Glyphosate and glyphosate-based herbicide exposure during the peripartum period affects maternal brain plasticity, maternal behaviour and microbiome

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Glyphosate and Glyphosate-based herbicide exposure during the peripartum period affects maternal brain plasticity, maternal behavior and microbiome

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ABSTRACT

Glyphosate is found in a large array of non-selective herbicides such as Roundup® and is by far the most widely used herbicide. Recent work in rodent models suggests that Glyphosate-based herbicides during development can affect neuronal communication and result in altered behaviors, albeit through undefined mechanisms of action. To our knowledge, no study has investigated the effects Glyphosate or its formulation in herbicide on maternal behavior and physiology. Here, relatively low doses of Glyphosate (5 mg/kg/d), Roundup® (5 mg/kg/day of Glyphosate equivalent), or vehicle were administered by ingestion to Sprague Dawley rats from gestational day (GD) 10 to postpartum day (PD) 22. The treatments significantly altered licking behavior toward pups between PD2 and PD6. We also show in the dams at PD22, Roundup exposure affected the maturation of doublecortin-immunoreactive new neurons in the dorsal dentate gyrus of the hippocampus of the mother. In addition, the expression of synaptophysin, was upregulated by glyphosate in the dorsal and ventral dentate gyrus and CA3 regions of the hippocampus, and downregulated in the cingulate gyrus. While a direct effect of glyphosate alone or its formulation on the central nervous system is currently not clear, we show here that gut microbiota is significantly altered by the exposure to the pesticides, with significant alteration of the phyla Bacteroidetes and Firmicutes. This is the first study to provide evidence that glyphosate alone or in formulation (Roundup) differentially affects maternal behavior and modulates neuroplasticity and gut microbiota in the mother.

KEYWORDS

Neurogenesis; Neuroplasticity, Hippocampus; Cingulate gyrus; Gut microbiota

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INTRODUCTION

Glyphosate (N-phosphonomethyl-glycine) is the active compound found in over 750 broad-spectrum herbicides and is widely used across the world [1]. In less than 20 years, Glyphosate use has significantly increased, from around 50,000 tons of bioactive compound sold in 1994 to over 825,000 tons in 2014, mostly due to the development of Glyphosate-resistant genetically modified corn and soy and pre-harvest application techniques [1,2]. Glyphosate inhibits the 5-enolpyruvyl shikimate-3-phophate synthase (EPSPS), a key enzyme in the shikimate pathway [3,4]. This metabolic pathway is required for the biosynthesis of aromatic amino acids (tryptophan, tyrosine and phenylalanine) in plants, as well as in bacteria and fungi. Because this pathway is absent in vertebrates, Glyphosate was considered safe for animals and humans [3,5]. However, a few studies link Glyphosate-based herbicide (GBH) exposure to some cancers in human [1,6] while others do not find any association [7–10], making risk assessment difficult for regulatory agencies. Indeed in 2015, Glyphosate was classified as "probably carcinogenic for humans" (Group 2A) by the International Agency for Research on Cancer [11] while the European Food Safety Authority [12] and the European chemical agency [13] concluded that Glyphosate is not carcinogenic, mutagen or genotoxic for reproduction. The debate on the safety of Glyphosate and Glyphosate-based herbicide in term of carcinogenicity is currently open [14–17].

More recently, an increasing number of studies suggest that GBH exposure might affect vertebrate development and physiology [18–20]. More precisely, several laboratories found that Glyphosate exposure could alter brain physiology and behavior, independently of its potential carcinogenic and mutagenic properties. Indeed, GBH induced alteration in neurotransmitter dopamine, norepinephrine, serotonin and glutamate levels and their metabolites in several brain regions such as the hippocampus, substantia nigra and prefrontal cortex in adult male rats [21,22] as well as neurotransmitter binding to their cognate receptor [23].

