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Neuronal development and axon growth are altered by glyphosate through a WNT non-canonical signaling pathway

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ABSTRACT

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. Glyphosate-containing herbicides are the most used agrochemicals in the world, particularly on genetically modified plants. Previous studies have demonstrated that glyphosate induces neurotoxicity in mammals. Therefore, its action mechanism on the nervous system needs to be determined. In this study, we report about impaired neuronal development caused by glyphosate exposure. Particularly, we observed that the initial axonal differentiation and growth of cultured neurons is affected by glyphosate since most treated cells remained undifferentiated after 1 day in culture. Although they polarized at 2 days *in vitro*, they elicited shorter and unbranched axons and they also developed less complex dendritic arbors compared to controls. To go further, we attempted to identify the cellular mechanism by which glyphosate affected neuronal morphology. Biochemical approaches revealed that glyphosate led to a decrease in Wnt5a level, a key factor for the initial neurite development and maturation, as well as inducing a down-regulation of CaMKII activity. This data suggests that the morphological defects would likely be a consequence of the decrease in both Wnt5a expression and CaMKII activity induced by glyphosate. Additionally, these changes might be reflected in a subsequent neuronal dysfunction. Therefore, our findings highlight the importance of establishing rigorous control on the use of glyphosate-based herbicides in order to protect mammals' health.

1. Introduction

Neurons are the most highly polarized cells, and their polarity is essential for informational processing and to ensure unidirectional signal flow. The formation of neural circuits depends on the morphological complexity of neurons and the establishment a proper neural connectivity. Neuronal differentiation and maturation are essential events which are acutely controlled by a combination of intrinsic pathways and a wide spectrum of extrinsic factors, such as neurothophins, semaphorins, sonic hedgehog (Shh), bone morphogenetic proteins (BMP) and Wnt proteins(Bovolenta, 2005; Salinas, 2005; Torroja et al., 2005). These extracellular signals bind to specific membrane receptors triggering signal cascades that likely affect neurite cytoskeleton and also modulate gene expression. Wnts are secreted signaling proteins that play important roles in neuronal development and plasticity (Rosso and Salinas, 2007; Chen et al., 2006; Salinas and Zou, 2008; Inestrosa and Arenas, 2010; Rosso and Inestrosa, 2013). The interaction of these proteins with receptors such as members of the Frizzled (Fz) family of seven-pass transmembrane proteins and/or molecules with tyrosine-kinase activity (Ryk; Ror2 and IGF-1R) triggers the activation of the Wnt signaling pathways (Oishi et al., 2003; Gordon and Nusse, 2006; Keeble and Cooper, 2006; Wang et al., 2006; Schulte, 2010; Ho et al., 2012; Bernis et al., 2013). In mammals, 19 Wnt members have been found and 10 members of the Fz family have been identified. Downstream Wnt receptors, three Wnt signaling pathways may be activated: the Wnt/β-catenin or canonical pathway that involves gene transcription; and the β-catenin-independent or non-canonical pathways inducing either an increase in intracellular calcium concentration and activation of Ca-sensitive kinases as PKC (protein kinase C) and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), or activation of the c-Jun-N-terminal kinase (JNK) cascade (Veeman et al., 2003; Gordon and Nusse, 2006; Angers and Moon, 2009).

Different parts of the CNS develop at different stages (Bayer et al., 1993; Rodier, 1994) and processes such as neurogenesis, cell

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proliferation, migration, differentiation, synaptogenesis, gliogenesis, myelination and apoptosis occur in a temporally- and regionally-dependent manner. Perturbations of these processes during development can result in long-term irreversible consequences that affect the structure and function of the nervous system. Developmental nervous system seems to be highly vulnerable to toxic insults caused by different environmental pollutants, *e.g.*, pesticides, solvents, inorganic heavy metals, polychlorinated biphenyls, among others. Many of those compounds are known to exert significant impacts on human health in a dose-dependent fashion. In this context, there is a large body of evidence showing the relationship between nervous system disorders and the exposure to those kinds of pollutants (Rosso et al., 2000; Zatta et al., 2003; Halliwell, 2006; Jomova et al., 2010; Parron et al., 2011; Bayrami et al., 2012; Giordano and Costa, 2012; Gascon et al., 2013).

Mammals and humans may be exposed to herbicide residues by agricultural practices (Acquavella et al., 2004) or when they enter the food chain (Takahashi et al., 2001; Glozier et al., 2012). Glyphosate-based herbicides contain acid glyphosate (*N*-(phosphonometyl) glycine) and different adjuvants, being the polyethoxylated tallowamine or POEA the predominant one (Acquavella et al., 1999; Williams et al., 2000), which has shown toxicity causing ocular disturbs, nausea, and diarrhea (Tsui and Chu, 2003). More evidence about the toxicity of glyphosate and its formulations has been reported in different animal species such as amphibians (Relyea, 2005) and other aquatic organisms (Marc et al., 2004; Bringolf et al., 2007; Langiano Vdo and Martinez, 2008). Furthermore, studies *in vitro* have shown that mammalian cells exposed to glyphosate disrupted hormone release (Benachour et al., 2007), increased cell death (Benachour and Seralini, 2009) and altered gene expression (Hokanson et al., 2007). Also, it has been reported teratogenic effects of glyphosate formulations in amphibians as evidenced by cranial deformities and mouth and eye abnormalities (Lajmanovich et al., 2003). Similar observations were shown in chicken embryos exposed to low concentrations of glyphosate-based herbicide. These effects were characterised by marked alterations in cephalic crest markers, reduction of optic vesicles and microcephaly caused by changes in retinoic acid (RA) and Sonic hedgehog (Shh) intracellular pathways (Paganelli et al., 2010).

In this work, we examined the effect of glyphosate exposure on the morphology and development of hippocampal cultured neurons.We observed that undifferentiated pyramidal neurons exposed to glyphosate showed a delay on development characterized by a decrease in axonal differentiation and neurite elongation and they also exhibited changes in the growth cones morphology. To go further, we attempted to elucidate the cellular mechanism by glyphosate affect neuronal morphology and development. Since Wnt signaling pathway play crucial roles on neuronal processes such as neuronal migration, axon differentiation, axon guidance, dendrite development, and synapses (Ciani and Salinas, 2005; Rosso et al., 2005; Inestrosa and Arenas, 2010; Rosso and Inestrosa, 2013) we evaluated the contribution of Wnt cascades on glyphosate effect. In this context, we observed that glyphosate treated neurons elicited a significant decrease in the Wnt5a expression, a key factor involved in axon outgrowth and guidance (Bovolenta et al., 2006; Salinas and Zou, 2008). The defects on neuronal morphology caused by glyphosate were reverted when recombinant Wnt5a was added to the medium. Furthermore, we identified the downstream effector of Wnt5a leading to the glyphosate neurotoxicity and found that glyphosate induced a significant decrease on CaMKII activity, a non-canonical Wnt pathway effector. We concluded that glyphosate exposure affects neuronal development and the ability of neurons to initiate their differentiation through changes in the Wnt pathway particularly through a decrease in CAMKII activity.

2. Materials and methods

2.1. Animals use and care

Wistar rats were group housed and maintained in air-conditioned room (about 22–25 °C) under a 12 h light–dark cycle (lights on at 07:00 am) with free access to food and water. All animal procedures were performed following approved protocols of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Approval to conduct the study was granted by the Animal Care and Ethics Committee of the School of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina.

2.2. Neuronal culture and transfection

Primary cultures of hippocampal pyramidal cells were prepared as describe previously (Rosso et al., 2004). Briefly, hippocampi from Wistar rats at embryonic day 18 were dissected aseptically in Hank's balanced salt solution (HBSS). Cells were dissociated in HBSS contain 0.25% (wt/vol) trypsin (Gibco) at 37 °C, followed by mechanical dissociation with glass Pasteur pipettes and plated onto poly-L-lysine (1 mg/ml, Sigma–Aldrich) coated coverslips at low density (7.5×10^4 cells/cm^2) or high density (1×10^5 cells/cm^2) in order to be used for immunoflurescence or biochemical determinations and cells transfections, respectively. To allow neurons attachment, they were incubated at 37 °C in a humidified incubator with 5% CO_2 during 2 h in DMEM (Gibco) containing 10% horse serum (Gibco). Afterwards DMEM was discarded and replaced with Neurobasal medium (Gibco) supplemented with 1% N2 and 2% B27 (Gibco). Cells were maintained according to previously described conditions. When indicated, cultures were treated with 4 mg/ml *N*-phosphonomethyl glycine (glyphosate, powder purity 95%, Sigma–Aldrich) 2 h after plating. Glyphosate was diluted in milliQ water and adjusted at pH 7.4 with NaOH. To evaluate the reversibility of the glyphosate effect, cultured hippocampal neurons were exposed to the herbicide for 24 h, after and then washed with PBS buffer (pH 7.4) and cultured in fresh medium for 1 more day. Neurons were fixed at 2 DIV and then analyzed by immunofluorescence and microscopy.

