

Full Length Article

Multiple effects of the herbicide glufosinate-ammonium and its main metabolite on neural stem cells from the subventricular zone of newborn mice

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ABSTRACT

The globally used herbicide glufosinate-ammonium (GLA) is structurally analogous to the excitatory neurotransmitter glutamate, and is known to interfere with cellular mechanisms involved in the glutamatergic system. In this report, we used an *in vitro* model of murine primary neural stem cell culture to investigate the neurotoxicity of GLA and its main metabolite, 4-methylphosphinico-2-oxobutanoic acid (PPO). We demonstrated that GLA and PPO disturb ependymal wall integrity in the ventricular-subventricular zone (V-SVZ) and alter the neuro-glial differentiation of neural stem cells. GLA and PPO impaired the formation of cilia, with reduced Celsr2 expression after PPO exposure. GLA promoted the differentiation of neuronal and oligodendroglial cells while PPO increased B1 cell population and impaired neuronal fate of neural stem cells. These results confirm our previous *in vivo* report that developmental exposure to GLA alters neurogenesis in the SVZ, and neuroblast migration along the rostral migratory stream. They also highlight the importance of investigating the toxicity of pesticide degradation products. Indeed, not only GLA, but also its metabolite PPO disrupts V-SVZ homeostasis and provides a novel cellular mechanism underlying GLA-induced neurodevelopmental toxicity. Furthermore, we were able to demonstrate a neurotoxic activity of a metabolite of GLA different from that of GLA active substance for the very first time.

1. Introduction

The reported number of neurological disorders, such as Alzheimer's and Parkinson's diseases, or autism spectrum disorders (ASD), has significantly increased over the last decades. Despite progress in the diagnosis of these pathologies, pure genetic origin has been implicated in only a small proportion of clinical cases, suggesting a gene-environment interaction as the most likely etiological factor. Indeed, there is now strong evidence that pesticides and other environmental pollutants are potent toxic agents, affecting neurodevelopment, and inducing long lasting neural changes contributing to the current increased prevalence of neurodevelopmental disorders (Grandjean and Landrigan, 2006; Mendola et al., 2002; Paradells et al., 2015; Shelton et al., 2014). This phenomenon, called the "silent pandemic", is directly related to the fact that the developing human brain is inherently much more vulnerable to

injury caused by toxic agents than the brain of adults (Grandjean and Landrigan, 2006, 2014). Here, we focused on neurodevelopmental toxicity induced by the broadspectrum herbicide, glufosinate-ammonium (GLA). GLA is an amino acid containing phosphorus, which is widely used in agriculture. It has a high degree of structural similarity to glutamate, the main excitatory neurotransmitter in the central nervous system (CNS) (Hack et al., 1994). The poisoning by massive ingestion of GLA following a suicide attempt affects the nervous system (Mao et al., 2012; Ohtake et al., 2001; Park et al., 2006). The inhalation exposure plays a significant role in GLA poisoning, notably in farmers and populations living near the application site. Recently, Aris and Leblanc reported the presence of GLA and its metabolites in maternal and fetal cord blood after pesticide exposure in genetically modified food, suggesting that pesticides in our environment have a direct impact on human health (Aris and Leblanc, 2011). Furthermore, a

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previous study has highlighted GLA neurotoxicity in mammals in terms of neurodevelopmental processes (Watanabe, 1997), which in turn leads to lasting behavioral defects in the offspring (Fujii, 1997).

More recently, we have been demonstrated that pre and postnatal exposure to low doses of GLA in mice induced molecular and behavioral alterations in the offspring, reminiscent of autism spectrum disorder-like symptoms (Laugeray et al., 2014). Interestingly, these alterations occurred concomitantly to neurogenesis abnormalities in the ventricular-subventricular zone (V-SVZ) leading to the migration of unsuitable neuroblasts along the rostral migratory stream, as well as abnormal cell proliferation (Herzine et al., 2016). Furthermore, a transcriptomic analysis revealed that several processes involved in the development of the neurogenic environment such as cell-cell adhesion (Celsr2, ANK3), cell proliferation (MKi67), cell differentiation (GFAP, DCX, CD24, FoxJ1) and glutamate homeostasis (GS, NMDAR) were dysregulated in the brain of GLA-exposed offspring (Herzine et al., 2016). Altogether, these results point to V-SVZ neurogenesis being a relevant target for the neurotoxic effects of GLA and probably other exogenous molecules, raising the possibility that disturbing V-SVZ homeostasis is likely to be an important pathological hallmark of GLA related neurotoxicity.

The V-SVZ is constituted of heterogeneous cell populations with various proliferation and differentiation capabilities (Ihrle and Álvarez-Buylla, 2011). The V-SVZ stem cells are multipotent B1 cells which can differentiate into different cell types such as astrocytes, oligodendrocytes, ependymal cells (E cells) and neurons (Doetsch et al., 1997; Menn et al., 2006; Ortega et al., 2013). B1 cells can give rise to transit-amplifying neural progenitor cells (C cells) that in turn generate migrating neuroblasts (A cells) (Alvarez-Buylla and Lim, 2004). E cells cover the ventricular surface of the V-SVZ, and surround the B1 cells, forming a pinwheel architecture which constitutes a functional barrier, protecting the brain from noxious substances that accumulate in the cerebrospinal fluid (CSF) (reviewed by Del Bigio, 1995). Due to this architecture, E and B1 cells are in direct contact with the CSF (Mirzadeh 2008). Given that the CSF transports environmental toxicants and their by-products, disturbing V-SVZ homeostasis through E cell or B1 cell alterations may contribute to their functional neurotoxicity (Pardridge, 2011). In line with this assumption, disruption such as proliferation and differentiation within the V-SVZ niche, has been reported following exposure to polluting agents (Franco et al., 2014; Paradells et al., 2015), and is suspected to contribute to various tumorigenic cancers (Gilbertson and Rich, 2007; Zong et al., 2015). Here, we investigated the possibility that GLA or its metabolite 4-methylphosphinyl-2-oxo-butanoic acid (PPO) might negatively affect V-SVZ homeostasis. Due to murine primary cultures of neural stem cells (NSCs) having the capacity to differentiate into either B1 cells or E cells, we were able to investigate whether GLA induces deleterious effects on the ependymal barrier and the neuro/gliogenesis. We also addressed the adverse effects that PPO, one of the degradation products of GLA, resulting from the rapid oxidative deamination of GLA, has on these processes (Droge-Laser et al., 1994; Yoshioka et al., 2011). These questions should be addressed urgently, as some of these compounds accumulate in the blood of pregnant women (Aris and Leblanc, 2011), and may thus induce neurodevelopmental defects.

The aim of the present study is to assess the impact of GLA and the metabolite PPO on NSC development using an *in vitro* model of V-SVZ. This model allows us to investigate deleterious effects on both ependymal cell integrity and neuro/gliogenesis by inducing their capacity to differentiate. We studied the effect of GLA and PPO on the neural precursors from the V-SVZ. We evaluated various cellular processes such as cell survival, proliferation and differentiation. Our data suggest that GLA and the metabolite PPO not only disturb the ependymal barrier, but also alter the differentiation potential of NSCs.

2. Material and methods

2.1. Animals

Janvier Labs provided the breeding adults (Le Genest-St-Isle, France), and standard conditions were maintained with *ad libitum* access to water, food, and 12-hour light/dark cycles. Animal care and experimentation were in accordance with the European Communities Council directive (2010/63/EU) and approved by the local Ethics committee (Approval C45-234-6).