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Consequently, these alterations in neurotransmission are likely to be the cause of deficits in locomotor activity and depressive- and anxiety-like symptoms observed in male mice and male and female rats following Glyphosate exposure [24–27]. Interestingly, while several studies have investigated the effect of perinatal exposure to GBH via the exposure to gestating and lactating females, there is no information about the mother's behavior and brain plasticity and how the dam herself could be affected by the treatment. In general, the vast majority of studies focus on the effects of the chemical environment on neurodevelopmental outcomes during the perinatal period, because of the intense neuroplasticity in the developing brain. However, the neural circuitry of adult females is also significantly remodeled during pregnancy and lactation to adapt her behavior to her offspring's needs and survival in a variable environment [28–31]. Amongst the various brain regions remodeled around the peripartum period, including the medial preoptic nucleus, the ventral tegmental area and the bed nucleus of the stria terminalis, the hippocampus has received much attention because of its persistent high level of neuroplasticity, including adult neurogenesis and because of its sensitivity to social and chemical environmental factors [32–34]. It should be added that the ventral and dorsal region of the hippocampus present physiological and functional differences, with the agreed consensus that the dorsal hippocampus is involved in spatial cognition and more cognitive performance and memory in general while the ventral part is involved in the regulation of emotions, including stress and anxiety [35,36]. Previous data suggest the GBH exposure affects hippocampal physiology and plasticity [22,26,37–39]. To our knowledge, there is no data on how Glyphosate could affect maternal behavior and the associated neuroplasticity.

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It should also be noted that most of these effects were observed with relatively high doses of GBH (over 50 mg/kg/day Glyphosate-equivalent) and only a handful of studies investigated the impact of exposure to low dose [40]. Probably as important, there is little work comparing the effects of Glyphosate alone and in GBH on neural and behavioral outcomes. This is important as a few studies suggest that some of the observed adverse effects of GBH are not triggered by Glyphosate itself but by some adjuvants, or "inert" molecules, found in the pesticide formulation, such as the polyethoxylated tallowamine [41,42].

Although it is hypothesized that the effects of Glyphosate in the brain might be linked to glutamate excitotoxicity and oxidative stress [21,26,38,43], the molecular target(s) of Glyphosate in the brain is/are not well defined. As introduced above, Glyphosate is best known for its direct inhibitory effects on EPSPS. While this protein is mostly investigated in plants for its clear economical reason, the shikimate enzymatic pathway is also present in most bacteria. Interestingly, every vertebrate (and invertebrate) organism is the host for billions of microorganisms (mostly bacteria), collectively named microbiota. Modification of microbiota by chemical exposure (including antibiotic, diet, pre or probiotics…) is known to be associated to significant alterations of the organism's physiology, including defect in brain neurodevelopment and neuroplasticity [44–46]. Recently, a few studies showed that GBH exposure can affect gut microbiota, a process known as dysbiosis [40,47–49] and such an alteration could result in mental health disorders, including anxiety and depression [24,25]. Alternatively, or in addition, Glyphosate possess chelating properties and binds divalent cations (e.g. Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}). This chelating effect *in vivo* is unclear and the outcome poorly investigated [50,51]. A reduction of these elements could lead to a significant alteration of most enzymatic cascades that usually require metallic cofactors but could also indirectly alter the microbiota.

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Therefore, the aim of the present study was to compare the potential effects of relatively low dose (5 mg/kg/day, 1:10th of the NOAEL, for "No Observable Adverse Effect Level") of Glyphosate and GBH on maternal behavior and maternal neuroplasticity, focusing on the hippocampus (dorsal and ventral) and cingulate gyrus. To measure changes in neurogenesis and cell structure, we focused on immunohistochemical techniques to investigate cell proliferation (Ki67-immunoreactivity(-ir)), immature neurons (doublecortin-ir), and pre- and post-synaptic protein densities (synaptophysin-ir and PSD95-ir) in the hippocampus. In the cingulate gyrus pre- and post-synaptic protein densities (synaptophysin-ir and PSD95-ir) were assessed. In addition, we also investigated signaling pathways that could be affected by Glyphosate exposure and lead to adverse neural outcomes. In parallel to brain and behavior analysis, we investigated whether Glyphosate alone or in formulation could affect the gut microbiota and/or modulate plasma concentration of several cations to affect neuroplasticity in the dam.