For stimulation assays, 100 ng/ml recombinant protein Wnt5a (R&D System) was added to the culture medium 6 h after plating. To analyze actin organization, 1 μM cytochalasin-D was added to 12 h neurons and cultures were fixed 12 h later. Neuronal transfections were performed 12 h after plating using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The transfection efficiency was between 35 and 50%, depending on the culture densities. cDNAs encoding shRNAs were inserted in a dicistronic vector pSuper.neo + GFP (pSuper RNAi System-OligoEngine). The target DNA sequence was ACTGTATCCAGCAGATCCT. A scrambled DNA target sequence (GCACTTCATGCCACTAGAT) was created using the siRNA WizardTM, InvivoGen. The resulting plasmids were referred to as CaMKII shRNA and scrambled sequence RNA (ssRNA). In same experiments, Wnt5a was added to the transfected cells 6 h post-transfection and fixed 18 h later (1.5 DIV, days *in vitro*).

2.3. MTT assay

This enzymatic test, based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma–Aldrich) into a blue-coloured product (formazan) by the mitochondrial enzyme succinate dehydrogenase was used to evaluate neurons cell viability. For these experiments neurons were cultured in 24-well plates at density of 9.5×10^4 cells/well using DMEM (supplemented as previously described). After 2 h, DMEM was replaced into Neurobasal medium (supplemented as previously described) and glyphosate was added at different concentrations (2, 4 or 6 mg/ml).

After appropriated time of treatment, medium was discarded and 250 μl of MTT solution (Cf = 0.5 mg/ml) was added and incubated for 4 h at 37 °C. Finally, MTT solution was discarded and 250 μl of dimethylsulfoxide (DMSO, Sigma–Aldrich) was added and the optical density was taken at 570 and 720 nm using a spectrophotometer (Jenway 7310, Bibby Scientific).

2.4. Immunofluorescence, image acquisition and analyses

Cell cultures were fixed at different times (mentioned in each experiment) for 20 min with 4% (wt/vol) paraformaldehyde (Merck) in phosphate-buffered saline (PBS) containing 4% (wt/vol) sucrose, and processed as previously described (Rosso et al.,

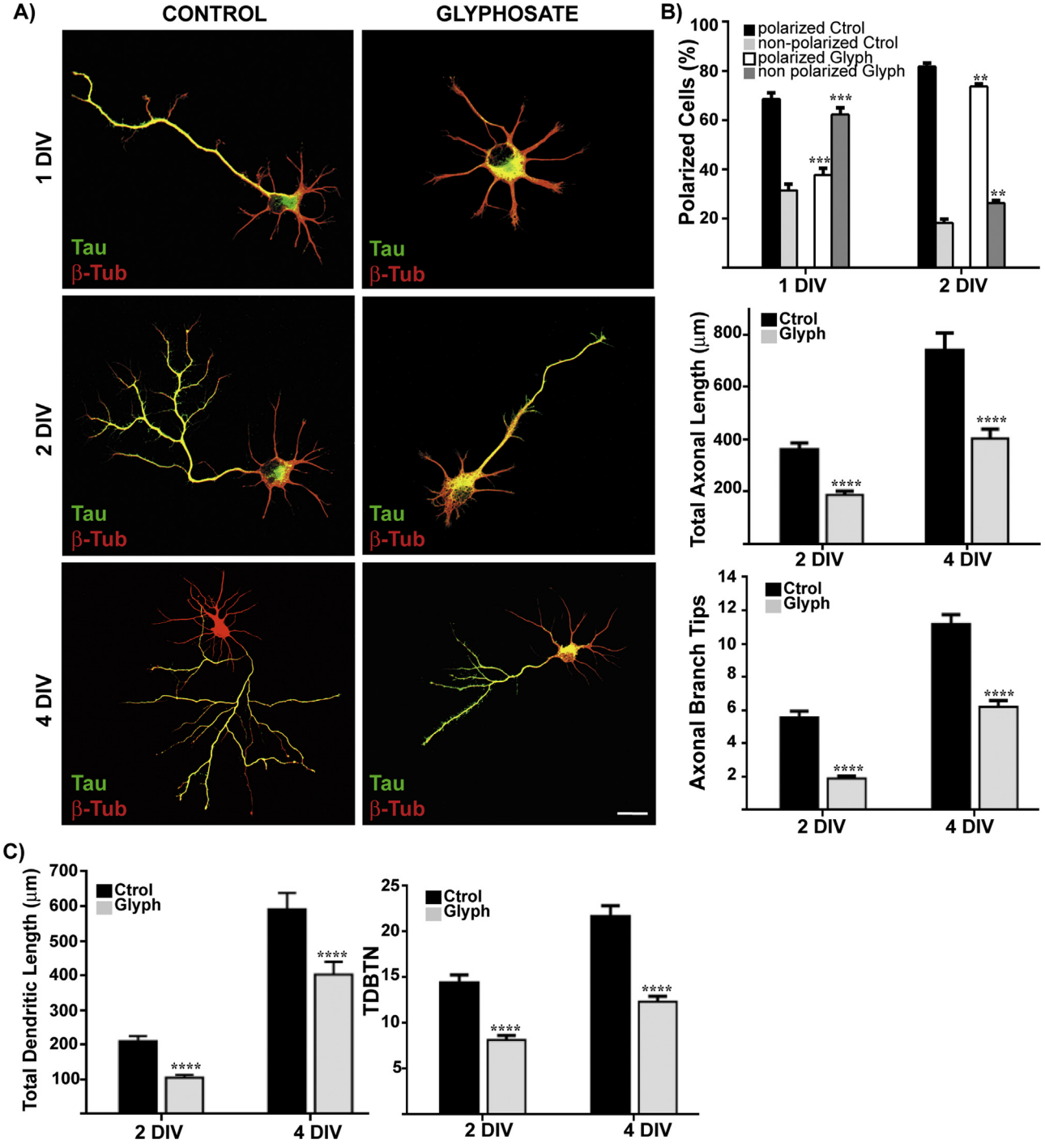

Fig. 1. Glyphosate treatment affected neuronal development and morphology.
(A) Representative confocal images showing control and glyphosate treated hippocampal pyramidal neurons. Cells were fixed at 1, 2 or 4 days *in vitro* and immunolabeled with the axonal marker Tau1 (green) and β-tubulin III (red). Glyphosate (final concentration 4 mg/ml) or vehicle was added to the culture after 2 h of plating. Scale bar 10 μm. (B) Percentage of neuronal polarization in 1 or 2 DIV control and treated cultures (top panel). For these experiments 150 cells were counting per condition from 3 independents experiments. Quantification shows the total axonal length (middle panel) and axonal branch tips (bottom panel) in 2 and 4 DIV in control or glyphosate (Glyph) treated neurons. In these experiments 60 cells were scored per condition from 3 independents experiments. (C) Graphics showing the quantifications of total dendritic length (left panel) and total dendritic branching tip number (TDBTN) (right panel) in 2 and 4 DIV control and glyphosate treated neurons (Glyph). In these experiments 60 cells were scored per condition from 3 independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ compared to controls. Errors bars represent mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2004). The primary antibodies used were: polyclonal rabbit anti-β-tubulin III (Sigma–Aldrich) diluted 1:5000, monoclonal antibody (mAb) mouse against tau protein (clone Tau-1, Chemicon International) diluted 1:600, mAb mouse anti-tyrosinated-α-tubulin (Tyr Tub) (clone TUB1A2, Sigma–Aldrich) diluted 1:5000, mAb mouse against Green fluorescent protein (GFP) (clones 7.1 and 13.1, Roche, Life Science) diluted 1:1500. The following secondary antibodies were used: Alexa Fluor 488 anti-mouse, Alexa Fluor 568 anti-mouse, Alexa Fluor 488 anti-rabbit, Alexa Fluor 568 anti-rabbit (Molecular Probes, Life Technologies) diluted 1:600 and for some experiments, rhodamine-labeled phalloidin (Molecular Probes) was included with the secondary antibody to visualize filamentous actin (F-actin), diluted 1:300. Coverslips were mounted with FluorSave Reagent (Merk Millipore) and cells were observed using a Nikon Eclipse TE-2000-E2 confocal microscope. Images were captured and digitized using EZ-C1 3.90 FreeViewer. After that, images were analyzed using *ImageJ* software, specifically, *NeuronJ* plugin was used to trace the neurites structure and measure their length, in μm. Measurements were performed by at least one researcher blind to the experiment conditions.

