl; 1.2 mM MgSO₄; 1.3 mM CaCl₂; 0.4 mM KH₂PO₄; 25 mM NaHCO₃) for 15 min in a Dubnoff metabolic incubator at 37 °C, pH 7.4 and gassed with O₂:CO₂ (95:5; v/v). After that, the medium was changed by fresh KRb with 0.1 µCi/mL ⁴⁵Ca²⁺ and incubated during 30 min in the absence (control group) or presence (treated group) of Roundup® at concentrations ranging from 0.00005 to 0.1%. In some experiments, L-type voltage-dependent channel blocker (10 µM nifedipine), antagonist of NMDA receptors (10 µM DL-AP5) or kinase inhibitors (10 µM H89, 20 µM Ro 31-8220, 10 µM KN93) were added during the preincubation and incubation periods (see figures). Extracellular ⁴⁵Ca²⁺ was thoroughly washed off in a washing solution containing 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO₄, 10 mM HEPES, 11 mM glucose, 10 mM LaCl₃, pH 7.3 (30 min in washing solution). The presence of La³⁺ during the washing stage was found to be essential to prevent release of the intracellular ⁴⁵Ca²⁺ (Zamoner et al., 2007). After washing, tissue slices were digested and homogenized with 0.5 M NaOH solution, 100 µL aliquots were placed in scintillation fluid and counted in a LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman Coulter, Boston, USA), and 5 µL aliquots were used for protein quantification as described by Lowry and colleagues (1951).

2.6. Measurement of lactate dehydrogenase (LDH) released

Hippocampal slices from 15-day-old male rats were preincubated in KRb in a Dubnoff metabolic incubator at 37 °C, pH 7.4 and gassed with O₂:CO₂ (95:5; v/v). After that, the medium was changed by fresh KRb and the slices were incubated during 30 min in the absence (control group) or presence (treated group) of Roundup® at concentrations ranging from 0.00005 to 0.1%. After incubation of the hippocampal slices in the absence or presence of Roundup®, the incubation medium was collected for determination of extracellular LDH activity by a spectrophotometric method. The estimation of LDH activity was carried out by measuring the oxidation of NADH and the results were expressed as control.

2.7. Glutamate uptake assay

For glutamate uptake the incubation medium was replaced by Hank's balanced salt solution (HBSS) containing 137 mM NaCl; 0.63 mM Na₂HPO₄; 4.17 mM NaHCO₃; 5.36 mM KCl; 0.44 mM

KH₂PO₄; 1.26 mM CaCl₂; 0.41 mM MgSO₄; 0.49 mM MgCl₂ and 5.55 mM glucose, pH 7.4. The hippocampal slices were exposed or not to 0.01% Roundup® during 23 minutes. Then, glutamate uptake assay was started by the addition of 0.1 mM L-glutamate and 0.66 µCi/mL L-[2,3-³H] glutamate. Incubation was stopped after 7 min by removal of the medium and rinsing the slices twice with ice-cold HBSS. Slices were then lysed in a solution containing 0.5 M NaOH (Cattani et al., 2013). Sodium-independent uptake was determined using N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the specific uptake. Radioactivity was measured with a scintillation counter.

2.8. Glutamate release assay

After the preincubation period to slice recovery (30 min), hippocampal slices were incubated in Hank's balanced salt solution (HBSS; composition in mM 1.29 CaCl₂, 136.9 NaCl, 5.36 KCl, 0.65 MgSO₄, 0.27 Na₂HPO₄, 1.1 KH₂PO₄, 2 glucose, and 5 HEPES). When present, Roundup® was incubated for 30 min. Glutamate (1 mM) was incubated for 15 min, and glutamate uptake was assessed by adding 0.33 µCi/mL D-[³H]aspartate with 100 µM unlabeled aspartate during the last 7 min and stopped by three ice-cold washes with 1 mL HBSS. D-[³H]-aspartate instead of L-[³H]glutamate was used in order to avoid glutamate metabolism in intracellular compartments, although similar results were obtained by using D-[³H]aspartate or L-[³H]glutamate. The slices were then further incubated for 15 min in HBSS, and the supernatant was collected to measure the amount of released D-[³H]aspartate. Slices were disrupted by overnight incubation with 0.1% NaOH/0.01% SDS, and aliquots of lysates were taken for determination of intracellular D-[³H]aspartate content. Intracellular and extracellular D-[³H]-aspartate content were determined through scintillation counting, calculated as nmol aspartate, and the amount of released aspartate was expressed as percentage of total D-[³H]aspartate.

2.9. Glutamine synthetase activity

Hippocampal slices were preincubated in HBSS at 37 °C and then incubated during 15 min in the absence or presence of 0.01% Roundup® during 30 minutes. Briefly, homogenate (0.1 mL) was added to 0.1 mL of reaction mixture containing 10 mM MgCl₂; 50 mM L-glutamate; 100 mM imidazole-HCl buffer (pH 7.4); 10 mM 2-mercaptoethanol; 50 mM hydroxylamine-HCl; 10 mM ATP and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 0.4 mL of a solution containing: 370 mM ferric chloride; 670 mM HCl; 200 mM trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate treated with ferric chloride reagent.

2.10. Neutral amino acid accumulation

For amino acid accumulation experiments, hippocampal slices were pre-incubated in KRb buffer for 30 min in a Dubnoff metabolic incubator at 37 °C, pH 7.4 and gassed with O₂:CO₂ (95:5; v/v). The slices were then incubated in fresh KRb buffer for 60 min. [¹⁴C] MeAIB (3.7 kBq/mL) was added to each sample during the incubation period (Silva et al., 2001; Cattani et al., 2013). The pesticide (0.01% Roundup®) was added in the last 30 min of incubation period. After incubation the slices were lysed in 0.5 M NaOH and the protein concentration was determined (Lowry et al., 1951). Then, 25 µL aliquots of tissue and external medium were placed in scintillation fluid and counted in a Beckman beta liquid scintillation spectrometer (model LS 6500; Fullerton, CA, USA) for radioactivity

measurements. The results were expressed as the tissue/medium (T/M) ratio: cpm/mL tissue fluid per cpm/mL incubation medium.

2.11. Reduced glutathione assay

The reduced glutathione (GSH) was determined using the reagent DTNB (5,5'-dithiobis 2-nitrobenzoic acid). After being centrifuged at $5000 \times g$ for 5 min, the supernatants from the acid extracts (TCA 12%, 1:10, w/v) were added to 2.5 mM DTNB in 0.2 M sodium phosphate buffer pH 8.0, and the formation of the thiolate anion was immediately measured at 412 nm. Determinations were expressed in $\mu\text{mol g}^{-1}$.