METHODS

Animals

Twenty-one adult pregnant (gestational day (GD) 8) Sprague-Dawley rats (239-340 g) were obtained from Janvier Laboratories, France, and singly housed upon arrival at our animal facility. They had access to rat chow and tap water *ad libitum* and were kept under standard laboratory conditions in a 12:12 light/dark cycle (light on at 07:00 am). All animal procedures were approved by the Ethics Committee of the French Ministry of Research (authorization number APAFIS#5294-2016050412068659 v3). The animal facility is licensed by the French Ministry of Agriculture (agreement D35-238-19). All efforts were made to minimize animal stress and pain.

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Pregnant females were randomly assigned to 3 groups (n = 7/group): Control (vehicle: milliQ sterilized water), Glyphosate (5 mg/kg/day of glyphosate isopropylamine salt, Dr. Ehrenstorfer GmbH, Germany) and Roundup® 3Plus (Roundup, 5 mg/kg/day glyphosate isopropylamine salt equivalent, S.A. Monsanto Europe L&G Dept., Belgium 170 g/L glyphosate acid, 229 g/L glyphosate isopropylamine salt) at GD9. Treatment was administered orally via biscuits [52] every morning between 8:30 am and 9:30 am from GD10 to the weaning of the pups at postpartum day (PD) 21. Females were fed 1/9th of a vanilla wafer cookie (Crousti fondante, Delacre®, Belgium) injected with treatment or vehicle. Injections were made into the vanilla portion of the wafer. Observations were made to ensure that the biscuit was completely ingested by the dam and not shared with the offspring. Cookies were consistently eaten in their entirety but the dams. Two females had problems during parturition and were euthanized (1 Control, 1 Glyphosate). Weight was recorded on GD10, PD1 and PD22. Figure 1 provides a timeline of the study.

Maternal behavior

One day after birth (PD1), litter was weighed, sexed and culled to 10 pups per litter (balanced sex ratio). Between PD1 to PD6, maternal behavior was scored during 5 minutes twice a day, directly in the cage without disturbing the animals. Scoring took place in the morning (between 09:30 and 13:30) and in the afternoon (between 14:00 and 16:30) with at least 1 h between the two sessions. An observer blind to the treatment scored the time the dams spent licking the pups, arched-back nursing (ABN), passive nursing, blanket nursing time the dams spent off the nest and the time the dams spent building the nest (NB). Data is reported as percentage of time spent for each maternal behavior. We analyzed maternal behavior on the first day following parturition independently from the other days, because the litter was handled and characterized, causing a short (~15 minutes) maternal separation and

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potentially affecting behavioral outcome. Therefore, maternal behavior was investigated separately on PD1 and aggregated across PD2-6 for additional analyses.

Microbiome

In addition to standard cage changing, the dams were transferred to new cages with clean bedding on GD9, GD19 and PD22, and feces collected with sterilized tweezer the following day and transferred to sterile 1.5 ml tubes for microbiota analysis. Analysis of the feces prior to treatment (GD9) and at the end of the study (PD22) was done. There were no differences between groups at GD9 and only differences at PD22 are reported. No samples were pooled. Samples from GD19 were not used.

16S rRNA amplicon generation for MiSeq sequencing The V3-V4 region was amplified from purified DNA with the primers F343 (CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG) and R784 (GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT) using 30 amplification cycles with an annealing temperature of 65°C. The amplicon lengths were about 510 bp (the exact length varies depending on the species). Because MiSeq sequencing enables paired 250-bp reads, the ends of each read overlap and can be stitched together to generate extremely high quality, full-length reads covering the entire V3-V4 region. Single multiplexing was performed using a home-made 6 bp index, which was added to the R784 primer during a second PCR with 12 cycles using the forward primer (AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC) and the modified reverse primer (CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGAGTTCAGACGTGT). The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer instructions. The quality of

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the run was checked internally using PhiX, and for further analysis, each pair-end sequence was assigned to its sample using the previously integrated index.