2.5. Gel electrophoresis and Western blot analysis

Protein extracts were prepared from rat hippocampal pyramidal neuron cultures. Cells were harvested with lysis buffer RIPA (50 mM Tris–HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS) plus proteases and phosphatases inhibitors (100 μl/ml PMSF, 1 mM sodium orthovanadate, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatine, 50 mMNaF). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to Nitrocellulose Blotting Membranes (Amersham) and developed with ECL on X-ray films as previously described (Rosso et al., 2004). Primary antibodies: polyclonal rabbit anti-β-tubulin III (Sigma–Aldrich) diluted 1:8000, rabbit polyclonal anti-Wnt5a (Abcam) diluted 1:1500, rabbit polyclonal anti-Phospho-CaMKII (Thr286) (Cell Signaling) diluted 1:1500, rabbit polyclonal anti-CaMKII (pan) (Cell Signaling) diluted 1:2000, rabbit mAB anti-Phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling) diluted 1:1500, rabbit mAB anti-cJNK (Santa Cruz Biotechnology) diluted 1:1500, mAB mouse anti-β-catenin (BD, BioScience) diluted 1:10000. Quantifications were performed by measuring optic density of bands with ImageJ.

2.6. Extraction of mRNA and RT-PCR

RNA was extracted from neuronal cultures at 12, 24 and 48 h *in vitro* using TRIzol (Invitrogen) following the manufacturer's protocol. cDNA was synthesized by adding 0.5 μg of oligodT primers (Biodynamics) to 2 μg of extracted RNA in nuclease free water. PCR was carried out using a 3 μl of cDNA, GoTaq DNA polymerase (Promega) and dNTPs with specific primer pairs. The forward (F) and reverse (R) primers were selected using the Primer3 free software (Rozen and Skaletsky, 2000). Primer sequences were: **Wnt3a F** 5′-TTTGGAGGAATGGTCTCTCG-3′; **Wnt3a R** 5′-CTTGAGGTGCATGT GACTGG-3′; **Wnt5a F** 5′-TCGAC-TATGGCTACCGCTTC-3′; **Wnt5a R** 5′-CGACCTGCTTCATTGT TGTG-3′; **Wnt7a F** 5′-CCCGAACCCTCATGAACTTA-3′; **Wnt7a R** 5′-TGTGGTCCAGCACGTCT TAG-3′; **Wnt7b F** 5′-AGCCAACATCATCTG-CAACA-3′; **Wnt7b R** 5′-GGCATTCATCGATAC CCATC-3′; **Wnt8b F** 5′-TCCCGAGCTTGGTTTAGAGA-3′; **Wnt8b R** 5′-TCCCGAGCTTGGTTTAG AGA-3′; **18S F** 5′-CGCTAGAGGTGAAATTCTTGG-3′; **18S R** 5′-CGGAACTACGACGGTATCTGA-3′. Quantifications were performed by measuring optic density of bands with ImageJ software.

2.7. Statistical analyses

Data are presented as the mean ± SEM and significant differences among means were considered at level of $p < 0.05$. Comparisons between two groups were made by two tails Student's *t* test or Mann–Whitney test. Graphs and statistical analysis were performed using GraphPad Prism statistical analysis software.

3. Results

3.1. Glyphosate effect on neuronal viability

We tested the potential cytotoxicity of growing concentrations of glyphosate on cultured hippocampal neurons treated for 1, 2 and 4 days *in vitro* (DIV). For that, different concentrations of the herbicide (2, 4 and 6 mg/ml) were added to the cultures 2 h after plating and then, at the proper time, MTTassay was used to evaluate the glyphosate effect on neuronal viability. Results showed that the lower concentrations, 2 and 4 mg/ml of glyphosate, did not affect neuronal survival at the evaluated periods (Suppl. Fig. 1). However, neurons exposed to the highest glyphosate concentration (6 mg/ml) showed a lethal effect after 4 DIV (Suppl. Fig. 1). In this context, a significant decrease in cell viability (around 25%) was observed compared to controls (Suppl. Fig. 1). These observations allowed us to choose a glyphosate concentration in which non-lethal effects were observed in order to perform the morphological studies.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2015.12.004.

3.2. Glyphosate affected neuronal differentiation and development

To study the glyphosate toxicity on developmental neurons, we initially examined its effect on the ability of neurons to polarize, resulting in an axon (positive for Tau marker) and several dendrites (positive for MAP2 marker) (Dotti et al., 1988). Our observations showed that most control neurons cultured for 24 h (1 DIV) generated a long, axon-like process that was enriched in Tau1 protein (Fig. 1A, upper panel). However, neurons cultured in the presence of glyphosate failed to form an axon and only short minor processes were present (Fig. 1A, upper panel) (Suppl. Fig. 2). Quantifications revealed that glyphosate treatment induced a significant decrease in the number of polarized neurons (Fig. 1B, upper panel). Thus, while in control conditions around 70% of 1 DIV neurons polarized, after glyphosate treatment only 38% of neurons differentiated showing a discernible axon (Fig. 1B, upper panel). To evaluate whether glyphosate induces a delay in axon differentiation we analyzed neuronal polarization after 2 DIV. Immunofluorescence studies revealed that even though glyphosate treated neurons polarized showing an axon labeled with Tau marker, the morphology of that axon was different from controls (Fig. 1A, middle panel). Quantifications showed that around 80% of control neurons polarized after 48 h in culture and at that time most of glyphosate treated neurons also polarized, since 70% of cells showed a clear axon (Tau+) (Fig. 1B, upper panel). These findings suggest that the herbicide treatment postpones neuronal differentiation, since treated neurons generated their axons later than controls. To go further, we analyzed axon morphology at 2 and 4 DIV in control and glyphosate exposed neurons (Fig. 1A, middle and bottom panels). We observed that control neurons developed a long and highly branched axon, while after glyphosate exposure; they elicited a simple axon, shorter and less branched than controls (Fig. 1A, middle and bottom panels). We scored these observations and found a significant reduction in the total axonal length and in the number of axonal branches after glyphosate treatment at 2 and

4 DIV (Fig. 1B, middle and bottom panels). Finally, we analyzed the effect of glyphosate on dendrite development. Quantifications showed that glyphosate induced a noticeable decrease in dendritic length compared to controls (Fig. 1C). Additionally, the complexity of the dendritic tree was altered after the herbicide treatment, since the number of dendritic branches was markedly diminished at 2 and 4 DIV respect to control cells (Fig. 1C).

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3.3. Glyphosate effect is not reversible

As was described, glyphosate treatment induces a delay in neuronal polarization and development after 1–2 DIV so we examined whether that effect may revert when the herbicide is removed. In order to evaluate the reversibility of glyphosate effects on neuronal development, cultured hippocampal neurons were exposed to glyphosate for 24 h, and then washed and cultured in fresh medium for 1 more day. Our observations revealed, as it was previously described, that 2 DIV glyphosate treated neurons developed shorter and less branched axons compared to controls. Interestingly, neurons exposed to glyphosate for 24 h and cultured in fresh medium for 1 more day showed a similar morphology to 2 DIV treated cells (Suppl. Fig. 3A, compare middle and right panels). Quantifications revealed that glyphosate exposure induced a significant decrease in axonal length and the number of axonal branches compared to controls (Suppl. Fig. 3B, $^{***}p < 0.001$, $^{****}p < 0.0001$), and this effect was observed in both treated groups (called: Glyph and Glyph removed). These findings suggest that glyphosate affects neuronal development and that this effect does not revert when the herbicide is taken out after 1 day of treatment.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2015.12.004.