2.12. Quantification of lipoperoxidation levels

The endogenous lipid peroxidation was evaluated in the hippocampus by detection of substances that react with thiobarbituric acid (TBARS), particularly malondialdehyde (MDA), according to the method described by Bird and Draper (1984). Briefly,

homogenate was precipitated with trichloroacetic acid (12% TCA) followed by the incubation with buffer 60 mM Tris-HCl pH 7.4 (0.1 mM DPTA) and 0.73% TBA, at 100°C , for 60 min. After cooling, the samples were then centrifuged (5 min at $10,000 \times g$) and the absorbance of the chromophore was measured at 535 nm. The values were expressed in nmol MDA g^{-1} .

2.13. Enzymatic activity of AST, ALT, GGT and G6PD

Slices of hippocampus were homogenized in cold 0.1 M Tris buffer, pH 8.5 (10% homogenate, w/v) to determine GGT activity or in 0.2 M Tris buffer, pH 7.4 to quantify acetylcholinesterase and G6PD activities. Sample aliquots were saved for total protein determinations (Lowry et al., 1951).

2.13.1. AST/ALT assay

The enzymatic activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was quantified in aliquots of the tissue homogenate by using the colorimetric method described

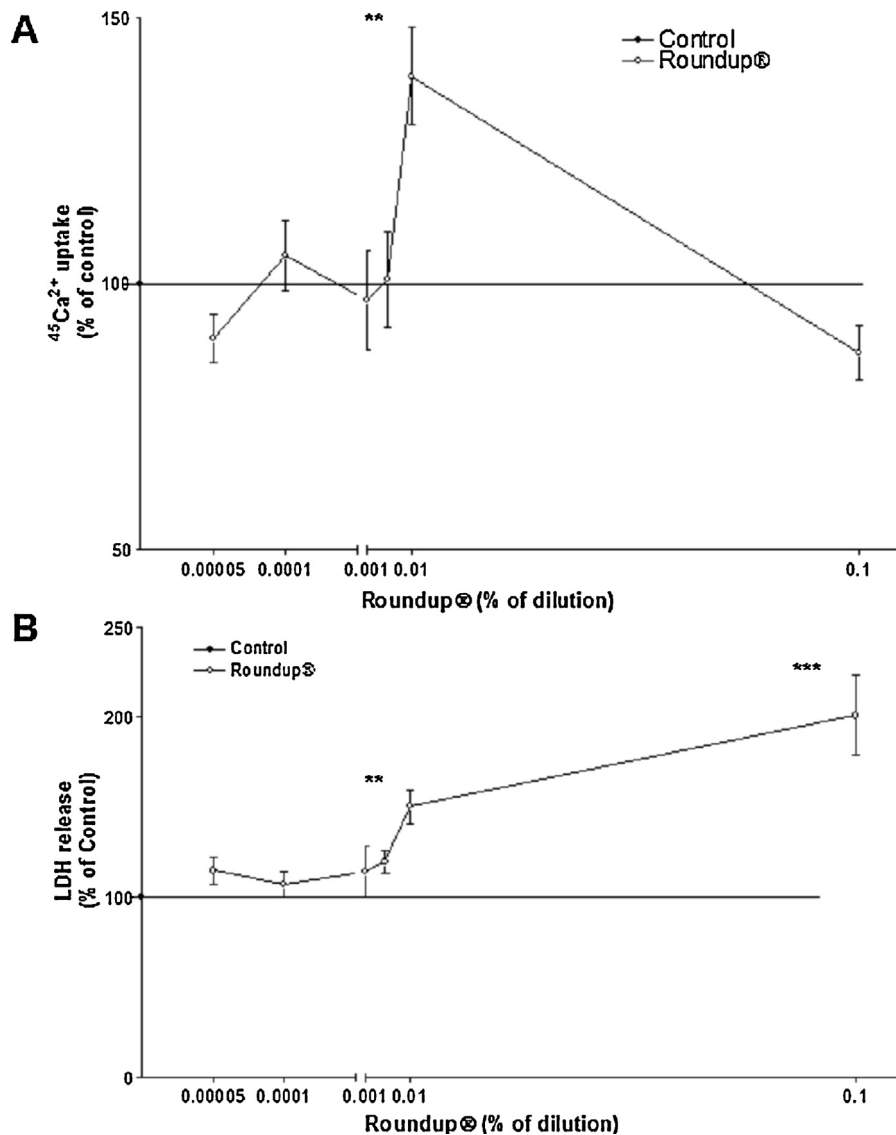


Fig. 1. Dose-response curve of Roundup® on $^{45}\text{Ca}^{2+}$ uptake (A) and on LDH release (B) on hippocampus from immature rats. Hippocampal slices from 15 day-old rats were pre-incubated for 15 min and then incubated for 30 min with 0.1 mCi/mL of $^{45}\text{Ca}^{2+}$ in the presence or absence of Roundup® at different concentrations (0.00005–0.1%). Slices from the each animal were used for control and treated groups. The radioactivity incorporated into control hippocampal slices from each animal was standardized as 100%. The control variability was 5.02% among animals. Values are means \pm S.E.M. of 8 animals. Statistically significant differences from controls, as determined by one-way ANOVA followed by Bonferroni multiple comparison test are indicated: ** $p < 0.01$, *** $p < 0.001$ compared with control group.

by [Reitman and Frankel \(1957\)](#), with modifications. Results for enzyme activity were obtained by using calibration curves based on non-chromogenic absorbance. The absorbance of the samples was determined in a plate reader (Tecan Infinite® 200 PRO) at 505 nm. The results were expressed as U/L/ μ g protein.

2.13.2. GGT assay

Gamma-glutamyl transferase (GGT) activity was measured with the use of the modified technique described previously by [Orlowski and Meister \(1963\)](#) using L- γ -glutamyl *p*-nitroanilide as substrate and glycylglycine as the acceptor molecule.

Aliquots of the tissue homogenate were incubated with the enzymatic substrate. The reaction was allowed to proceed for 60 min at 37 °C and the enzymatic reaction was stopped by addition of acetic acid. The absorbance of the samples was determined in a plate reader (Tecan Infinite® 200 PRO) at 530 nm. The results were expressed as IU/L/ μ g protein.

2.13.3. G6PD assay

For measuring the glucose-6-phosphate dehydrogenase (G6PD) activity, hippocampus slices were incubated in the presence of NADP⁺ leading to the oxidation of glucose-6-phosphate to 6-phosphoglutamate. The NADPH produced was measured in a kinetic mode during 10 min. The results were calculated by assessing the increase of the optical density per minute (slope) of the sample against the “slope” of standard G6PD enzyme activity.