2.2. Synthesis of 4-methylphosphinyl-2-oxo-butanoic acid (PPO), a GLA intermediate metabolite

GLA (0.1 g, 0.5 mmol) was dissolved in 0.1 M sodium acetate buffer, pH 5 (2.5 mL). Pyridoxal phosphate (PLP) (0.145 g, 0.55 mmol) and CuCl₂ (0.034 g, 0.25 mmol) were added and the pH was adjusted to 5 using 1 M NaOH. The mixture was heated at 100 °C for 70 min. After cooling to room temperature, the mixture was poured onto a column of dowex 50WX8 (200-400mesh, H⁺ form, and 50 mL). The column was eluted with H₂O. The PPO containing fractions were combined and concentrated under reduced pressure. PPO was isolated as slightly yellow oil (0.073 g, 81%). Nuclear Magnetic Resonance (NMR) analyses in deuterated acetone showed PPO to be an 87:13 mixture of ketone and enol forms. ¹H NMR (400 MHz, acetone-d₆) ketone form: δ1.66 (3H, d, J = 15 Hz), 2.18 (2H, m), 2.95 (2H, m); Enol form: δ 1.59 (3H, d, J = 14.5 Hz), 2.89 (2H, dd, J = 8.5 and 19.5 Hz), 5.69 (1H, m), 8.5 (1H, s); ¹³C NMR (100 MHz, acetone-d₆) ketone form: δ14.2 (d, J = 93 Hz), 23.4 (d, J = 96 Hz), 32.1, 162.7, 194.2 (d, J = 14 Hz); Enol form: δ13.8 (d, J = 94 Hz), 28.2 (d, J = 92 Hz), 102.4, 144.5 (d, J = 12 Hz), 165.5 (d, J = 3 Hz); ³¹P NMR (162 MHz, acetone-d₆) δ44.81; MS (ES⁻) calc. *m/z* for C₅H₈O₅P: 179.0110, found: 179.0109

2.3. Primary culture of NSCs from the forebrain of newborn mice

In vitro murine primary NSC cultures were performed on postnatal days 1–5 (P1 to P5) C57BL/6JRj mice and NSCs were cultured according to the modified experimental procedures of Delgehr and colleagues (Delgehr et al., 2015). Briefly, newborn mice were sacrificed by decapitation, and brains were transferred to a Petri dish containing cold complemented Hank's solution (Hank's balanced salt solution, HBSS, SIGMA H6648; 1 M HEPES, SIGMA H3537; 1% sodium bicarbonate; 1000units/mL Penicillin-Streptomycin, P/S, GIBCO 15070-063). The olfactory bulbs and hippocampal were removed, and the meninges were peeled off at the surface of the telencephalon. The dissected telencephalon was mechanically dissociated and transferred with Hank's solution to a 15 mL Falcon tube. The supernatant was removed and 1 mL of the enzymatic digestion solution was added per tube (DMEM Glutamax-I SIGMA D5671 containing 10% Fetal Bovine Serum, FBS, GE Healthcare HyClone[™]; 1000units/mL P/S, GIBCO 15070-063; 2 mM L-Glutamine, GIBCO 25030-024; 39.2 mg/mL Papain, Worthington 3126; 12 mg/mL L-Cysteine, SIGMA C7352; 10 mg/mL DNase I, SIGMA DN25). After an incubation of 45 min at 37 °C, 5% CO₂, samples were centrifuged for 1 min at 110 g (4 °C), and 1 mL of the stop solution was added per tube (Liebovitz's L-15 medium, SIGMA L5520, containing 10 mg/mL DNase I). After a centrifugation (1 min 110 g 4 °C), the cells were rinsed with 5 mL of L15 medium. The cells were centrifuged for 5 min at 110 g 4 °C and were suspended mechanically in 1 mL of the NSCs growth medium (DMEM Glutamax-I medium; 10% FBS; 1000units/mL P/S; 2 mM L-Glutamine). The cells were inoculated into poly-L-Lysine (PLL; 5 µg/mL, BD Bioscience) precoated 25cm² flasks (one brain per flask). A humidified incubator maintained cell cultures at 37 °C with 5% CO₂ until confluence. The media was replaced the

following day.

2.4. Cell differentiation and treatments

When the cells reached confluence, usually 5 days after plating, the flasks were shaken at 250 rpm overnight at room temperature, in order to remove differentiated cells. The next day, the flasks were rinsed with sterile $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Dulbecco's phosphate-buffered saline (DPBS, SIGMA D8537). 1 mL of 0.05% Trypsin-EDTA (1X, GIBCO 25300-054) was added to each flask to remove cells, and flasks were incubated for under 15 min. After shaking briefly, the cells came to the surface, and 1 mL of NSC growth medium was added. The cells were centrifuged for 7 min at 110 g 4 °C, then suspended at high density ($1\text{--}2.10^6$ cells/mL or 2.10^5 cells per 20 μL for immunocytochemistry). Up to 6 cell suspensions should be grouped together to avoid the individual effect. The plates were incubated for 1 h in a cell incubator (37 °C 5% CO_2) to provide cell adhesion at high density before adding 1 mL of NSC growth medium per well. The culture now consisted of neural progenitor cells that differentiated progressively into neuroglia or ependymal cells depending on the culture medium used. The NSC growth medium was used for less than 7 days *in vitro* (DIV) to initiate spontaneous differentiation into neuroglial cells. A DMEM FBS-free medium (DMEM Glutamax-I; 1000 units/mL P/S; 2 mM L-Glutamine) was used for 10-15 DIV to obtain ependymal cells. The medium was removed every second day.

Our exposure model was a chronic treatment at various concentrations of GLA or PPO (1, 3, 10 and 100 μM). GLA (Glufosinate-ammonium, PESTANAL™, analytical standard, Sigma-Aldrich 45520) or PPO were added at the same time as the culture medium renewal, every second day. The GLA concentrations used during chronic exposure were in accordance with the work of Lantz and his collaborators (Lantz et al., 2014). Due to the lack of PPO data, the same concentrations as for GLA were used to allow comparison at equivalent doses.

2.5. Immunocytochemistry

Cell proliferation and differentiation were analyzed using an immunocytochemical staining method. NSCs were plated at a concentration of 2.10^5 cells per 20 μL into 24-well plates containing PLL precoated coverslips. 20 μL of the suspension cell was dropped onto a PLL-coated coverslip, and the treatment was repeated at each change in culture medium every two days. At the end of the exposure period, the medium was removed, and the coverslips were washed with TrisBuffer Saline 1X (TBS, pH 7.6, Tris–HCl 50 mM, NaCl 150 mM), fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. After washing, cells were permeabilized with 10% FBS; 0.2% Triton X-100 (Euromedex, ref 2000A) and 2% Bovine Serum Albumin (BSA Fraction V, ICN Biomedicals, ref 9048-46-8) for 15 min at room temperature. Coverslips were then incubated overnight at room temperature using the following primary antibodies: anti-Ki67 (1:500, polyclonal IgG rabbit, ABCAM ab15580) for cell proliferation; antiNestin (1:500, monoclonal IgG1k mouse, ABCAM ab11306) for B1 and C cells; anti-GFAP (1:500, monoclonal IgG1 mouse, SIGMA G3893 or 1:500, polyclonal IgG rabbit, Dako Z0334) for B1 cells and astrocytes; anti-S100beta (1:100, monoclonal IgG1 mouse, SIGMA S2532) for mature astrocytes; antiDoublecortin (1:500, polyclonal IgG rabbit, ABCAM ab77450) for A cells; anti-Olig2 (1:500, polyclonal IgG rabbit, Millipore AB9610) for oligodendrocyte precursor cells; antiCD24 [m1/69] (1:500, monoclonal IgG2b rat, ABCAM ab64064) and antiPolyglutamylated tubulin (1:500, monoclonal IgM mouse ABCAM ab11324) for ciliated ependymal cells. Coverslips were rinsed with TBS1X and incubated with the appropriate secondary antibodies (antirabbit, antimouse or anti-rat Alexa-488 or TRITCconjugated) for 45 min at room temperature in 2% BSA and 10% FBS solution. Coverslips were rinsed and then DAPI was used to counterstain the cell nuclei. Coverslips were mounted on glass slides in the mounting medium Fluoromont-g®.

Microscopic images were obtained using a fluorescent microscope Leica DM6000B with Metamorph® software with 20x, 40x or immersed 100x magnification.

Quantitative analysis was performed on the four wells used for each combination of double immunostaining. Each well corresponded to a pool of up to six individual suspension cells allowing homogenizing NSC cultures. Microscopic images of 5–6 fields per slide were analyzed using ImageJ® software. Each field of microscopic images represented a mean of 200 cells. Values were averaged across the fields to render a single value for each condition. Results are expressed as percentage of control group.