3.4. Growth cone morphology was altered by glyphosate treatment

Axonal growth cone is a very dynamic structure at the tip of the neurite. The organization and re-arrangement on actin cytoskeleton within this structure have been indicated as one of the most important factors underling the establishment of neuronal polarity. In cultured hippocampal pyramidal neurons, axon formation is preceded by the appearance of a large and

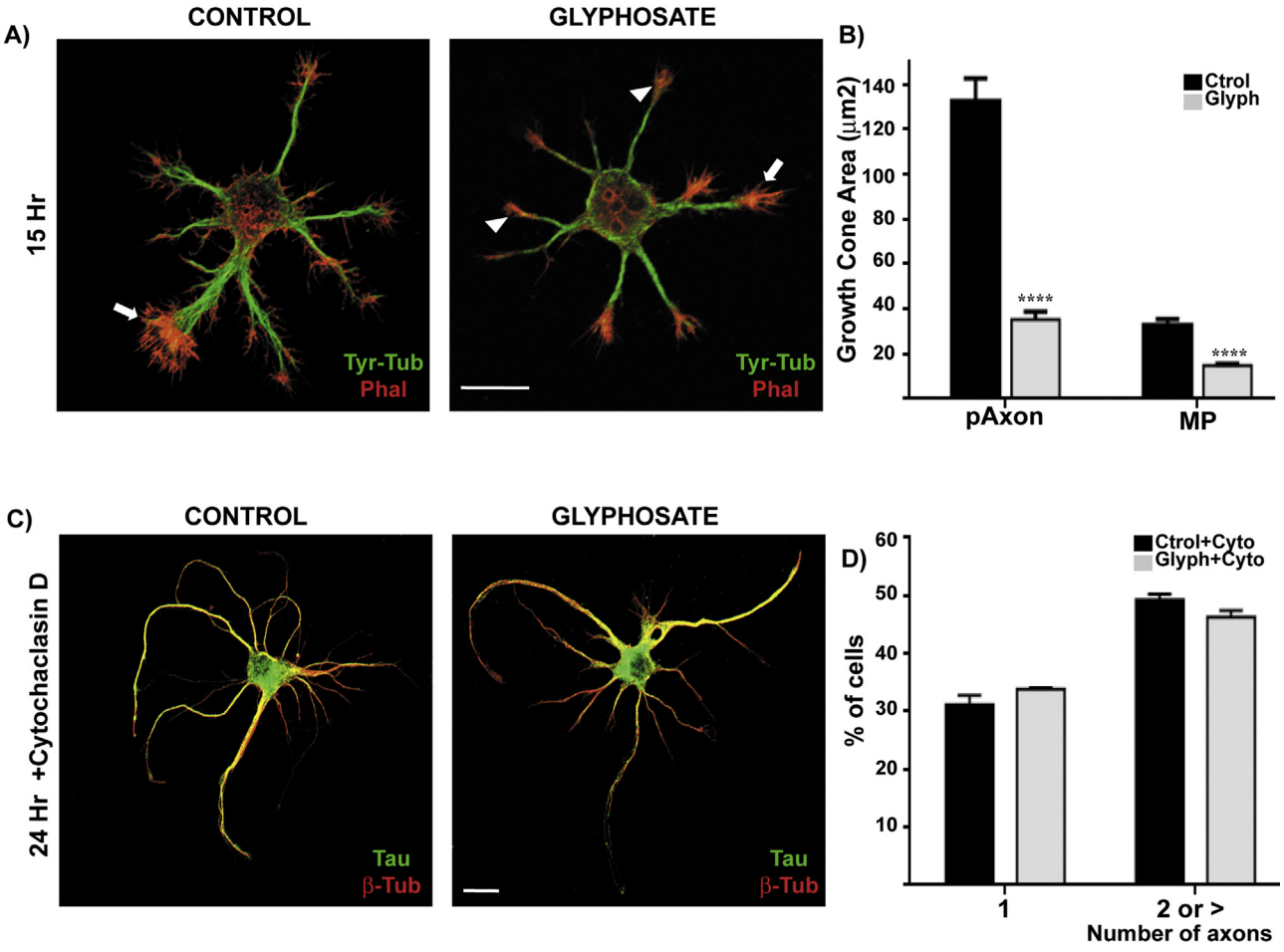

Fig. 2. Neuronal growth cone size and morphology were affected by glyphosate treatment.
(A) Immunofluorescence images showing hippocampal cultured neurons fixed at 15 h. Neurons were incubated with vehicle or glyphosate from 2 h of plating. Cells were double-labeled with anti-tyrosinated α-tubulin (green) and rhodamine-phalloidin (red). The growth cones corresponding to minor processes (MP) were indicated by white arrow head and those corresponding to prospective axons (pAxon) were indicated by white arrow. Scale bar 10 µm. (B) Quantification shows the average growth cone area from MP and pAxon from control or glyphosate exposed neurons. For this graphic we scored 40 cells per condition from 3 independent experiments, $^{****}p < 0.0001$ compared to control condition. Errors bars represent mean ± SEM. (C) Images of neuronal cells incubated with cytochalasin D (final concentration 1 µM). Glyphosate or vehicle was added to the medium after 2 h of plating and cytochalasin D was added at 12 h of culture. Neurons were fixed at 24 h and they were immunolabeled with Tau1 (green) and β-tubulin III (red) antibodies. (D) Quantification shows the percentage of cells with 1, 2 or more axons in control or glyphosate treated neurons in the presence of cytochalasin D. We scored 100 cells per condition from 3 independent experiments. Errors bars represent mean ± SEM. Scale bar: 10 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

highly dynamic growth cone in one of multiple neurites, which probably will become the future axon (Bradke and Dotti, 1997, 1999; Paglini et al., 1998). In order to elucidate whether the glyphosate effect on axon outgrowth is due to changes in the growth cones morphology, we evaluated growth cone size and shape, as well as the distribution of microfilaments after glyphosate treatment. At this point, most neurons have extended several short neurites known as minor processes (stage 2) (Dotti et al., 1988). We observed that control neurons developed a neurite with a large growth cone that likely becomes in axon (Fig. 2A, upper panel). However, most of glyphosate treated neurons did not show a prominent growth cone since all neurites evidenced small growth cones (Fig. 2A, upper panel). Confocal microscopy of 15 h-control neurons labeled against tyrosinated α-tubulin (Tyr-Tub) and rhodamine phalloidin (Phal) revealed that the largest growth cone has a microtubule region surrounded by a peripheral area formed by short actin ribs (Fig. 2A) as was previously described (Dotti et al., 1988; Kunda et al., 2001). However, after glyphosate treatment, all growth cones were significantly smaller than controls. Also, they elicited a very compacted actin arrangement on the tip of the neurites (Fig. 2A). Quantitative analysis revealed that glyphosate treatment markedly affected growth cones size of minor processes (MP) and prospective axons (pAxon) in stage 2 hippocampal neurons (Fig. 2B). Thus, glyphosate treated neurons showed a reduction of around 70% and 54% in the size of growth cone from pAxon and MP, respectively, compared to controls (Fig. 2 B). To go further, we examined whether glyphosate affected actin stability, by treating neurons with cytochalasin D (Cyto), an actin-depolymerizing drug, inducing multiple axon-like neurites (Bradke and Dotti, 1999; Kunda et al., 2001). Moreover, disruption of the actin network in one individual growth cone induces its neurite to become the axon (Bradke and Dotti, 1999). Therefore, local instability of the actin network restricted to a single growth cone is a physiological signal that specifies neuronal polarization. In our study, the application of cytochalasin D to undifferentiated neurons treated with glyphosate allowed us to evaluate whether actin cytoskeleton is involved in the mechanism of glyphosate's neurotoxicity. For that, control and glyphosate exposed cells were treated with a single dose of cytochalasin D (1 μM) and fixed 12 h later. Then, cultures were analyzed for immunofluorescence. Results showed that destabilization of actin by cytochalasin D abolished the glyphosate effect on axon differentiation since glyphosate treated neurons displayed multiple axon-like neurites (Tau+) (Fig. 2C) as well as controls. Quantifications revealed that equivalent percentages of cells generating one, two or more axons were detected in control and glyphosate exposed neurons treated with cytochalasin D (Fig. 2D). This evidence indicates that glyphosate treatment affects the actin organization and stability in growth cones of immature neurons.

3.5. Glyphosate induced changes in Wnt5a expression

Considering the findings described above and the important role of Wnt factors in the formation of neural circuits, we evaluated whether glyphosate affects neuronal development through changes in Wnt signaling pathways. For this study, we analyzed the level of those Wnt molecules that are highly expressed in the nervous system during early development, such as Wnt3a, Wnt5a, Wnt7a, Wnt7b and Wnt8b (Shimogori et al., 2004) after glyphosate treatment by RT-PCR assays. Our observations revealed that the treatment led to a decrease in mRNA-Wnt5a level after 24 h compared to controls, whereas no changes in others Wnt species were observed (Fig. 3A). Quantifications revealed that glyphosate induced a significant decrease in relative Wnt5a compare to control level (Fig. 3A). Moreover, significant reductions in mRNA-Wnt5a were observed in cultures exposed to glyphosate for 12 and 48 h (Fig. 3B). Next, we analyzed the Wnt5a protein level by electrophoresis and Western blot and found that glyphosate induced a decrease in Wnt5a protein at 24 and 48 h. Quantifications showed that the herbicide treatment led to a significant diminution of Wnt5a (around 25%) respect to controls (Fig. 3C).