Bioinformatics analysis Sequences were trimmed for adaptors and assembled with Flash1.6.2 [53]. PCR primers were removed and sequences with sequencing errors in the primers were excluded (Mothur) [54]. 12000 reads were randomly selected for each sample. Chimera were removed with UCHIME [55] and Mothur [54] software. Reads were clustered into Operational Taxonomic Units (OTUs) at the 97% identity level using Esprit-tree [56]. A reference sequence was picked for each OTU and assigned it at different taxonomic levels (from phylum to species) using the Greengenes database (release 13-5) [57] and the RDP classifier [58].

After rarefaction of all communities to even sampling depths, the abundances of all families were computed by agglomerating the OTUs assigned to those families. For each such family, Kruskall Wallis test with BH correction [59] were performed to detect the combinations (treatment) that were significantly different in terms of abundance. The same procedure was applied for each genus and for each OTUs. All analyses were done using R (R Core Team, 2015, R Foundation for Statistical Computing, Vienna, Austria).

Euthanasia

At PD22, one day after weaning of the offspring, dams were deeply anaesthetized with sodium pentobarbital (*i.p*, 150 mg/kg BW, Ceva Santé Animale) and euthanized by a rapid decapitation. Trunk blood was collected and kept on ice until processing within 8 h. Blood samples were centrifuged at 2375 x g for 10 minutes at 4°C. Serum was stored at -80°C until use. The brains were quickly removed, immersion-fixed in 4% paraformaldehyde at 4°C for 24 h. Brains were then transferred in 30% sucrose/phosphate-buffered saline

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solution for 7 days for cryoprotection before snap-freezing on dry ice. Brains were stored at -80°C until processing (see below).

Serum Analysis

Serum transaminases analysis. Serum alanine (ALT) and aspartate (AST) transaminases were measured according to the *International Federation of Clinical Chemistry* (IFCC) primary reference procedures using Olympus AU2700 Auto-analyser® (Olympus Optical, Tokyo, Japan). Serum transaminases levels in dams were measured to evaluate potential hepatotoxic effect [60].

Interleukin 6 (IL-6). Serum levels of interleukin 6 (IL-6) were determined using a commercial ELISA kit with pre-coated plates (Legend Max™, BioLegend Inc) and performed according to the provider instructions. All samples were analyzed in triplicate. The plates were read with iMark microplate reader (Bio-rad) and analyzed with MPM6 software (Bio-rad). The assay had a minimum detectable concentration of 5.3 pg/mL.

Cations. Serum levels of calcium (Ca), iron (Fe), magnesium (Mg) and manganese (Mn) were quantified by ICP/MS/MS at the LERES analytical platform. Briefly, plasma was acidified with HNO3 (1% final concentration) and diluted 1/10 in ultra-pure water before analysis on quadrupole-based ICP-MS instrument (ICP-MSMS 8800, Agilent) equipped with an extra octopole-containing cell to remove interferences by selective reaction with a reactive gas (e.g., H2, O2, NH3) or collisions of the ions with a non-reactive gas (e.g., He). Technical triplicate for each sample were measured following a calibration curve (8 points for each ions) and internal standards (Iridium, Scandium, Germanium, Indium, Rhénium and Rhodium).

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Brain histology

Brains were sliced with a cryostat (Microm HM560) at 40 μm, in series of 10. Sections were stored at -16°C in ethylene glycol-based antifreeze solution until immunohistochemical processing.