2.6. Real-time quantitative polymerase chain reaction

After treatment, total RNA was extracted using TRIzol reagent (Ambion, Life technologies). The reverse transcription was performed on 1 μg of total RNA using Superscript III Reverse Transcriptase (Invitrogen, Thermo Scientific). All cDNA samples were diluted by one-fifth using 200 nM of each TaqMan® primer using the Expression Master Mix (Applied, Life technologies). Real-time quantitative polymerase chain reactions (RT-qPCR) were carried out using Stratagen Mx3005 P Agilent material (Agilent Technologies). The amplifications were performed by an initial denaturation step at 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 seconds and at 60 °C for 1 min. TaqMan® primer sequences used were *Celsr2* (Mm 00457515-m1), *FoxJ1* (Mm 01267279-m1) and *Ngn2* (Mm 00437603-g1).

Each sample was evaluated in triplicate and each triplicate corresponded to a pool of up to six individual suspension cells allowing homogenizing NSC cultures. Three independent experiments were carried out for each condition. Data were analyzed using the $\Delta\Delta C_t$ method and normalized to the reference standard *RNA18S* (Mm 03928990-g1). Three independent experiment of RT-qPCR were carried out.

2.7. Cell viability

Cell viability was assessed using tetrazolium dye 3-(4, 5-dimethylthiazol-2-yl)2, 5-diphenyl-2H-tetrazolium bromide (5 mg/mL MTT) according to the manufacturer's instructions (Vybrant® MTT Cell Proliferation Assay Kit, ThermoFisher Scientific). Cells were plated onto PLL precoated 96-well plates and treated with GLA and PPO for 48 h. Wells were rinsed with HBSS and the MTT was added to the medium (12 mM stock solution). After 4 h of incubation at 37 °C and 5% CO_2 , the medium was removed, and the formazan end-product was solubilized in SDS-HCl. After 2 h at 37 °C and 5% CO_2 , formazan production was determined by spectrophotometry (CLARIOstar, BMG LABTECH) at a wavelength of 562 nm.

Each sample was evaluated in triplicate and each triplicate corresponded to a pool of up to six individual suspension cells allowing homogenizing NSC cultures. Three independent experiments were realized for each condition.

2.8. Statistics

Data was analyzed using a one-way ANOVAs followed by Dunn's multiple comparisons 262 test, using XLSTAT® for Windows (XLSTAT 2018: Data Analysis and Statistical Solution for 263 Microsoft Excel. Addinsoft, Paris, France (2018)). Each mean was compared with the control 264 mean. All data shown in the graphs are expressed as mean \pm SEM. SEM is used as statistical 265 measure as it assesses the estimated means of groups of measurements relate to each other; 266 instead of describe the variability within a group of individual measurements such as the SD 267 (Pleil, 2016). The values were considered significantly different in comparison to the control 268 group in which the p-value was < 0.05.

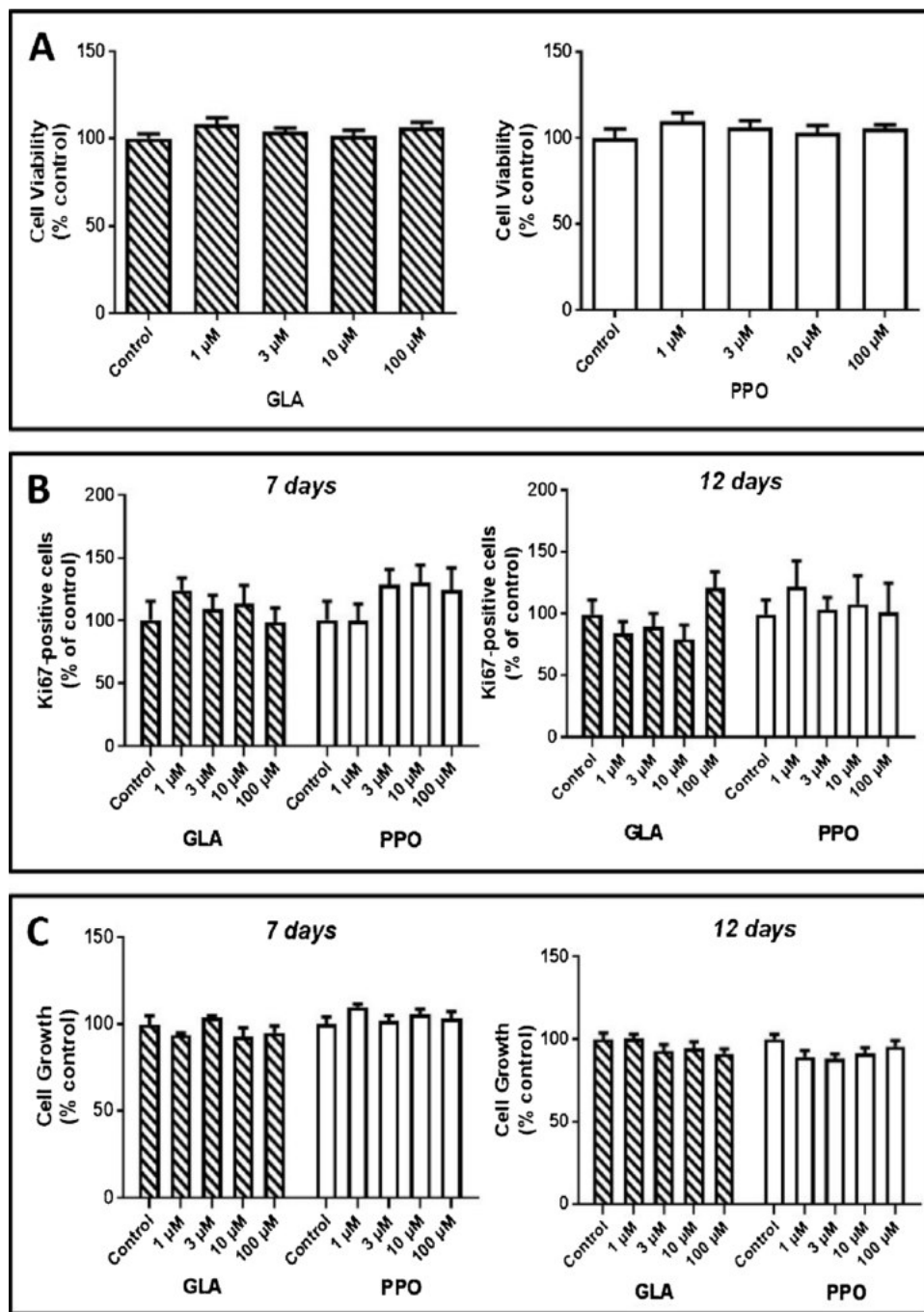


Fig. 1. Neither GLA nor PPO induced toxicity in cultured V-SVZ NSCs.

Cell viability, proliferation and cell growth were quantified by MTT assay, Ki67-immunostaining and DAPI-stained nuclei respectively on NSCs exposed at different concentrations of GLA or PPO (1, 3, 10 and 100 μM). (A) MTT assay was performed on NSCs following 48-hours of exposure (n = 4) (ns p > 0.05). (B) Cell proliferation was measured using Ki67 immunocytochemistry at 7DIV (neuro-glial cells) or 12DIV (ependymal cells) after exposure to GLA or PPO. Cells were seeded at high density with 2.10⁵ cells / well and Ki67 expression was quantified using ImageJ®. The percentage of Ki67-positive cells was calculated as the ratio of Ki67-positive cells compared to the total number of cells with DAPI-stained nuclei (n = 4; an average of 200 cells analyzed per replicate) (ns p > 0.05). (C) NSCs were differentiated and exposed to GLA or PPO for 7DIV or 12DIV then cell growth was measured by DAPI-stained nuclei (n = 4; an average of 200 cells analyzed per replicate) (ns p > 0.05).

3. Results

3.1. Neither GLA nor PPO induced toxicity in cultured V-SVZ NSCs

V-SVZ is the main neurogenic niche in which NSCs constitute a heterogeneous population with various proliferation and differentiation capabilities. Some cells are in direct contact with the CSF, making them potential targets for exogenous components. We were interested in verifying whether GLA and its metabolite PPO exerted toxicity on V-SVZ NSCs.