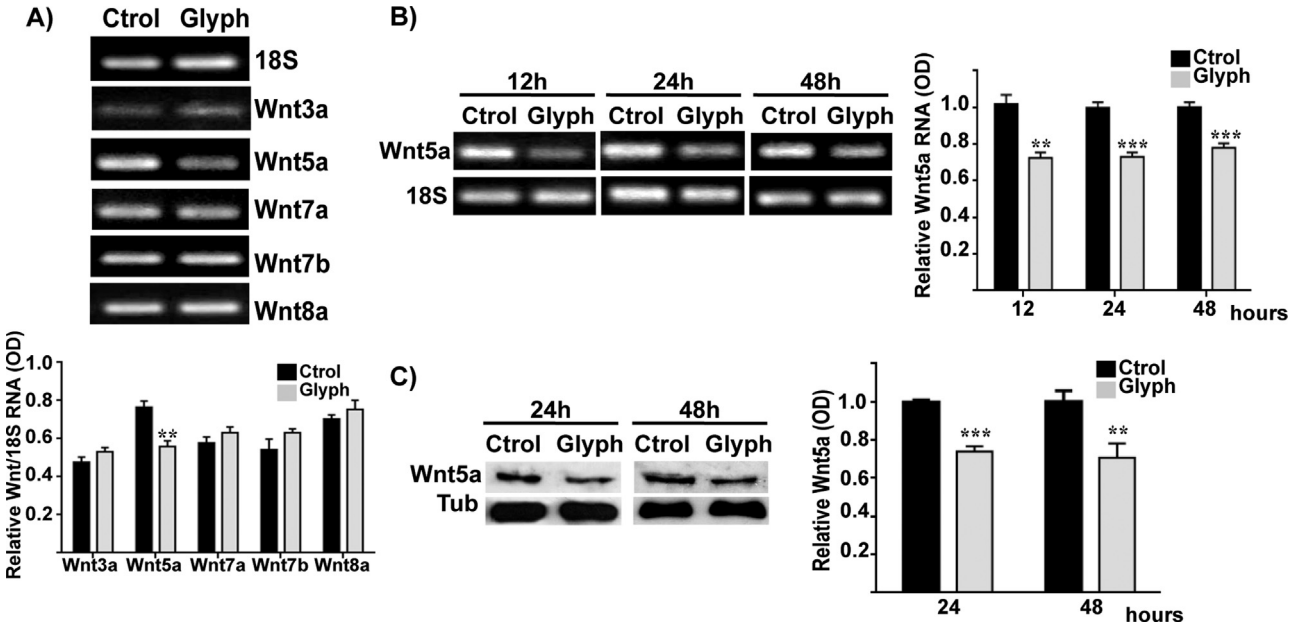

Fig. 3. Glyphosate treatment affected Wnt5a expression in neuronal cultures.
(A) mRNA levels of Wnt molecules from 1 DIV control and treated neurons by RT-PCR assay. The 18S mRNA level was used as loading control. Quantification shows the relative level of Wnt/18S mRNA. (B) Images showing the Wnt5a and 18S mRNA in control and glyphosate treated neurons cultured for 12, 24 and 48 h. Quantification shows the relative optical densities of Wnt5a/18S mRNA at different times. (C) Representative Western blots showing the Wnt5a protein level and β-tubulin III in cultured neurons for 24 and 48 h. Neurons were incubated with vehicle or Glyph from 2 h of plating. Quantification shows the relative optical densities of Wnt5a/β-tubulin III. Data was obtained from 3 independent experiments, $^{**}p < 0.01$; $^{***}p < 0.001$. Errors bars represent ± SEM.

3.6. Wnt5a abolished the glyphosate effect on neuronal morphology

In order to analyze whether the effect of glyphosate on neuronal morphology was due to changes in Wnt5a expression, we performed assays adding recombinant Wnt5a protein (final cc 100 ng/ml) to 2 h glyphosate treated neurons and then, cells were fixed at 1 or 2 DIV. As we already described, 1 DIV glyphosate treated neurons did not polarized since they remained in undifferentiated stage showing only several minor processes (Fig. 4A, upper panel). However, when neurons were cultured in the presence of glyphosate and recombinant Wnt5a, they showed a similar morphology to controls, characterized by a long axon (Tau +) and several minor processes (Fig. 4A, bottom panel). Quantifications showed that the presence of Wnt5a abolished the impairment on neuronal polarization caused by glyphosate treatment (Fig. 4B, upper panel) and it also blocked the glyphosate effect on axonal length (Fig. 4B, bottom panel). In accordance with other studies, we observed that Wnt5a promoted axonal growth since neurons cultured in the presence of Wnt5a generated a long and highly branched axon compared to controls (Fig. 4B, bottom panel) (Bodmer et al., 2009). Interestingly, Wnt5a alone did not affect the number of axons per cell since neurons showed only one axon-like neurite (Tau+) (Fig. 4B upper panel). To go further, we analyzed the effect of exogenous Wnt5a on 2 DIV glyphosate treated neurons (Fig. 5). These neurons exhibited a longer and more branched axon and more complex dendritic arbours than the herbicide treated cells (Fig. 5A). Quantitative analysis revealed that Wnt5a stimulation of glyphosate treated neurons restored the axonal and dendritic length and complexity to control levels (Fig. 5B and C).

3.7. Glyphosate impaired Wnt5a non-canonical pathway through changes in CaMKII activity

Wnt ligands can signal through different pathways: the canonical or β-catenin pathway and the non-canonical Wnt pathways (β-catenin independent) such as the planar cell polarity (PCP) and the Ca^{2+} pathways (Ciani and Salinas, 2005; Rosso and Inestrosa, 2013). In this context, we analyzed the activation of the three Wnt cascades in cultured hippocampal neurons after Wnt5a stimulation. We evaluated the level of β-catenin, and the activity of two downstream kinases: JNK (p-JNK) for PCP pathway and CaMKII (p-CaMKII) for calcium pathway using electrophoresis and Western blot techniques (Fig. 6A). Results showed that Wnt5a stimulated neurons did not exhibit any change in the level of β-Catenin or p-JNK at 1 DIV compared to controls (Fig. 6A left and middle panel, respectively). However, we observed that Wnt5a induced an increase in the level of phospho-CaMKII (p-CaMKII), used as a read out of CaMKII activity (Fig. 6A right panel). In this context, quantifications revealed that Wnt5a produced an increase