2.14. Statistical analysis

The results are means \pm S.E.M. Fifteen-day-old animals from different litter were treated as a single statistical replicate. When multiple comparisons were performed, evaluation was done using

one-way ANOVA followed by Bonferroni's multiple comparison test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Ca²⁺ uptake participates in Roundup®-induced neurotoxicity to rat hippocampus

Hippocampal slices from immature rats were exposed for 30 min to different concentrations of glyphosate-Roundup® ranging from 0.00005 to 0.1% and ⁴⁵Ca²⁺ uptake as well as cell viability were investigated. [Fig. 1A](#) shows that 0.01% Roundup® leads to ⁴⁵Ca²⁺ uptake and induces cell death, as demonstrated by the increased LDH release from hippocampal cells ([Fig. 1B](#)). Moreover, 0.1% Roundup®, the concentration currently used in agricultural activities, causes the highest LDH release without affect ⁴⁵Ca²⁺ uptake, suggesting that necrotic cell death could be directly related to Roundup®-induced neurotoxicity. The subsequent experiments were carried out with 0.01% Roundup®, a concentration able to induce ⁴⁵Ca²⁺ uptake and decrease cell viability. Similar results were observed in male reproductive tissue exposed to Roundup® ([de Liz Oliveira Cavalli et al., 2013](#)). Therefore, in the present study, we used some pharmacological approaches in order to investigate the mechanisms involved in Roundup®-induced neurotoxicity, emphasizing its action on glutamatergic system.

3.2. Signal transduction pathways involved in the Roundup®-induced ⁴⁵Ca²⁺ uptake

[Fig. 2A](#) showed that either AP5 (a NMDA receptor antagonist) or KN-93 (a Ca²⁺/calmodulin-dependent protein kinase II selective inhibitor) prevented Roundup®-induced ⁴⁵Ca²⁺ influx. Moreover,

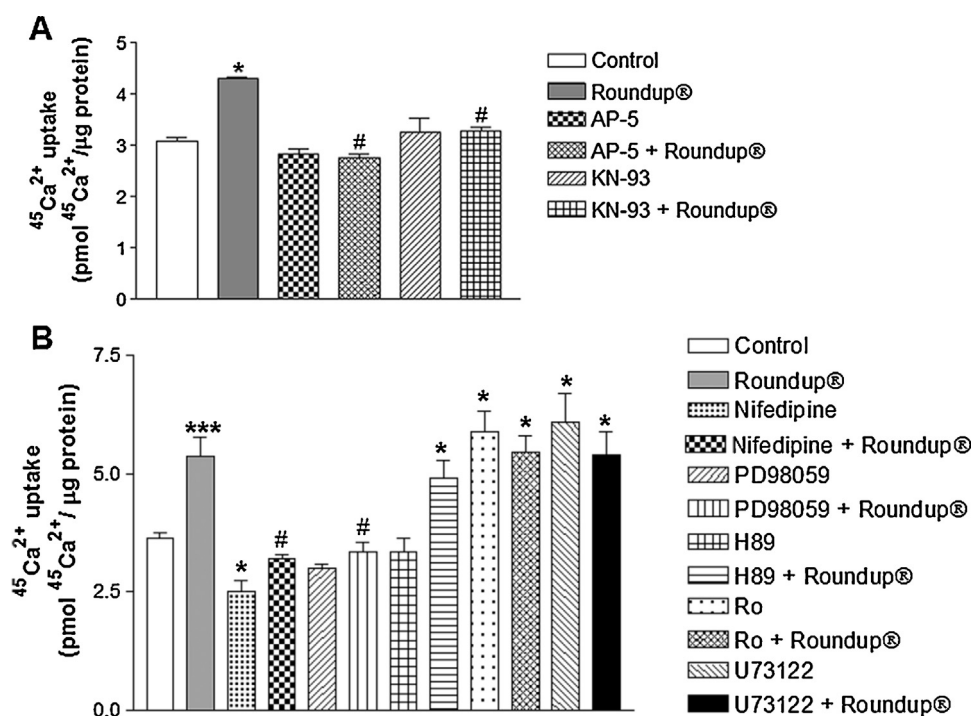


Fig. 2. Signaling mechanisms underlying Roundup®-induced ⁴⁵Ca²⁺ influx. Hippocampal slices were pre-incubated for 15 min with or without 10 μ M AP-5 (a competitive NMDA receptor antagonist), or 10 μ M KN93 (CaMKII inhibitor) (A), or 10 μ M nifedipine (L-VDC blocker), or 10 μ M H89 (PKA inhibitor), or 10 μ M U73122 (PLC inhibitor), or 20 μ M RO 31-8220 (PKC inhibitor), or 10 μ M PD 98059 (MAPK inhibitor) (B). After that, the slices were incubated in the presence of the drugs described above with or without 0.01% Roundup® for 30 min (incubation) in the presence of 0.1 mCi/mL of ⁴⁵Ca²⁺. Values are means \pm S.E.M. of 8 animals. Statistically significant differences from controls, as determined by one-way ANOVA followed by Bonferroni multiple comparison test are indicated: * $p < 0.05$, *** $p < 0.001$ compared with control group. # $p < 0.01$ compared with Roundup® group.

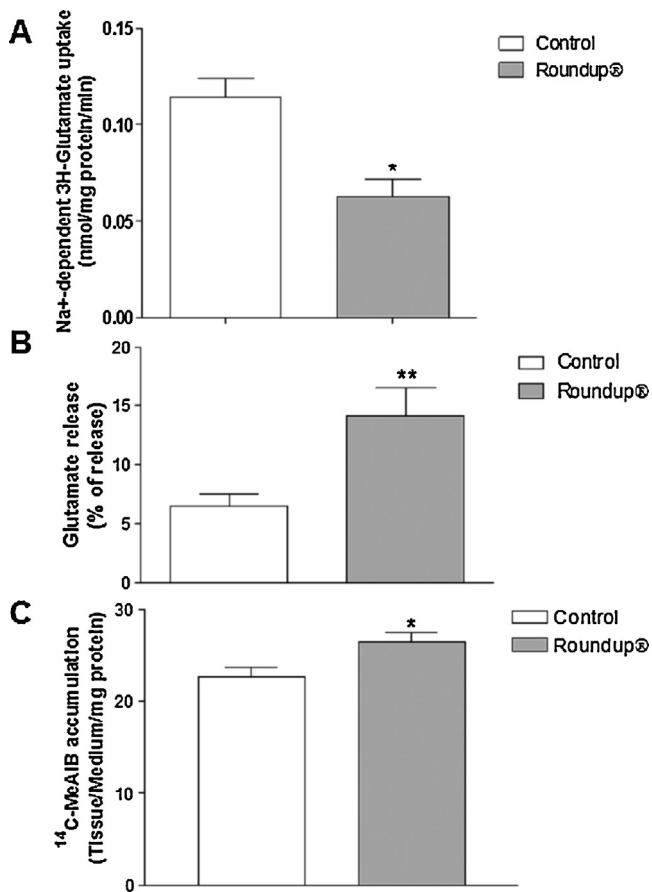


Fig. 3. Effect of Roundup[®] exposure on [³H]-glutamate uptake and release (A and B, respectively) and on neutral amino acid accumulation (C) in hippocampal slices from 15 day-old rats. Data are reported as means ± S.E.M. of 8 animals in each group. Statistically significant differences from controls, as determined by Student's *t*-test were indicated: ***P* < 0.001; **P* < 0.01.