With this aim, we investigated the impact of these molecules on cell viability, growth and proliferation. Our results showed that none of these compounds had deleterious effects on cell viability measured by the MTT test (Fig. 1A). We also assessed proliferative abilities of

cultured VSVZ NSCs through Ki67 immunocytochemical staining at 7DIV (NSCs-to-macrogial cells differentiating time point) and 12DIV (NSCs-to-ependymal cells time point). To assess the effect on cell proliferation, Ki67-positive cells were counted after immunocytochemistry staining. In our experimental conditions, neither GLA nor PPO treatments had any effect on the proliferation of macrogial cells (7DIV) or ependymal cells (12DIV) (Fig. 1B). Cell growth was then analyzed by measuring the total number of nuclei in GLA or PPO exposed cells in comparison to the control. In line with the absence of effect on proliferation, neither GLA nor PPO affected the total cell number in NSC cultures compared to the control (Fig. 1C).

Altogether, the data indicate that neither GLA nor PPO affected viability nor proliferative parameters of NSC primary cultures.

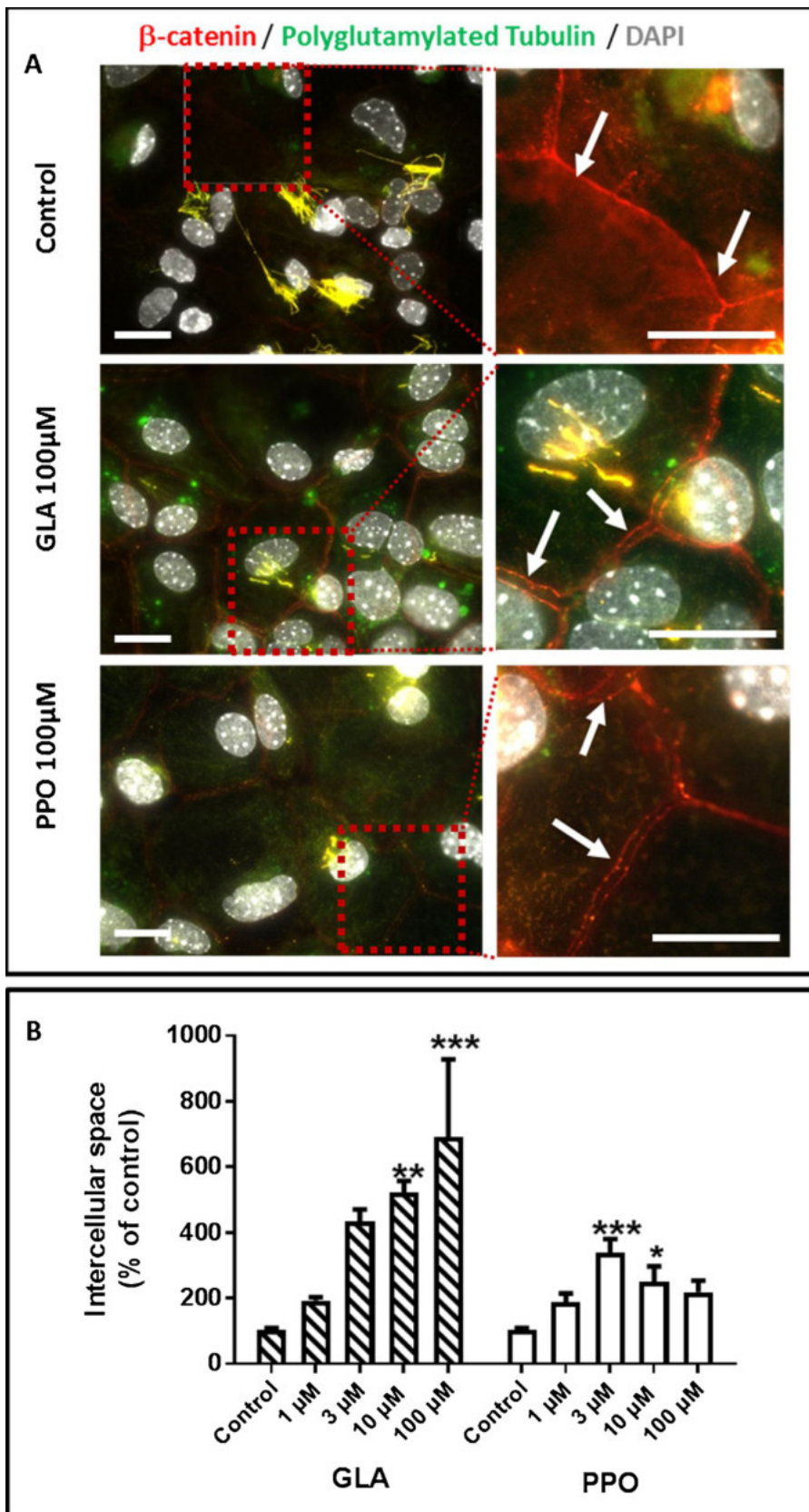


Fig. 2. GLA and PPO disrupt cell-cell adhesion in differentiated V-SVZ NSCs.

Immunocytochemistry showing β -catenin/Poly glutamylated tubulin on endymal cells with analysis of intercellular space at 12DIV after GLA or PPO exposure. (A) Microscopic images of intercellular junctions between endymal cells with β -catenin (red), polyglutamylated tubulin (green), DAPI (white) at magnification 60x (scale bar 20 μ m). Arrows point to intercellular space. (B) Measurement of intercellular space in endymal cells with β -catenin staining showing that GLA, and to a lesser extent PPO, significantly disrupt the cellular adhesion in endyma (n = 4; an average of 200 cells analyzed per replicate) (*p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001).

3.2. GLA and PPO disrupt cell-cell adhesion in differentiated V-SVZ NSCs

NSCs are capable of differentiating into different neuroglia cell types, namely neurons, astrocytes, oligodendrocytes and E cells. E cells constitute the front line between the CSF and the brain parenchyma. They protect the brain from exogenous agents through the constitution of a well-formed epithelium *via* adherens junctions and cell-cell contacts (Mirzadeh et al., 2008).

We have previously demonstrated that perinatal exposure to GLA induced potent alterations of the cytoskeleton and cell-cell adhesion process. We hypothesized that GLA may act through alterations of the integrity of the ependymal epithelium. To test this hypothesis, NSCs differentiated into E cells were exposed to either GLA or PPO, and cell-cell adhesion was investigated by beta-catenin immunocytochemistry staining and intercellular space measurement (Fig. 2A). Our results show a significant dose-dependent increase in intercellular space after GLA exposure (691.5 ± 237 at $100\mu\text{M}$; $***p < 0.005$). To a lesser extent, PPO treatment also induced an increase in intercellular space with a peak effect at $3\mu\text{M}$ PPO (338 ± 42.73 ; $***p < 0.005$) (Fig. 2B).

These results show that GLA and PPO induce a loss of cell-cell interaction in ependymal epithelium that may subsequently lead to a disruption of the underlying neurogenic zone.

3.3. GLA and PPO impact the capacity of ependymal cells to produce cilia

One of the main functions of ependymal cells is to propel the flow of CSF in the brain ventricles using the cilia extending from their apical surface. Cilia production being strongly dependent on cytoskeleton integrity, we quantified the number of multiciliated tufts using tubulin/polyglutamylated tubulin immunocytochemistry (Fig. 3A). Polyglutamylated tubulin is a post-translational modification of the C-terminal tail of tubulin indispensable for microtubule stabilization (Janke and Kneussel, 2010). Our results showed a significant dose-dependent decrease in the number of multiciliated clusters following GLA or PPO treatments in comparison to the control condition (GLA $100\mu\text{M}$ 23.25 ± 4.62 and PPO $100\mu\text{M}$ 20.50 ± 1.05 ; $****p < 0.001$) (Fig. 3B). To investigate whether this resulted from a differentiation defect of NSCs into E cells, we used CD24 as a specific E cell marker. There was no change in the number of CD24-positive cells following GLA or PPO exposure (Fig. 3C). These results indicated that GLA and PPO do not alter the ependymal differentiation of NSCs, but disrupt cilia production. To support this hypothesis, we quantified the relative gene expression of *FoxJ1*, a transcription factor necessary and sufficient for motile cilia biosynthesis (Jacquet et al., 2009; Yu et al., 2008). We also analyzed the expression pattern of *Celsr2*, a planar cell polarity cadherin controlling the development of cilia (Feng et al., 2012; Tissir et al., 2010). While *FoxJ1* expression was unaltered following GLA or PPO treatment, there was a significant decrease in *Celsr2* gene expression following treatment with PPO $100\mu\text{M}$ (55.25 ± 14.16 ; $*p < 0.05$) (Fig. 3D).