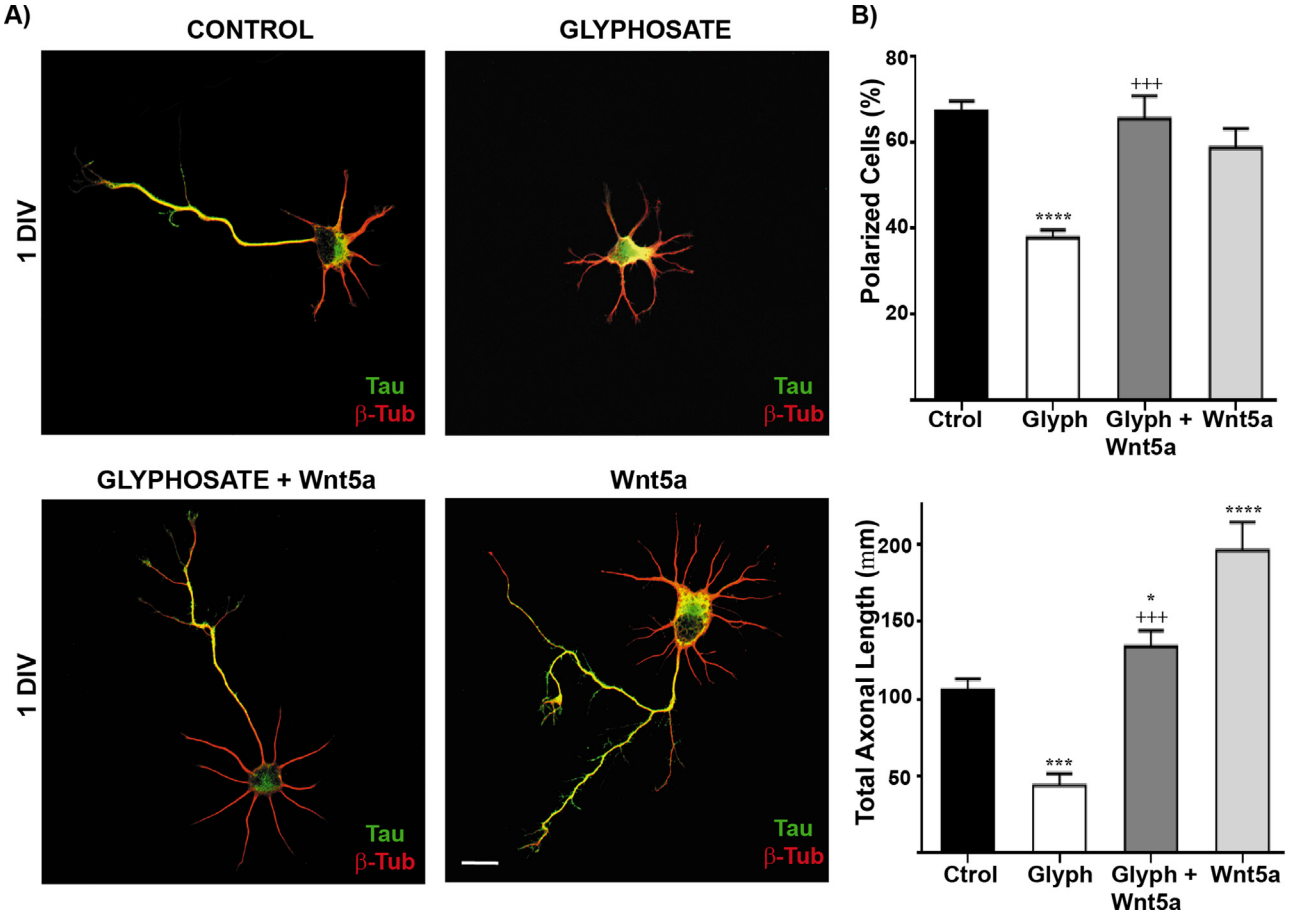

Fig. 4. Exogenous Wnt5a reverted glyphosate effects in 1 DIV neurons.
(A) Confocal images showing neurons incubated under different conditions: control, glyphosate 4 mg/ml, glyphosate 4 mg/ml + Wnt5a 100 ng/ml or Wnt5a 100 ng/ml. Cells were fixed after 24 h and immunolabeled with Tau1 (green) and β-tubulin III (red) antibodies. In all cases, vehicle or glyphosate (4 mg/ml) were added to the cultures 2 h after plating and Wnt5a was added 4 h later. (B) Quantification evidences the percentage of polarized neurons (upper panel) (150 cells were counted per condition, $n = 3$) and the total axonal length (bottom panel) (60 cells were counted per conditions, $n = 3$) from 1 DIV cultured neurons, $^{*}p < 0.05$; $^{***}p < 0.001$; $^{****}p < 0.0001$, compared to controls and $^{+++}p < 0.001$ compared to glyphosate condition. Error bars represent mean ± SEM. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

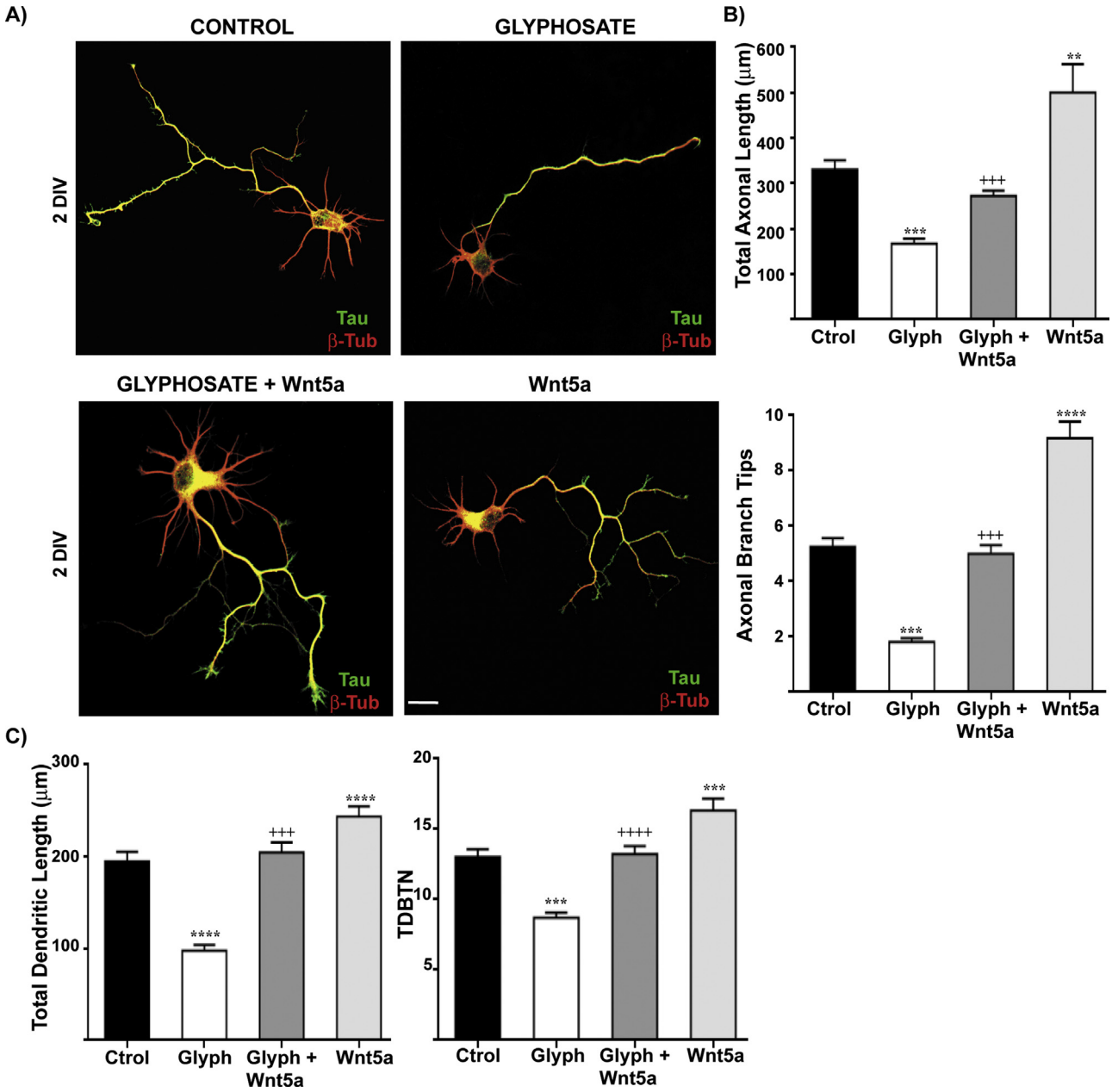

Fig. 5. Addition of Wnt5a to differentiated treated neurons induced the recovery of neuronal complexity.
(A) Representative images showing neurons cultured during 48 h under different conditions (control, glyphosate 4 mg/ml, glyphosate 4 mg/ml + Wnt5a 100 ng/ml or Wnt5a 100 ng/ml). Vehicle or glyphosate (4 mg/ml) were added to the medium 2 h after plating and Wnt5a was added 4 h later. Cells were immunolabeled with Tau1 (green) and β-tubulin III (red) antibodies. (B) Measurementsof total axonal length (upper panel) and axonal branch tips (bottom panel) for all conditions, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; compared to controls and $+++p < 0.001$ compared to glyphosate treated cells. Error bars represent mean ± SEM. (C) Quantification of dendritic development through total dendritic length (left panel) and total dendritic branching tip number (TDBTN) (right panel), to evaluate dendrite complexity, for each condition (Ctrol, Glyph, Glyph + Wnt5a or Wnt5a), $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared to controls and $+++p<0.001$ and $++++p < 0.0001$ compared to glyphosate treated neurons. Error bars represent mean ± SEM. All graphics were made by scoring 60 cells per condition from 3 independent experiments. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of CaMKII activity (around 55%) compared to controls. These findings would suggest that Wnt5a signals through the Wnt/Ca^{2+} non-canonical pathway in hippocampal neurons.