the use of nifedipine and PD98059 allowed us to set the implication of L-VDCs and MAPKs, respectively, in the mechanism of Roundup[®] which is leading to ⁴⁵Ca²⁺ influx (Fig. 2B). However, even in the presence of H89, a cell permeable inhibitor of PKA, Roundup[®] was able to increase ⁴⁵Ca²⁺ uptake in hippocampal slices. On the other hand, U73122 and Ro-31-8220 (PLC and PKC inhibitors, respectively) had an effect *per se* stimulating calcium uptake. In addition, the effect of Roundup[®] triggering ⁴⁵Ca²⁺ influx was unaltered by its coinubation with U73122 or Ro-31-8220. These results do not permit a final conclusion on the involvement of PLC/PKC cascade on Roundup[®]-induced ⁴⁵Ca²⁺ influx.

3.3. Effect of Roundup[®] on glutamate metabolism and on neutral amino acid accumulation in hippocampus of immature rats

Fig. 3A showed that the 30 min exposure to Roundup[®] evokes an increase in glutamate release in hippocampus of immature rats. In accordance with these results, the herbicide also decreased the Na⁺-dependent glial [³H]-glutamate uptake (Fig. 3B), important to remove the bulk of the neurotransmitter from the synaptic cleft and to prevent neurotoxic damage. Once internalized in glial cells, glutamate might be metabolized to glutamine-by-glutamine synthetase. Then, glutamine might be released to the synaptic space and taken up by neurons. System A transporter is the major pathway involved in glutamine uptake inside neurons (Kanamori and Ross, 2004; Jenstad et al., 2009) controlling glutamine/glutamate equilibrium within astrocytes and neurons. Taking into account these findings, the increased [¹⁴C]-MeAIB accumulation in rat

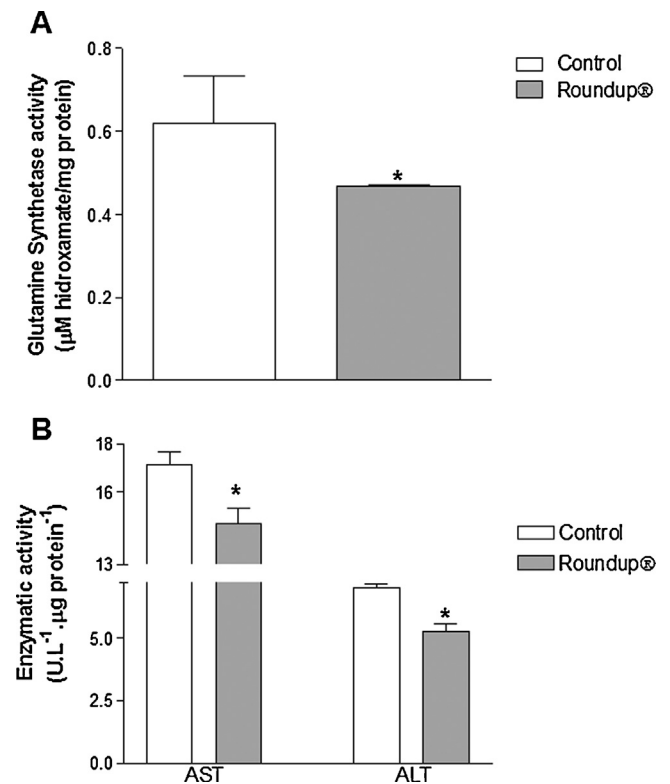


Fig. 4. Effect of Roundup[®] on enzymatic activity of glutamine synthetase (A), aspartate aminotransferase (AST) and aspartate aminotransferase (ALT) (B). Hippocampal slices were preincubated for 15 min and then incubated in the presence or absence of 0.01% Roundup[®] for 30 min. Data are reported as means ± S.E.M. of 6 animals from each group. Statistically significant differences from controls, as determined by Student's *t* test, are indicated. **P* < 0.01.

hippocampus (Fig. 3C) suggested higher glutamate uptake by neurons, a prerequisite for the formation of glutamate inside these cells.

In addition, the present study was conducted to determine the effect of Roundup[®] on the activities of the enzymes glutamine synthetase, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are involved in glutamate metabolism. Results showed that the activities of all of them were inhibited by Roundup[®] in rat hippocampus (Fig. 4A and B).

3.4. In vivo exposure to Roundup[®] during pregnancy and lactation might cause excitotoxic damage

Chronic exposure to Roundup[®] during pregnancy and lactation did affect neither dam's body weight nor daily water consumption over pregnancy (data not shown). Based on female body weight and on daily water consumption we estimated that each dam ingested 70 mg/Kg body weight/day. This dose was equivalent to 1/2.5 of the limits of NOAEL. Fig. 5A showed that maternal exposure to Roundup[®] (70 mg/Kg body weight) decreased the body weight gain of pups from 10- to 15-day old. Moreover, chronic maternal exposure to Roundup[®] leads to similar excitotoxic events observed during short-term *in vitro* treatments in hippocampal cells. Results showed decreased the Na⁺-dependent glial [³H]-glutamate uptake (Fig. 5B) and glutamine synthetase inhibition (Fig. 5C), suggesting excess of glutamate in synaptic cleft associated with decreased neurotransmitter metabolism. Corroborating the glutamatergic excitotoxicity, the chronic exposure to Roundup[®] leads to increased ⁴⁵Ca²⁺ uptake in hippocampal slices of immature pups (Fig. 5D). Also, we observed increased glutamine uptake inside