Immunohistochemistry. Immunohistochemistry was performed as previously published [52,61–63]. Briefly, 1 series of sections was used for each marker (Ki67, Doublecortin, Synaptophysin and PSD95; see Table 1 for details). Sections were brought to room temperature and extensively rinsed in PBS before processing. For PSD95 staining only, sections were incubated in 10 mM sodium citrate buffer (pH 6.0) for 10 min at 80°C for antigen retrieval then rinsed in PBS before further processing. For all markers, endogenous peroxidase activity was blocked in 0.6 % H_2O_2 for 30 min at room temperature. Sections were then incubated at room temperature in normal donkey serum (NDS, Sigma 5% in PBS containing 0.1% of triton (PBST), for Ki67 and Doublecortin) or in normal goat serum (NGS, 5%, synaptophysin or 2%, PSD95) for 30 min as described in Table 1. Tissue was then incubated at 4°C in primary antibody (Ki67, DCX, synaptophysin, PSD95, as described in Table 1). Sections were rinsed in PBST then incubated in secondary biotinylated antibody. Signal amplification was performed using avidin-biotin-horseradish peroxydase complex (Vectastain® ABC Elite kit; 1:1000; Vector Laboratories) for 2 h at room temperature and signal was visualized using DAB (3,3-diaminobenzidine) peroxidase substrate kit (SK-4100, Vector Laboratories). Sections were mounted on SuperFrost Plus slides (Thermo Scientific), dried overnight, counterstained with Cresyl Violet acetate (only for Ki67), dehydrated and cover with Permount (Fischer Scientific).

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Plasticity measures

Hippocampal volume. Cresyl-stained sections were observed under 2X magnification using an Olympus AX70 TRF and a DP71 digital camera with Cell^F software (Olympus). Areas of the granular cell layer and subgranular zone (GCL/SGZ) throughout the dentate gyrus rostro-caudal extent were measured using ImageJ software [64]. Areas were summed and multiplied by the sampling interval (10) to derive an estimate of volume.

***Ki67 and DCX quantification*.** DCX- and Ki67-positive cells were counted under X40 objective (Olympus BX60 optic microscope) throughout the dentate gyrus (GCL/SGZ) by an observer blind to the experimental treatment. Ki67-positive cells were also counted in the hilus. The number of cells counted was multiplied by 10 (1 series of sections analyzed out of 10) to obtain an estimate of the total number cells in the dentate gyrus.

We also quantified and characterized DCX-positive cells based on their morphology in 2 dorsal and 3 ventral sections. A minimum of 60 DCX-positive cells were counted per brain section and categorized as either proliferative (no process or short process), intermediate (medium process with no branching) or post-mitotic neurons [65,66]. Figures are expressed as a percentage of total DCX-ir cells. For representative photomicrographs of Ki67 and DCX-positives cells see Figure 3A (Ki67) and 4 A-D (DCX).

Synaptophysin and PSD95 quantification. Photomicrographs from 3 separate sections in the dorsal and ventral dentate gyrus, dorsal CA3 and the cingulate gyrus were taken to determine the optical density of synaptophysin and PSD95. Six and nine photomicrographs (respectively for PSD95 and synaptophysin) were taken in the dorsal dentate gyrus, 3 photomicrographs in dorsal CA3 region bregma -2.64 mm to -5.16 mm; 9 photomicrographs in ventral dentate gyrus bregma -5.16 mm to -6.48 mm; and 6

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photomicrographs in the cingulate gyrus (CG) bregma 2.76 mm to -0.12 mm [67] were taken under 40X objective using a Olympus AX70 TRF and a DP71 digital camera with Cell^F software (Olympus). For representative photomicrographs of synaptophysin density see Figure 5A.

Optical densities (OD) were quantified with ImageJ (NIH) software. The relative optical density was calculated as the difference between optical density (grey level after calibration) of the area of interest and the background of an adjacent region [52,61].

Statistical analysis

Data were analyzed using the software Statistica 13 (Dell Inc.) and GraphPad Prism version 5.03. One-way ANOVAs were computed on separate maternal behaviors on PD1 and across PD2-6, litter characteristics, hepatic toxicity (AST/ALT), hippocampal volume, measures of synaptic plasticity (synaptophysin, PSD95), neurogenesis and serum analysis of cations and IL6. Repeated measures ANOVAs were done on maternal weight, Ki67-ir cells in the GCL/SGZ and hilus as well as for the morphology of DCX-ir cells. Post hoc comparisons were performed using Fisher LSD when necessary. Due to the high variance between groups for maternal behavior on PD1, values were normalized before statistical analysis but untransformed data are presented in the bar graph (figure 2) to feature natural individual variability. For all statistical analysis, $p < 0.05$ was considered significant.