Considering that glyphosate affected Wnt5a expression, we then investigated whether glyphosate treatment induced changes in the CaMKII activity. For this, cultured hippocampal neurons were exposed to glyphosate (4 mg/ml) for 24 h and the level of p-CaMKII was analyzed by Western blot technique. Our observations revealed that glyphosate treated neurons showed a reduction in the p-CaMKII respect to controls (Fig. 6B). No changes in p-JNK or β-catenin were observed after glyphosate treatment (data not shown). Quantifications showed that glyphosate induced a decrease in the p-CaMKII level of around 25% respect to controls and similar results were observed from 2 days treated neurons (data not shown). These findings indicate that glyphosate exposure affects CaMKII activity in cultured hippocampal neurons. To corroborate whether the effect on CaMKII activity was a consequence of the glyphosate effect on Wnt5a expression, we performed experiments adding recombinant Wnt5a. Thus, glyphosate treated neurons were stimulated by Wnt5a (100 ng/ml) from 6 h and then they were lysed after 24 h. As shown in Fig. 6B, glyphosate treated neurons stimulated by Wnt5a exhibited similar

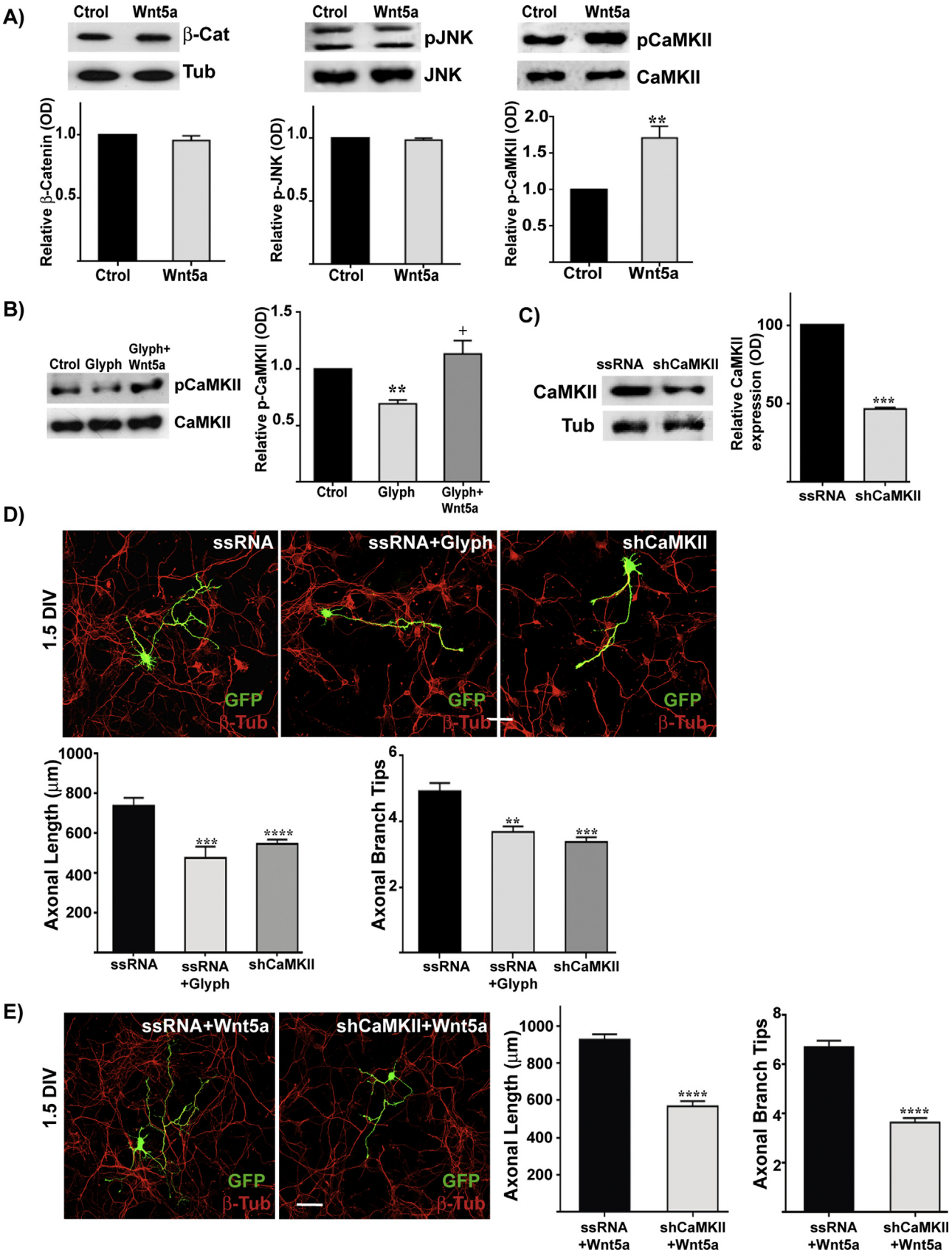

Fig. 6. Glyphosate treatment affected Wnt5a/pCaMKII pathway in hippocampal neurons.
(A) Representative Western blot images and quantifications showing the expression of β-catenin, pJNK and pCaMKII in control and Wnt5a stimulated neurons at 2 DIV. Wnt5a (100 ng/ml) was added to the medium at 24 h, $^{**}p < 0.01$ respect to control condition. Error bars represent mean ± SEM, $n = 3$ independent experiments. (B) Western blot and quantification showing pCaMKII level in 1 DIV hippocampal neurons under different conditions: control, glyphosate 4 mg/ml or glyphosate 4 mg/ml + Wnt5a 100 ng/ml. Vehicle or glyphosate was added to the cultures 2 h after plating and Wnt5a was added 4 h later, $^{**}p < 0.01$ compared to control and $+p < 0.05$ compared to glyphosate

levels of p-CaMKII to controls, suggesting that Wnt5a blocks the effect of glyphosate on CaMKII activity.

To further investigate the role of CaMKII on neuronal morphology we carried out assays to examine the effect of CaMKII loss of function expressing a specific shRNA-CaMKII in high density cultures. Firstly, we analyzed the ability of shCaMKII to affect the expression of endogenous CaMKII in cultured neurons (Fig. 6C). Our observations showed that the expression of shRNA-CaMKII induced a decrease of about 50% in the CaMKII level in 1.5 DIV neurons compared to ssRNA controls (Fig. 6C). Then, we analyzed the morphology of shCaMKII expressing neurons by immunofluorescence. Confocal images revealed that 1.5 DIV CaMKII-silenced neurons showed a defect in their axonal development, since they elicited a shorter and less complex axon than ssRNA expressing neurons (Fig. 6D). In addition, we noted that these neurons developed similar morphology to ssRNA expressing cells treated with glyphosate for 1.5 days (Fig. 6D). Quantifications showed that the axonal length and the number of axonal branches of CaMKII silenced neurons were similar to those from glyphosate treated neurons. Under these conditions, these neurons showed a decrease in the axonal length and complexity (around 40%) compared to ssRNA expressing cells. These findings suggest that the defects on axonal development may be due to a decrease in the CaMKII activity induced by glyphosate.

Finally, we performed assays to corroborate whether Wnt5a requires CaMKII activation to promote axon growth and elongation. We evaluated the Wnt5a effect on 1.5 days-hippocampal neurons expressing the shRNA-CaMKII. As we already showed in low density cultures (Figs. 4 and 5), Wnt5a induced a positive effect on axonal development since ssRNA expressing cells stimulated by Wnt5a evidenced longer and more complex axons than controls (compare Fig. 6E and D). Importantly, this Wnt5a effect on axonal morphology failed when neurons expressed the shCaMKII indicating that Wnt5a requires CaMKII activation to modulate axonal growth and elongation (Fig. 6E).

4. Discussion

Humans are daily exposed to complex mixtures of potentially neurotoxic compounds from occupational and residential environments. Among these compounds, pesticides are used indiscriminately with restricted control from environmental agencies. Glyphosate-based herbicides are worldwide used to control the weeds growth on genetically modified soybeans. Although there is growing evidence describing toxic effects of glyphosate (Richard et al., 2005; Benachour and Seralini, 2009; Gasnier et al., 2009), limited data is found about its neurotoxicity and the mechanisms leading to the potential damage on nervous system. It has recently been demonstrated that glyphosate based herbicide causedneurotoxicity in immature rats due to acute and chronic exposition that markedly affected the extracellular glutamate levels and consequently induced glutamate excitotoxicity and oxidative stress in rat hippocampus (Cattani et al., 2014).