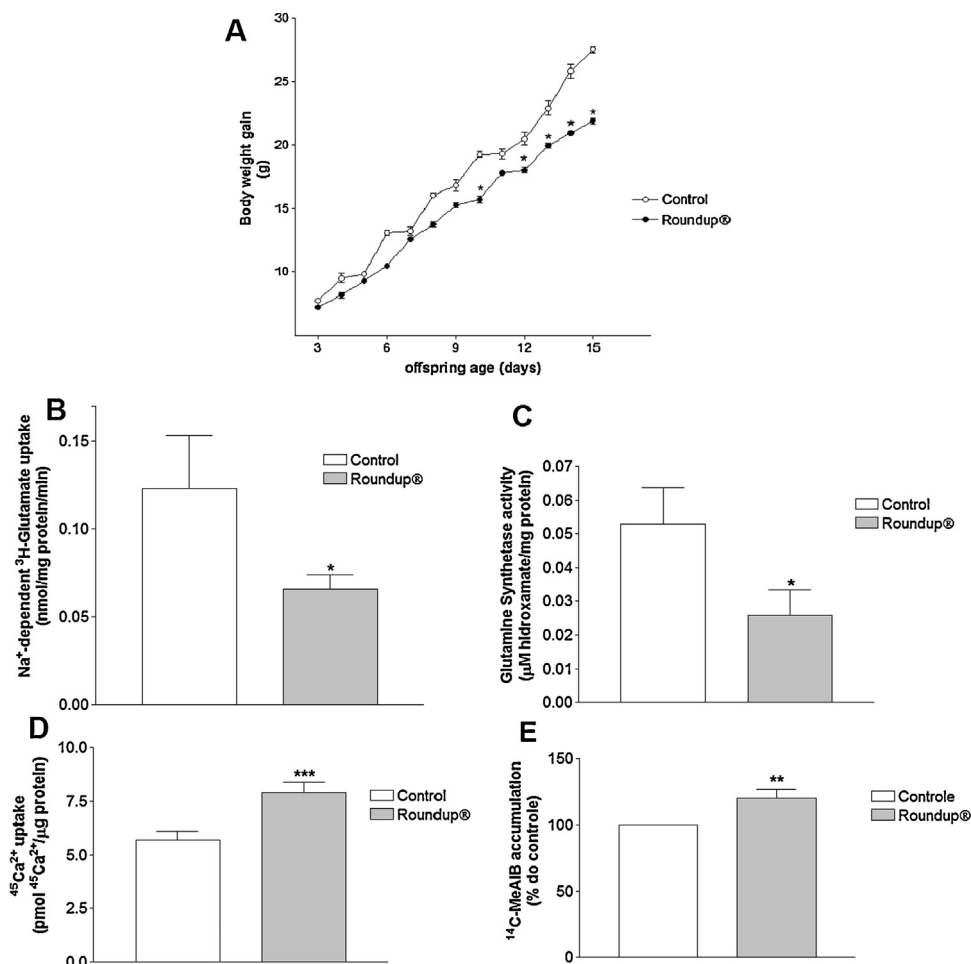


Fig. 5. Effect of maternal exposure to glyphosate-Roundup® during pregnancy and lactation on offspring body weight gain (A), [³H]-glutamate uptake (B), on glutamine synthetase activity (C), on ⁴⁵Ca²⁺ uptake (D) and on neutral amino acid accumulation (E) in hippocampal slices from 15 day-old rats. Pregnant female Wistar rats were exposed to 1% Roundup® (corresponding to 0.36% glyphosate) in the drinking water from gestation day 5 and continually up to lactation day 15. Pups are exposed to the pesticide that cross the placental barrier during gestational period and/or that is given by the mother through breast milk during suckling period. Data are reported as means ± S.E.M. of 8 animals in each group. Statistically significant differences from controls, as determined by Student's *t*-test were indicated: ***P* < 0.001; ****P* < 0.01.

neurons, as demonstrated by increased [¹⁴C]-MeAIB accumulation (Fig. 5E), reinforcing alterations in glutamate/glutamine cycle.

3.5. Roundup® exposure might lead to oxidative stress

Lipid peroxidation was assessed by the formation of thiobarbituric acid-reactive substances (TBARS). Results showed that Roundup® exposure increased TBARS levels (Fig. 6A) and decreased GSH content in rat hippocampus (Fig. 6B), suggesting the participation of oxidative damage in the mechanism of pesticide-induced neurotoxicity. In addition, the enzymatic activities of GGT and G6PD (involved in glutathione metabolism) were inhibited in the hippocampus exposed to Roundup® (Fig. 7A and B).

3.6. Signaling mechanisms involved in the gamma-glutamyl transferase inhibition caused by Roundup®

Fig. 8A showed that NMDA/glycine (NMDA receptor agonist) inhibited GGT activity, without any additional inhibitory effect when co-incubated with Roundup®. On the other hand, the effect of Roundup® inhibiting GGT activity was prevented by AP-5 a competitive NMDA receptor antagonist. Moreover, MK-801, a selective non-competitive NMDA receptor antagonist that acts at the NMDA receptor-operated ion channel as an open channel blocker also prevented the inhibition of GGT activity provoked by Roundup®.

Activation of glutamatergic system by Roundup® was also attested by using MCPG, a non-selective metabotropic glutamate receptor antagonist, which also prevented the effect of herbicide (Fig. 8B). The use of AP5, MK801 and MCPG clearly demonstrate the involvement of glutamatergic receptors in the mechanisms underlying Roundup®-induced neurotoxicity. In order to attest that similar mechanisms are involved in Roundup®-induced calcium uptake and GGT inhibition, PD98059 (MEK inhibitor), U73122 (PLC inhibitor) and KN93 (CaMKII inhibitor) were also used. Results showed that CaMKII inhibition is unable to affect the Roundup® effect on GGT activity. Interestingly, ERK and PLC pathways are demonstrated to be involved in GGT inhibition provoked by Roundup® (Fig. 8C). Despite the activity of PLC was not involved in the effect of Roundup® on calcium influx, this enzyme participates in the mechanism of action of the herbicide leading to GGT inhibition. The mechanisms involved in these events need to be clarified.

4. Discussion

The present study demonstrated that glyphosate-Roundup® affects glutamate uptake, release and metabolism within neural cells leading to Ca²⁺ influx through NMDA receptors and L-VGCC in hippocampus from immature rats. These events implicate the involvement of glutamatergic excitotoxicity and oxidative

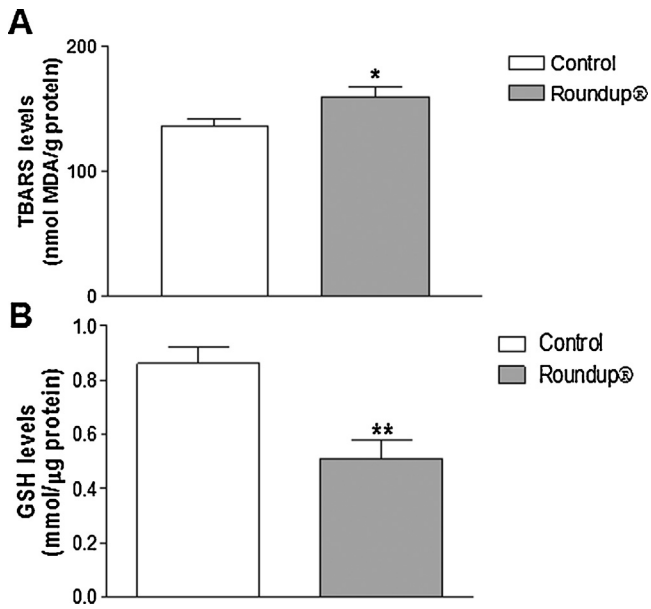


Fig. 6. Effect of Roundup® on lipid peroxidation and GSH levels in immature rat hippocampus. After preincubation, hippocampal slices were incubated in the presence or absence of 0.01% Roundup® for 30 min. Data from thiobarbituric acid-reactive substances (TBARS) measurement of lipid peroxidation and GSH levels are reported as means \pm S.E.M. of 8 animals from each group. Statistically significant differences from controls, as determined by Student's *t* test, are indicated. * $P < 0.05$, ** $P < 0.01$.

damage in the mechanism of Roundup®-induced neurotoxicity during development. Exposure to environmental toxicants during pregnancy and suckling periods has the potential to affect embryo and fetal development (Brent, 2004). In our experimental model, we induce a maternal exposure to the glyphosate-Roundup® and consequently, the offspring are exposed to the pesticide that cross the placental barrier during gestational period and/or that is given by the mother through breast milk during suckling period.