These results indicate that either GLA or PPO impaired the capacity of ependymal cells to produce cilia, with compound-specific underlying mechanisms.

3.4. GLA and PPO exposure lead to abnormal neuro-glial differentiation of NSCs

Underlying the ependyma, B1 cells play the role of primary progenitors giving rise to neurons, astrocytes and oligodendrocytes in the forebrain SVZ (Doetsch et al., 1999; Menn et al., 2006). Disturbances in B1 cell homeostasis are thus likely to modify the differentiation towards gliogenic and neurogenic lineage. To investigate such a possibility, we labelled and quantified the different populations of V-SVZ precursor cells using specific cell markers. B1 cells are GFAP and Nestin double-positive whereas C cells are Nestin-positive cells (Fig. 4A). GFAP and Nestin correspond to intermediate filaments mainly found in astrocyte-

like stem cells. Mature astrocytes were co-labelled with GFAP and S100 β proteins, two specific markers of astroglia lineage (Fig. 4B). Doublecortin (DCX) is a specific marker for A cells (Fig. 4C). Olig2 is a basic helix-loop-helix transcription factor of oligodendrocyte progenitor cells (OPC) in the V-SVZ (Fig. 4C). Furthermore, in our *in vitro* model, the different populations of V-SVZ 339 precursor cells express the receptors NMDAR and mGluR5 (supplementary data).

Our results show that GLA exposure increased the *Neurogenin2* (*Ngn2*) expression, a transcription factor inducing the neuronal fate (Fig. 5D) (Imayoshi and Kageyama, 2014), in accordance with the significant increase of A cell production (173.50 ± 31.50 at $100\mu\text{M}$; $*p < 0.05$) (Fig. 5C). Interestingly, GLA did not modify the number of B1 cells (Fig. 5A) but significantly reduced the pool of progenitor C cells (69.75 ± 5.12 ; $*p < 0.05$) (Fig. 5B). GLA exposure had no impact on astrocytes (Fig. 5E). Interestingly, we found a dose-dependent increase in Olig2-positive cells after GLA exposure (286.7 ± 33.30 at $100\mu\text{M}$; $***p < 0.005$) (Fig. 5F). These results suggest that GLA maintains B1 and C cells and promotes the differentiation of neuronal and oligodendroglial lineages (Fig. 5G). The Fig. 5H represents the proportion of each cell type within the neuroglial-differentiated cultures for the control compared to GLA $10\mu\text{M}$ condition (Fig. 5H).

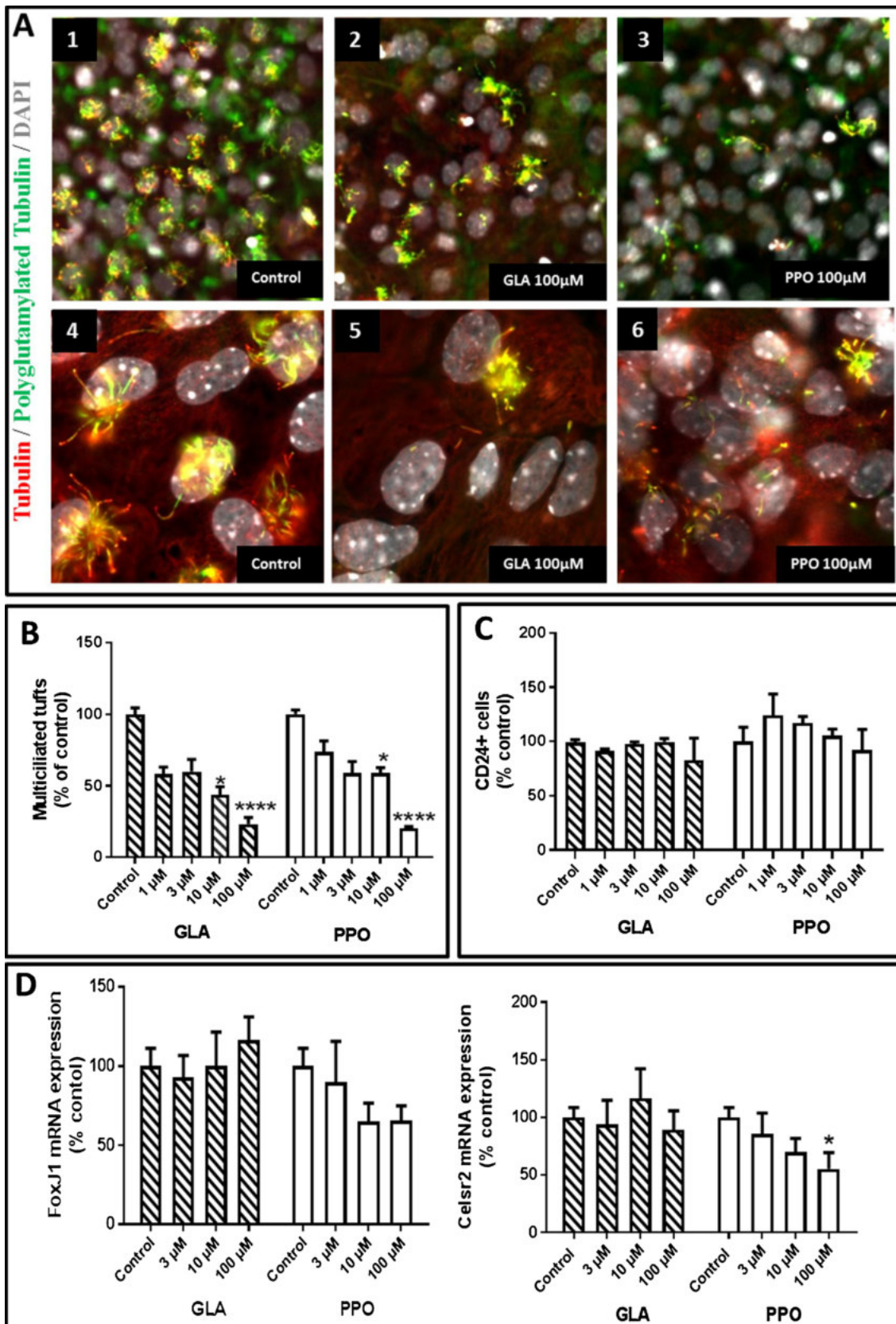
Following PPO treatment, we noted a significant increase of B1 cell population (158 ± 12.6 at $100\mu\text{M}$; $***p < 0.005$) (Fig. 6A). No difference was observed for C cells (Fig. 6B). However, we quantified a significant and dose-dependent decrease of A cells after PPO exposure (23.41 ± 5.06 at $100\mu\text{M}$; $***p < 0.005$) (Fig. 6C). PPO disturbed *Ngn2* expression (Fig. 6D). PPO exposure had no effect on astrocytes (Fig. 6E), but significantly increased the Olig2-positive population in comparison to the control condition (163.3 ± 5.20 at $10\mu\text{M}$; $***p < 0.005$) (Fig. 6F). This data shows that PPO exposure reduced A cell production and increased the proliferation of B and OPC compared to GLA (Fig. 6G). Our data suggest that PPO significantly enhances the B1 cell population but clearly impairs the neuronal fate of NSCs in favor of the oligodendroglial lineage. Similarly, to GLA, the Fig. 6H represents the proportion of each cell type within the neuroglial-differentiated cultures for the control compared to PPO $10\mu\text{M}$ condition (Fig. 6H).

Altogether, these data demonstrate that both GLA and PPO affect cell differentiation in the V-SVZ, leading to putative alterations in cell patterning and cell fate specification in the developing brain.

4. Discussion

The results of the present study show GLA and PPO exposure have a significant impact on the formation and the maintenance of the V-SVZ neurogenic niche *in vitro*. Our data indicate that exposure to GLA or its main metabolite PPO interferes with the formation of the ependymal epithelium and modulates the neuro-glial differentiation of B1 cells without influencing their ability to grow and proliferate.