In our study, we described the specific effects of glyphosate exposure on neuronal polarization and development, and identified the cellular mechanism by which glyphosate would affect the capacity of neurons to differentiate and reach the mature phenotype. Considering that none of the tested doses affected neuronal viability after 1 or 2 days of treatment (Suppl. Fig. 1), we performed morphometric analysis using an intermediate glyphosate concentration (4 mg/ml, 23 mM) that was added to the medium after cell plating. Previous studies have demonstrated that glyphosate-based herbicides were toxic for a human placental cell line (Richard et al., 2005) and that they also had endocrine-disrupting potential on estrogen synthesis, at concentrations below that recommended for agriculture use (suggested concentration: 1–2%, containing 21–42 mM glyphosate) (Benachour et al., 2007). In that work, authors have compared the effect of glyphosate and Roundup formulation and they showed that the effect after the Roundup exposure was significantly higher than the effect produced by glyphosate alone, probably due to the presence of different adjuvants in the formulation (Richard et al., 2005). In this context, the glyphosate concentration used in our study (a single dose of 4 mg/ml, 23 mM) did not evidence any lethal effect on hippocampal cultured neurons. Despite this, we observed that glyphosate treatment affected the ability of neurons to polarize and generate the axon. Thus, 1 DIV treated neurons remained in stage 2 (undifferentiated stage) (Dotti et al., 1988), and they did not show an axon-like neurite (Fig. 1) (Suppl. Fig. 2). However, later studies revealed that most 2 DIV treated neurons developed an axon-like neurite suggesting that glyphosate treatment induced a delay in axonal differentiation. At that time of culture, glyphosate affected the overall neuronal development since neurons exhibited a short and unbranched axon and also impaired dendrite growth and complexity. These observations indicated that early neuronal exposure to glyphosate induces a delay in neuronal development that could be related to a defect in neuronal connectivity and synaptic function in mature neurons. In cultured hippocampal neurons, axon formation is preceded by the appearance of a large growth cone in one of the minor neurite containing a very labile actin network. Thus, the regulation of actin organization and dynamic within a selected growth cone underlies the establishment of neuronal polarity (Bradke and Dotti, 1997, 1999). Considering the glyphosate effect on neuronal polarization, we analyzed the morphology of the growth cones (Fig. 2) in control and in treated undifferentiated neurons. We found that treated neurons developed several and identical growth cones, which were significantly smaller than controlones. Furthermore, we observed that in those neurons, actin organization surrounding the growth cones was markedly affected since fluorescence images revealed more compacted actin rearrangement compared to controls (Fig. 2). At the same time, control and glyphosate-treated neurons developed multiple axons-like neurites after cytochalasin D challenge (Fig. 2C and D) suggesting that cells preserve the molecular machinery necessary to generate the axon. It is well known that cytochalasin D allows microtubules to penetrate any neuritic tip devoid of actin filaments and hence leads to multiple axon formation (Forscher and Smith, 1988; Bradke and Dotti, 1999). These observations would suggest that changes in the actin organization in growth cones induced by glyphosate can postpone the ability of neurons to polarize and generate a clear axon.

Wnt proteins are crucial for neuronal development and maturation. Wnts are involved in regulating axon guidance, dendrite morphogenesis, and synapse formation (Rosso et al., 2005; Inestrosa and Arenas, 2010; Budnik and Salinas, 2011; Rosso and Inestrosa, 2013). Among the effects of Wnt proteins on nervous system, several studies indicated that Wnts secreted molecules may act as potent axonal branching factors (Ciani and Salinas,

treatment. Error bars represent mean ± SEM, $n = 3$ independent experiments. (C) CaMKII level from ssRNA or shRNA-CaMKII expressing neurons at 1.5 DIV. (D) Representative images showing the morphology of 1.5 DIV ssRNA, ssRNA + glyphosate or shCaMKII expressing neurons. Cells were transfected at 0.5 DIV and for the glyphosate condition (middle panel) the herbicide was added 2 h after plating. Fixed neurons were immunolabeled using GFP and β-tubulin antibodies. Scale bar 10 μm. Graphics show total axonal length (μm) and the number of axonal branch tips for each experiment. For quantifications we scored 60 cells per condition from 3 independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ respect to controls. (E) Confocal images showing 1.5 DIV ssRNA or shCaMKII expressing neurons. Wnt5a was added to the cultures 6 h after transfection and cells were immunolabeled with GFP and β-tubulin antibodies. Scale bar 10 μm. Quantification shows total axonal length and axonal branch tips for each condition. Graphics were made scoring 50 cells per condition from 3 independent experiments, $^{****}p < 0.0001$ respect to ssRNA + Wnt5a. Error bars represent mean ± SEM.

2005). Wnt7a expressed by cerebellar granule cells induces growth cone enlargement and axonal spreading of mossy fibers (Lucas and Salinas, 1997; Hall et al., 2000). Other evidence showed that Wnt3a mediates axon specification, morphology and also microtubule organization in immature neurons (Krylova et al., 2002; Purro et al., 2008; Bernis et al., 2013). In addition, it has been identified Wnt5a as a key downstream effector of NGF in mediating axonal branching and growth in developing sympathetic neurons (Bodmer et al., 2009). Also, Wnt5a has been implicated in cortical axon guidance (Li et al., 2014) and neurite development in olfactory bulb interneuron (Pino et al., 2011) mainly through activation of a non-canonical pathway. In this context, it has been described that Wnt5a increases axon outgrowth by reorganization of dynamic microtubules (Li et al., 2014) and it promotes axon growth and repulsion by the activation of CaMKII pathway and Ryk receptor in dissociated cortical neurons (Hutchins et al., 2011). Our results showed that glyphosate treatment affects the Wnt5a expression in 1 and 2 DIV hippocampal neurons without affecting the expression of others Wnts molecules. Glyphosate exposure induced a decrease in the Wnt5a level in cultured neurons and that diminution may contribute to a delay in axon elongation and growth. To corroborate that issue, we attempted to rescue the glyphosate effect on neuronal morphology by adding recombinant Wnt5a to cultured neurons. Glyphosate treated neurons stimulated by Wnt5a for 1 day were able to polarize and generated a long and branched axon. Importantly, the stimulation of control cells led to a markedly effect on axon elongation and branching. Moreover, 2 days glyphosate treated neurons stimulated by Wnt5a elicited complex axons and elaborate dendrites similar to controls. These findings may imply that morphological defects induced by glyphosate treatment could be due to a decrease in the Wnt5a expression, a key factor for the initial neurite development and maturation. These observations highlight the critical role of Wnt secreted proteins to mediate neuronal development.

Finally, we identified the intracellular Wnt5a mediated pathway by which glyphosate affected neuronal development. Firstly, we evaluated the specific effectors for the three Wnt cascades after Wnt5a neuronal stimulation. We found that Wnt5a signals through the Wnt/Ca^{2+} pathway since it induced an increase of p-CaMKII level (Fig. 6A). Several points such as: (i) glyphosate induced CaMKII inhibition and this effect was reverted after Wnt5a stimulation (Fig. 6B); (ii) neuronal cultures exposed to glyphosate developed an axonal morphology similar to shRNA-CaMKII expressing cells, and (iii) the Wnt5a effect on axonal growth and branching was inhibited when a shRNA-CaMKII was expressed (Fig. 6D), led us to postulate that glyphosate induces a delay in neuronal development through a cellular mechanism involving the downregulation of Wnt5a-CaMKII signaling pathway. In this context, previous studies demonstrated that CaMKII plays an important role in the regulation of neuronal morphology since CaMKII binds to the actin filaments inducing changes in actin organization and dynamics (Shen et al., 1998; Lin and Redmond, 2008). Furthermore, it has been shown that CaMKII, mainly its β-subunit, induced LIMKI activation, a key factor to modulate actin polymerization and neurite formation through cofilin inactivation (Rosso et al., 2004; Endo et al., 2007; Saito et al., 2013). These findings suggest that the glyphosate-induced CaMKII down-regulation may promote actin disorganization at the growth cones affecting the neurite elongation. The effect of glyphosate exposure on LIMK1 activity and actin stability will be widely analyzed in future studies.

Considering the limited data regarding glyphosate effect on the central nervous system, in this work we demonstrate that glyphosate exerts neurotoxicity on developmental hippocampal neurons and more importantly, we identified the cellular mechanism by which it affects neuronal development.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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