The first demonstration that the herbicide glyphosate may affect Ca^{2+} homeostasis was demonstrated by Olorunsogo (1990), which

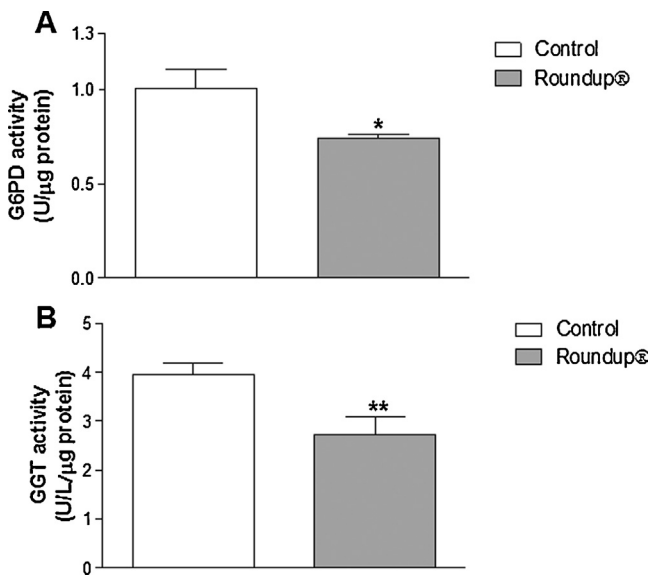


Fig. 7. Effect of Roundup® on the activities of glucose-6-phosphate dehydrogenase (G6PD), and gamma-glutamyl transferase (GGT) in immature rat hippocampus. Hippocampal slices were incubated for 30 min in the presence or absence of 0.01% Roundup®. Data are reported as means \pm S.E.M. of 8 animals from each group. Statistically significant differences from controls, as determined by Student's *t* test, are indicated. * $P < 0.01$, ** $P < 0.001$.

showed increased mitochondrial membrane permeability to protons and Ca^{2+} . In our study we demonstrated that 0.01% Roundup® (corresponding to 0.036 g/L glyphosate) induced $^{45}Ca^{2+}$ uptake and decreased cell viability in hippocampal slices from immature rats. It is important to emphasize that the concentration of Roundup® used in agricultural working activities ranges from 1% to 2%, concentrations 10,000–20,000 times larger than those used in our experimental protocols. In this context, we have recently demonstrated that the same concentrations used in this study were able to increase calcium uptake and decrease Sertoli cell viability, clearly demonstrating that Roundup® might affect male reproductive cells (de Liz Oliveira Cavalli et al., 2013). The present study demonstrates that both *in vitro* (acute) and *in vivo* (chronic) exposure to Roundup® induced $^{45}Ca^{2+}$ uptake in hippocampal cells. Interestingly, our results demonstrated that modifications in $^{45}Ca^{2+}$ influx were related with cell death at 0.01% Roundup®. However, the higher Roundup® concentration used (0.1% Roundup®, corresponding to 0.36 g/L glyphosate) leads to increased cell death while decreases $^{45}Ca^{2+}$ influx, suggesting that high pesticide doses might induce necrotic cell death. Thus, we propose that the decreased cell viability induced by the pesticide might compromise the plasma membrane activity and permeability, and therefore affects calcium uptake into hippocampal cells.

The Roundup®-induced Ca^{2+} influx occurs through NMDA receptor activation and L-VDCC opening. The Roundup®-induced excitotoxicity in rat hippocampus also involves the recruitment of signal transduction pathways leading to the activation of kinase cascades including ERK. Activation of the ERK pathway might account for neuroprotection under excitotoxic conditions in hippocampal neurons (Almeida et al., 2005). Roundup®-induced ERK activation probably involves a compensatory effect to the excitotoxic damage caused by the pesticide. In this context, Gomes et al. (2012) demonstrated a BDNF-induced activation of ERK under excitotoxic condition. The authors suggest that upon excitotoxic stimulation the kinase activity probably requires multiple components that may be lost and/or redistributed within the cell. Moreover, previous results from our group demonstrated that Roundup® disrupts male reproductive functions by triggering calcium-mediated cell death in male reproductive cells (de Liz Oliveira Cavalli et al., 2013).

Astrocytes play a key role in removing glutamate from the synaptic cleft and metabolizing it to glutamine. Then, glutamine is released from these cells and then taken up by neurons, to serve as a glutamate precursor (Danbolt, 2001). On the other hand, Torres et al. (2013) have recently demonstrated that once in astrocytes, glutamate is preferentially used as a fuel in the tricarboxylic acid (TCA) cycle instead of being converted into glutamine. They suggested that oxidation of glutamate could buffer excitotoxic conditions due to high glutamate concentrations. Our results demonstrated that either *in vivo* or *in vitro* exposure to Roundup® reduced glutamate uptake and metabolism within glial cells, associated with increased release of this neurotransmitter in the synaptic cleft. Moreover, Roundup® leads to glutamine synthetase, AST and ALT enzymatic activity inhibition, attesting that either glutamate uptake or its metabolism is impaired in hippocampal astrocytes exposed to the herbicide. Taken together these results demonstrated that Roundup® might lead to excessive extracellular glutamate levels and consequently to excitotoxic condition in rat hippocampus.