Multiciliated E cells cover the lateral wall of lateral brain ventricles shaping a protective interface between the CSF and the brain parenchyma (Del Bigio, 2010, 1995). Cilia are dynamic structures at the apical surface of E cells that express a broad range of membrane transporters and receptors for various signal molecules present in the CSF (Prothmann et al., 2001; Svanidze et al., 2012). Hence, E cells maintain the V-SVZ neurogenic microenvironment and the disruption of E cell differentiation and ciliogenesis can indirectly affect V-SVZ progenitors (Grondona et al., 2013; Jacquet et al., 2009; Peng et al., 2013). E cells are derived from radial glial cells, primary stem cells in the embryonic brain, and become mature during the early post-natal period (Spassky, 2005). Due to their origin, E cells harbor some glial features notably the expression of glutamine synthetase (GS), and glutamate transporters such as EAAT3 and xCT (Akimoto et al., 1993; Del Bigio, 1995). GS is an important enzyme for amino acid metabolism. Furthermore, it is well known that the herbicidal action of GLA is related to the irreversible inhibition of GS due to its structural analogy



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Fig. 3. GLA and PPO impair the capacity of ependymal cells to synthesize cilia.

GLA and PPO induce a modification in the number of multiciliated E cells and do not disturb ciliogenesis. An immunocytochemistry and a RT-qPCR analysis were performed on E cells following GLA and PPO treatment during 12DIV. (A) Microscopic images of multiciliated clusters stained with antibodies specific for tubulin (red) or polyglutamylated tubulin (green) to visualize cilia at magnification 20x (A1-3) and 100x (A4-6). (B) The number of multiciliated tufts was quantified using Tubulin/PolyE tubulin-positive cilia at 12DIV after pesticide exposure. GLA and PPO treatment induced an important decrease in the number of multiciliated clusters ($n = 4$; an average of 200 cells analyzed per replicate) ($*p < 0.05$; $****p < 0.001$). (C) CD24-positive cell quantification at 12DIV after GLA or PPO exposure. GLA and PPO treatment induced no significant impact on the number of ependymal cells ($n = 4$; an average of 200 cells analyzed per replicate) ($ns p > 0.05$). (D) RT-qPCR analysis of ciliogenesis gene expression in ependymal cells 12DIV after pesticide exposure ($*p < 0.05$).

with glutamate (Ebert et al., 1990).

Here, we showed that GLA or PPO exposure significantly reduced the number of multiciliated tufts, without altering the ependymal differentiation of E cells, but with a significant loss of cell adhesion. We focused on the effect of GLA or PPO exposure on ciliogenesis by investigating mRNA *FoxJ1* and *Celsr2* expression. *FoxJ1* is essential and sufficient for progenitor cell differentiation into E cells during development until the first postnatal weeks, and *FoxJ1* expression is a prerequisite for postnatal neurogenesis in the olfactory bulb (Jacquet et al., 2011, 2009). *FoxJ1* also interacts with Ankyrin 3 (ANK3), a larger adaptor molecule of the Ankyrin family of proteins in mammals, and a deletion of this pathway leads to the complete loss of the SVZ niche structure and results in a dramatic depletion of neurogenesis (Paez-Gonzalez et al., 2011). *Celsr2* is the cadherin of the planar cell polarity (PCP) complex. PCP is a multistep mechanism process organized by motile cilia in E cells, a critical element for the ciliary beating (Mirzadeh et al., 2010). A defect in *Celsr2* expression leads to ependymal function abnormality but without alteration of mobility (Tissir et al., 2010). Our data revealed no alteration in mRNA *FoxJ1* expression after pesticide exposure. Nevertheless, we noted a significant decrease

of mRNA *Celsr2* expression after E cells were exposed to a high dose of PPO *in vitro*. We concluded that E cells still express the essential factors for the biosynthesis of mobile cilia following GLA and PPO exposure, but that E cells fail to produce cilia as in control conditions. Our results showed no ependymal differentiation defect, but in fact a failure in cilia formation. Consequently, we hypothesized that GLA and PPO may interfere with a down-stream mechanism of ependymal ciliogenesis. During cilia formation, tubulin polyglutamylation, a mechanism involving glutamate, could be targeted by GLA or PPO and therefore disrupted. This phenomenon is important in axonemal maturation, and mainly occurs in neural cells (Janke et al., 2008). GLA and PPO are structural analogues of glutamate, and may take the place of glutamate and thus disrupt tubulin polyglutamylation. However, whether GLA or PPO interfere with polyglutamylation enzymatic mechanisms is still unknown.

GLA or PPO exposure could thus result in the disruption of ependymal integrity and lead to disorders in the underlying neurogenic niche. In fact, E cells and B1 cells are strongly associated and both bring physical and chemical factors to the regulation of B1 cell self-renewal and multipotentiality (Chojnacki et al., 2009). This close relationship

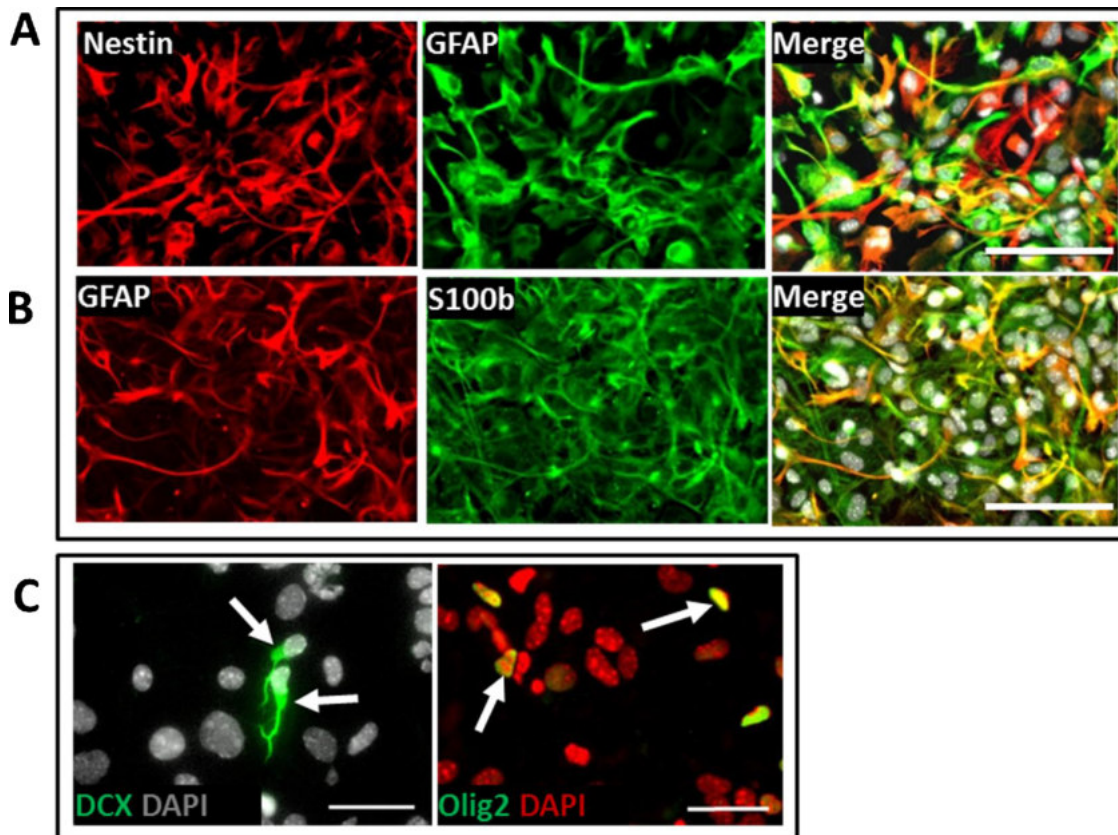


Fig. 4. Specific markers of different populations of V-SVZ precursor cells.

Immunocytochemistry of NSCs at magnification 20x (scale bar = 50 μ m). (A) B1 cells were colabelled for Nestin (red), a prototypic marker for undifferentiated NSCs, and GFAP (green) whereas C cells were identified as Nestin-positive and GFAP-negative immunostained cells (scale bar = 100 μ m) (B) Astrocytes were double-positives for GFAP (red) /S100b (green) (scale bar = 100 μ m). (C) Neuroblasts and oligodendrocyte progenitor cells were immunolabelled for Doublecortin (DCX) (green) and Olig2 (yellow) marker respectively (scale bar = 50 μ m). Arrows point to DCX and Olig2-positive cells.

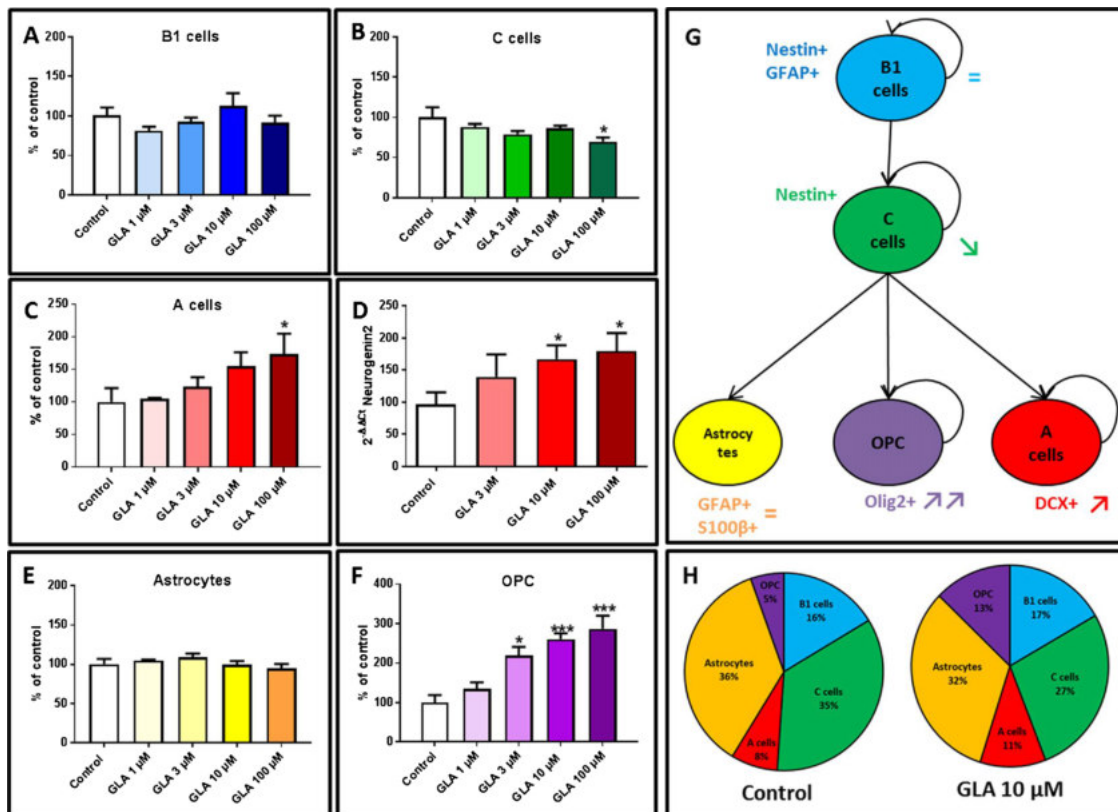


Fig. 5. GLA impairs NSC neuro-glial differentiation.

Percentage of differentiated NSCs identified by specific markers in immunocytochemistry. NSCs cultured in culture medium supplemented with FBS for 7DIV in macroglial cells after GLA or PPO exposure. (A) Type B1 cells were monitored for the presence of the stem cell marker, Nestin, and the astrocytic marker, GFAP. GLA seems to maintain the pool of primary progenitors (n = 4; an average of 200 cells analyzed per replicate) (ns p > 0.05). (B) The number of type C cells, measured by immunocytochemistry Nestin-positive and GFAP-negative cells, decreased significantly following GLA exposure (n = 4; an average of 200 cells analyzed per replicate) (*p < 0.05). (C) The number of type A cells, quantified by DCX-stained cells, increased significantly following GLA exposure (n = 4; an average of 200 cells analyzed per replicate) (*p < 0.05). (D) *Neurogenin2* gene expression quantified by RT-PCRq increased significantly after GLA exposure (n = 3; independent experiments). (E) Astrocytes were counted by Nestin-negative and GFAP-positive cells and were not modified by GLA exposure (n = 4; an average of 200 nuclei per replicate) (ns p > 0.05). (F) Oligodendrocytes were analyzed by Olig2-stained cells immunocytochemistry and data revealed that GLA promoted the oligodendrocyte lineage (n = 4; an average of 200 cells analyzed per replicate) (*p < 0.05; ***p < 0.005). (G) Schematic representation of the effects of GLA on NSC neuro-glial differentiation. (H) The proportion of each cell type within the neuro-glial-differentiated cultures for the control and GLA 10 μM conditions.

makes the ependyma an important modulator of SVZ cell populations. Moreover, E cells organize the SVZ through the production of trophic factors and metabolic regulation, including glucose uptake and ion homeostasis, thus creating a favorable neurogenic environment for the underpinning SVZ (Del Bigio, 2010, 1995). A disruption of the ependyma can lead to an overall disorganization of the neurogenic niche, with a loss of B1 cells, and a clear decrease in neural progenitors, leading to several disorders such as neural tube defects, ciliary dyskinesia and hydrocephalus (Jiménez et al., 2009, 2014).

Underneath the ependymal epithelium, the neurogenic niche constitutes a pool of multipotent B1 cells which can differentiate into different cell types such as astrocytes, oligodendrocytes, E cells and neurons (Doetsch et al., 1997; Menn et al., 2006; Ortega et al., 2013). B1 cells can give rise to transit-amplifying neural progenitor cells (C cells) that in turn generate migrating neuroblasts (A cells) (Alvarez-Buylla and Lim, 2004).

We questioned the potential impact of GLA and PPO on B1 cells and their progenitors. We observed a proliferation of intermediate progenitor cells (OPC and A cells) without affecting B1 and C cell pools after GLA exposure. B1 cells exhibit potential targets of GLA inherited from radial glial cells such as GFAP, GS, metabotropic glutamate receptors and transporters (Ciceroni et al., 2010; Melchiorri et al., 2007; Sánchez-Mendoza et al., 2017). However, B1 population does not seem to be affected by GLA. In fact, in another study, glutamate was not cytotoxic for B1 cells and increased their survival and proliferation

(Brazel et al., 2005). Similarly, astroglial cell population does not seem to be affected by GLA exposure. Intermediate progenitor A cells and OPCs express metabotropic and ionotropic functional receptors (Faigle and Song, 2013; Platel et al., 2010a) and are thus potential targets of GLA. We did observe a significant increase in OPCs and A cells after GLA exposure. Our data thus suggest that GLA favors the OPC lineage. This effect could be due to the disturbance of glutamatergic signal. In accordance with several studies, glutamate and its receptors have a beneficial effect on the proliferation and differentiation of neural progenitor cells derived from the SVZ niche (Brazel et al., 2005; Fan et al., 2012; Jansson and Åkerman, 2014; Lai et al., 2016; Platel et al., 2010b, 2007). Notably, the action of glutamate on glutamate receptors positively modulates the production of immature progenitor cells, mainly on ionotropic receptors expressed by OPCs and A cells. Furthermore, an oxidative stress mediated by glutamate can lead to an excitotoxicity. However, neurogenesis and oligodendrogenesis are enhanced following an oxidative stress (Pérez Estrada et al., 2014). The variation in C population after exposure to GLA 100μM is likely to be correlated to the increase in OPCs and A cells.