The glial-neuronal metabolism is controlled by several metabolic pathways. Both in astrocytes and in neurons glucose is converted through glycolysis in pyruvate, which could be converted to lactate by lactate dehydrogenase, alanine by ALT, oxaloacetate by pyruvate carboxylase or acetyl CoA by pyruvate dehydrogenase. The acetyl CoA enters the TCA cycle and the TCA cycle intermediates can exit the cycle at the α -ketoglutarate step and then

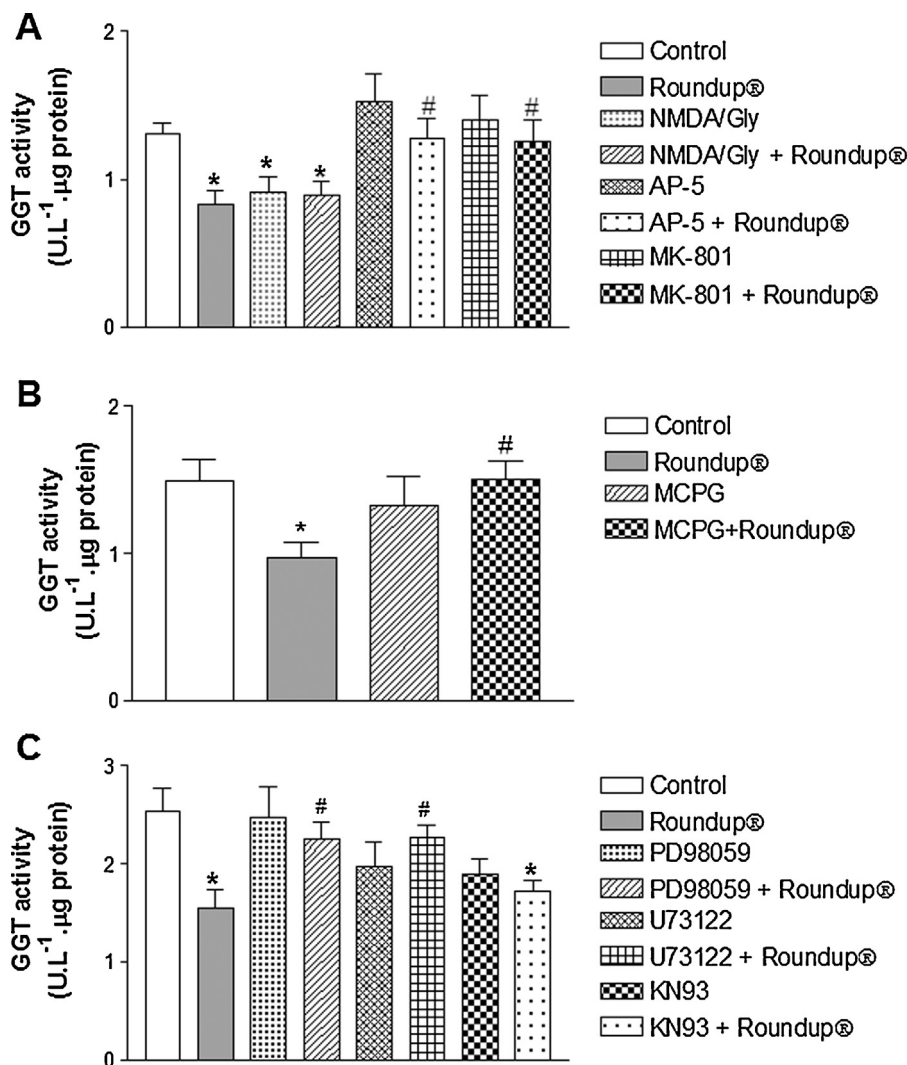


Fig. 8. Involvement of glutamatergic system and kinase pathways on the mechanism of GGT inhibition by Roundup® in immature rat hippocampus. Hippocampal slices were pre-incubated for 15 min with or without NMDA/gly (NMDA glutamate receptor agonist), or 10 μM AP-5 (a competitive NMDA receptor antagonist), or 10 μM MK-801 (a selective non-competitive NMDA receptor antagonist), or MCPG (a metabotropic glutamate receptor antagonist), 10 μM KN93 (CaMKII inhibitor), or 10 μM PD 98059 (MAPK inhibitor) or 10 μM U73122 (PLC inhibitor). After that, the slices were incubated with or without 0.01% Roundup® for 30 min (incubation) in the presence or absence of the drugs described above. Values are means ± S.E.M. of 8 animals. Statistically significant differences from controls, as determined by one-way ANOVA followed by Bonferroni multiple comparison test are indicated: **p* < 0.01 compared with control group. #*p* < 0.01 compared with Roundup® or glyphosate group.

form glutamate by transaminases such as AST and ALT (Alvestad et al., 2011). Moreover, the glutamate released into the synaptic cleft is taken up into astrocytes at which it can be converted to glutamine or enter the TCA cycle. Then, glutamine can be transported back to the neurons and regenerate glutamate. Considering that Roundup® affects the activity of important enzymes involved in neural cell metabolism, our results suggested that the herbicide causes energetic deficit in addition to excitotoxic damage in hippocampus from immature rats. The ineffective glutamate transport into astrocytes supports the decreased glutamate oxidation under Roundup®-induced neurotoxic condition. On the other hand, the glutamine transport into neurons was increased both after *in vitro* and chronic Roundup® exposure, as demonstrated by the high ¹⁴C-MeAIB accumulation observed in herbicide-treated hippocampus. Corroborating these findings, Morken et al. (2013) have recently demonstrated that the transfer of glutamate from neurons to astrocytes was much lower in neonatal rat brain than in adult one, while transfer of glutamine from astrocytes to glutamatergic neurons was relatively higher. Corroborating the present

results, we have recently demonstrated that congenital hypothyroidism also leads to stimulation in system A transporter which is associated with decreased glutamate uptake and increased Ca²⁺ influx in rat hippocampus (Cattani et al., 2013). These interconnected events compromise glutamate-glutamine cycle accounting for oxidative stress in hypothyroid hippocampus (Cattani et al., 2013) and cerebral cortex (Zamoner et al., 2008) accounting for glutamate excitotoxicity.

Supporting our findings Pérez-De La Cruz et al. (2008) demonstrated that excitotoxicity and energy deficit caused by the co-administration of quinolinate and 3-nitropropionate lead to excessive cytoplasmic calcium levels which mediate oxidative damage in brain synaptosomal membranes. In this context, excessive calcium influx might potentiate lethal metabolic pathways, which in turn involves increased formation of reactive oxygen species and mitochondrial dysfunction (Rami et al., 1997). Moreover, increased calcium influx might augment the release of excitatory amino acids (such as glutamate), and hence propagate excitotoxic cell damage through a positive feedback, further