We showed that PPO, the first metabolite of GLA, had different effects on V-SVZ cells. In contrast to GLA, the effects of PPO on the glutamatergic system were previously unreported, but our data clearly demonstrated a modification of cell differentiation associated with a neurogenesis defect after PPO exposure. Using our *in vitro* model, we observed a significant increase in B1 cell and OPC population, whereas

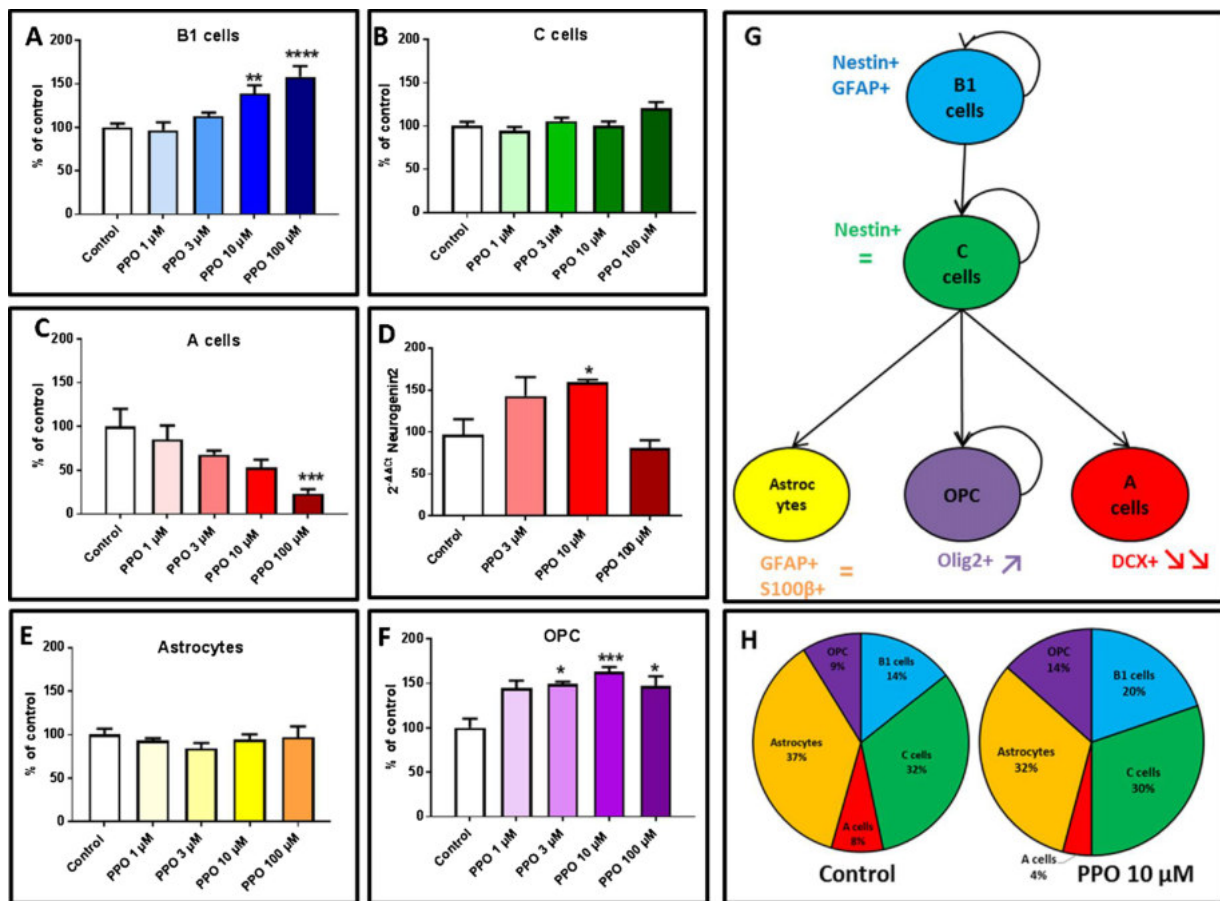


Fig. 6. PPO impairs neuro-glial differentiation of NSCs. Percentage of differentiated NSCs identified by specific markers as determined by immunocytochemistry. NSCs cultured in culture medium supplemented with FBS for 7DIV in macroglial cells after GLA or PPO exposure. (A) Type B1 cells were monitored for the presence of the stem cell marker Nestin, and the astrocytic marker, GFAP. At higher doses, PPO seemed to promote the generation of type B1 cells (n = 4; an average of 200 cells analyzed per replicate) (**p < 0.01; ****p < 0.001). (B) Type C cells, using immunocytochemistry to determine Nestin-positive and GFAPnegative cells, were not affected by PPO treatment (n = 4; an average of 200 cells analyzed per replicate) (ns p > 0.05). (C) The number of type A cells, quantified by DCX-stained cells, decreased significantly following PPO exposure (n = 4; an average of 200 cells analyzed per replicate) (***p < 0.005). (D) *Neurogenin2* gene expression, quantified by RT-PCRq was altered after PPO exposure (n = 3; independent experiments) (*p < 0.05). (E) Astrocytes, Nestin-negative and GFAP-positive cells, were promoted by PPO (n = 4; an average of 200 cells analyzed per replicate) (ns p > 0.05). (F) Oligodendrocytes were analyzed using Olig2-stained cell immunocytochemistry, and data revealed that PPO also promoted the oligodendrocyte lineage (n = 4; an average of 200 cells analyzed per replicate) (*p < 0.05; ***p < 0.005). (G) Schematic representation of the effects of PPO on NSC neuro-glial differentiation. (H) The proportion of each cell type within the neuro-glial-differentiated cultures for the control and PPO 10 μM conditions.

neurogenesis was significantly decreased. Indeed, few studies addressed the cellular or molecular mechanisms involved in the neurotoxic action of pesticides and their metabolites on the CNS (Franco et al., 2010; Mathieu et al., 2017). Most studies documented the metabolism of active substances by determining the concentration of metabolites in the placenta, urine or semen (Bradman et al., 2011; Komsky-Elbaz and Roth, 2017; Melgarejo et al., 2015; Richardson et al., 2014). Here, we reported direct deleterious cellular effects of PPO on neural stem cells for the first time. Our results clearly show the need for further study into the molecular mechanisms targeted by this GLA metabolite. We demonstrated a neuroactive effect of PPO GLA metabolite. However, the PPO mode of action remains unknown. Hack and colleagues showed that the compound PPO was reversibly aminated to GLA, but was unstable (Hack et al., 1994). This instability was due to the spontaneous decarboxylation of PPO in aqueous solution leading to the formation of a second GLA metabolite, the 3-methylphosphinopropanoic acid (MPP). MPP was found to be a more stable end product of plants' metabolic pathways than PPO (Hack et al., 1994). The potential conversion of PPO to MPP or to GLA in mammal cells cannot be excluded. Thus, PPO may have a multifold action, with either similar or different molecular targets to GLA itself. Therefore, further studies should aim at

defining the molecular actors involved in the observed neuroactive effects of GLA or its metabolites. In the future, a detailed knowledge of the different regulatory pathways involved in the regulation of SVZ cell fate under normal conditions and following pesticide exposure will be primordial for studying the CNS responses to environmental cues.

5. Conclusions

By specifically targeting stem cells and following their progeny *in vitro*, our model will be suitable for screening potential effects of xenobiotics on the V-SVZ neurogenic niche. These stem cells express major features of *in vivo* V-SVZ cells and differentiate into ependymal or neuro-glial cells. Overall, our results fit into the conceptual framework of modern toxicology currently in development. These *in vitro* models are new powerful tools for screening substances present in the environment. Our findings demonstrated the possibility that GLA and its main metabolite PPO influenced neural stem cell differentiation for the first time. We quantified the deleterious effect on the ependymal epithelium and the modification of neural stem cell fate following GLA and PPO exposure. Previous studies documented the *in vivo* effects of GLA by behavioral assessment, although no data analyzed its actions at

cellular or molecular levels. In this study, we highlighted the neural deleterious effects of PPO, the first GLA metabolite, at cellular level for the very first time. It is now important to explore cellular effects of PPO in more detail, including its currently unknown mechanism and molecular targets. In this context, our data will contribute to the establishment of a suitable method for assessing the neurotoxicity of GLA, its metabolites such as PPO, and other substances in the category "amino acid structural analogues".

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Conflicts of interest

The authors indicate no potential conflicts of interest.

Authors contributions

JFV and SM developed the concepts and designed the experiments; JFV performed *in vitro* experiments with the help of VL, GD, CM and AH; JFV analyzed and discussed results with the help of SM, VL, GD, OR, CMD. TG provided study materials. JFV, VL, AL, VQ, CMD, SM wrote the paper; all authors edited the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.neuro.2018.10.001>.

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