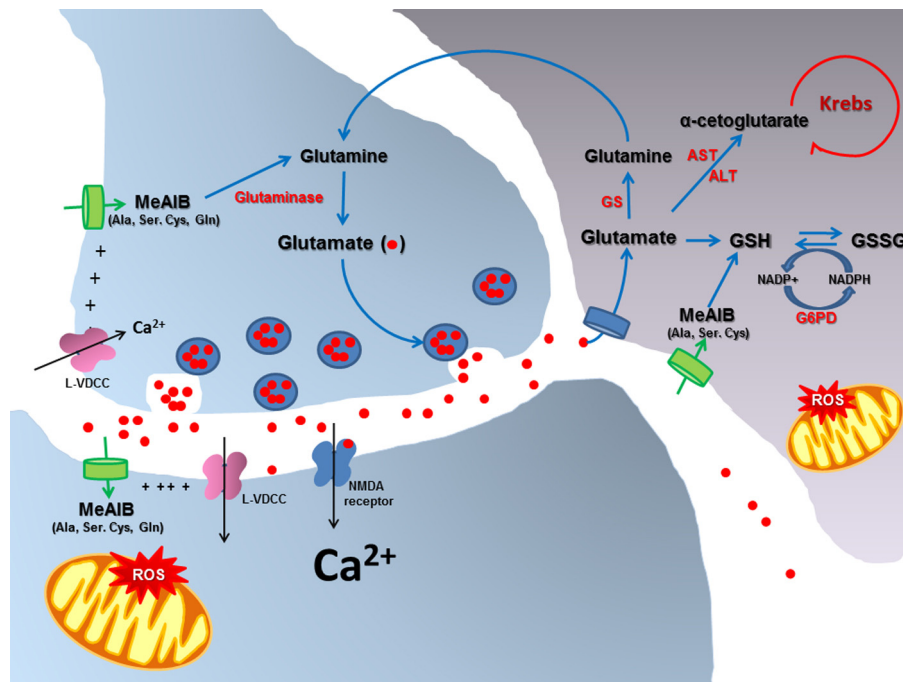


Fig. 9. Proposed mechanism underlying hippocampal neurotoxicity of Roundup®. The pesticide causes Ca^{2+} influx by activating NMDA receptors and L-type voltage-dependent Ca^{2+} channels (L-VDCC) setting off oxidative stress and cell death. The mechanisms underlying Roundup® neurotoxicity involve the activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and extracellular signal-regulated kinase (ERK). Astrocytes play a key role in removing glutamate from the synaptic cleft and metabolizing it to glutamine, which serve as a glutamate precursor in neurons. Our results demonstrated that Roundup® reduced glutamate uptake and metabolism within glial cells, associated with increased release of this neurotransmitter in the synaptic cleft. Moreover, Roundup® leads to glutamine synthetase, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymatic activity inhibition, attesting that either glutamate uptake or its metabolism is impaired in hippocampal astrocytes exposed to the herbicide. Moreover, Roundup® reduced GSH levels and increased the amounts of thiobarbituric acid reactive species (TBARS), characterizing oxidative damage. Also, exposure to the pesticide decreased the activity of gamma-glutamyl transferase (GGT) and glucose-6-phosphate dehydrogenase, supporting the depletion of GSH. In this context, the GGT inhibition induced by Roundup® could decrease the amino acid availability to GSH *de novo* synthesis and the decreased activity of G6PD may reduce NADPH levels, necessary to reduce glutathione. Taken together these results demonstrated that Roundup® might lead to excessive extracellular glutamate levels and consequently to excitotoxic condition and energetic deficit in rat hippocampus.

leading cells to death (Pérez-De La Cruz et al., 2008). Taking into account these findings, we could ascribe that oxidative damage, demonstrated by increased lipid peroxidation and GSH depletion, is probably dependent on the high intracellular calcium concentrations induced by Roundup® in hippocampal slices.

The oxidative damage induced by Roundup® in rat hippocampus is also confirmed by the inhibition of the enzymatic activity of either GGT or G6PD. It was described that in neonatal brain more glucose is prioritized to pentose phosphate pathway and pyruvate carboxylation than in adult one. These events may have implications for the capacity to protect the neonatal brain against excitotoxicity and oxidative stress (Morken et al., 2013). The rate-limiting enzyme of the pentose phosphate pathway, G6PD, has been implicated not only to promote reduced glutathione (GSH) but also enhance oxidative stress in specific cellular conditions. In this context, Zhao et al. (2012) have demonstrated that G6PD plays a role in either oxidative neuronal damage or neuroprotection during ischemic reperfusion period in CA1 pyramidal neurons. The G6PD enzymatic inhibition might decrease the NADPH availability to reduce the glutathione. The GSH depletion, an important marker of oxidative stress, in Roundup®-exposed hippocampus and could be a consequence of G6PD inhibition. Therefore, Roundup® exposure can decrease the protective action of G6PD against oxidative damage in immature rat brain.

GGT is involved in extracellular breakdown of GSH, providing precursor amino acids for GSH *de novo* synthesis (Lee et al., 2004). Therefore, the GGT inhibition in Roundup®-exposed hippocampus might cause a decrease in the glutamate reservoir to GSH synthesis leading to a decrease in the levels of this important antioxidant in brain. The present study investigated the mechanisms involved

in the inhibition of this enzyme by Roundup® in rat hippocampus. Moreover, results showed that NMDA receptors and ERK activation participate in the mechanisms underlying the inhibition of GGT activity in this brain structure. Consistent with our findings, Dang et al. (2011) demonstrated that after 30 min of treatment with acrolein it was observed GSH depletion and ERK activation in cultured astrocytes. Although CAMKII activation participates in Roundup®-induced Ca^{2+} influx, this kinase is not involved in GGT modulation. Moreover, the activation of CaMKII and ERK1/2 contributes to the potentiation of Ca^{2+} response elicited by capsaicin in rat dorsal root ganglion neurons. Results indicate that the potentiation is a Ca^{2+} -modulated process that is mediated by intracellular signaling pathways involving activation of CaMKII and ERK1/2, but not by activation of PKC or PKA (Zhang et al., 2011). Moreover, interactions between Ca^{2+} , CAMKII e ERK1/2 signaling pathways were previously demonstrated in neural cells (Ji and Woolf, 2001; Choe and Wang, 2002; Zamoner et al., 2008).

5. Conclusions

The proposed mechanism underlying Roundup®-induced neurotoxicity is summarized in Fig. 9. In conclusion, our results showed that the herbicide Roundup® leads to glutamatergic excitotoxicity and energy deficit in hippocampal cells from immature rats. The mechanisms underlying Roundup® neurotoxicity involve activation of kinase cascades as well as misregulation of glutamatergic synapses, Ca^{2+} influx through NMDA and L-VDCC, energetic deficits and oxidative damage in rat hippocampus. Thus, we propose that Roundup® induced glutamatergic excitotoxicity which culminate in neural cell death.

Funding

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil)/Edital Universal 14/2011/research grant #479483/2011-6; Fundação de Apoio à Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC) research grant “chamada pública FAPESC 04/2011 – Apoio a infra-estrutura de CT&I para jovens pesquisadores” #11,338/2012-7; PGFAR; PPGBQA; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil) and CAPES-REUNI.

Conflict of interest

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Transparency document

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Acknowledgements

We acknowledge FAPESC, CNPq-Brazil, CAPES, CAPES-REUNI, PGFAR and PPGBQA for grants and research scholarships. We also thank INTERCIENTÍFICA (São José dos Campos, SP, Brazil) for the generous gift of G6PD kits. Daiane Cattani was registered on Pharmacy Postgraduate Program/UFSC-Brazil (PGFAR), Vera Lúcia de Liz Oliveira Cavalli was registered on Biochemistry Postgraduate Program/UFSCBrazil (PPGBQA).

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