Microbiome statistical analysis. Following rarefaction of all communities to even sampling depths, the abundances of all families were computed by agglomerating the Operational Taxonomic Units (OTUs) assigned to those families. For each comparison between all groups, Kruskal-Wallis tests with BH correction [59] were carried out to identify the significant effect of treatment on abundance at Phylum, family, genus and OTU levels. Probabilities were considered significant when $p < 0.05$.

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RESULTS

General toxicity

Several parameters were measured to define potential general toxic effects of Glyphosate or Roundup. As expected, the weight of the dams was significantly different during pregnancy and the postpartum period ($F_{(2,32)}$ = 13.24, p < 0.0001; Table 2). But there was no significant effect of treatment ($F_{(2,16)}$ = 0.9118, p = 0.4217) and not interaction between treatment and reproductive state ($F_{(4,32)}$ = 2.114, p = 0.1020) on dam weight. In addition, treatment did not alter litter size ($F_{(2,16)}$ = 0.4579, p = 0.6406) or litter weight ($F_{(2,16)}$ = 0.2770, p = 0.7616; Table 2). A trend toward a change in sex ratio was observed ($F_{(2,16)}$ = 3.559, p = 0.0526; Table 2), with Roundup treatment showing a slight increase in the number of male offspring compared to female offspring. There were also no significant effects of treatment on AST ($F_{(2,16)}$ = 1.857, p = 0.1883) or ALT ($F_{(2,16)}$ = 1.347, p = 0.2881) or serum levels of cations and IL-6 serum (Table 2).

Maternal behavior

On PD1, treatment affected pup licking behavior in dams but this did not reach significance ($F_{(2,16)}$ = 2.9103, p = 0.08357, Figure 2A). No other maternal behaviors were significantly affected on PD1 (ABN $F_{(2,16)}$ = 0.68192, p = 0.51975; blanket $F_{(2,16)}$ = 0.42441, p = 0.66131; passive $F_{(2,16)}$ = 0.58698, p = 0.56754; off nest $F_{(2,16)}$ = 0.77431, p = 0.47755; nest building $F_{(2,16)}$ = 0.18884, p = 0.82974, Figure 2A).

On PD2-6, there was also a significant main effect of treatment on pup licking behavior ($F_{(2,16)}$ = 5.0637, p = 0.01978, Figure 2B), with post-hoc tests revealing that Roundup treated dams spent significantly more time licking offspring compared to control or Glyphosate-treated dams (p's < 0.04). There were no other significant main effects on additional maternal behaviors (ABN $F_{(2,16)}$ = 1.5988, p = 0.23281; blanket $F_{(2,16)}$ = 0.62086, p

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= 0.54994; passive $F_{(2,16)}$ = 0.28858, p = 0.75314; off nest $F_{(2,16)}$ = 1.9319, p = 0.17720; nest building $F_{(2,16)}$ = 0.35596, p = 0.70591, Figure 2B).

Hippocampal Plasticity

Hippocampal volume. There were no significant effects of treatment by Glyphosate or Roundup on the volume of the dentate gyrus ($F_{(2,16)}$ = 0.2633, p = 0.7718, Table 3). Therefore, total number of cell counts were used throughout.

Hippocampal cell proliferation (Ki67). Using repeated-measures ANOVAs between the GCL/SGZ and hilus, there were no significant effects of treatment and no interaction between the treatment and the area on the number of Ki-67-ir cells in the dorsal region of the GCL/SGZ and hilus (respectively $F_{(2,\ 16)}$ = 0.=65158, p = 0.5345 and $F_{(2,\ 16)}$ = 0.15107, p = 0.8610, Figure 3A). As expected there were significantly more proliferative cells in the GCL/SGZ than in the hilus ($F_{(1,\ 16)}$ = 147.65, p < 0.0001).

In the ventral region of the GCL/SGZ, there was strong tendency toward an interaction between the treatment and area ($F_{(2,\ 16)}$ = 3.6168, p = 0.05059, Figure 3B). Similarly to the dorsal hippocampus, significantly more Ki67-ir cells were found in the ventral GCL/SGZ compare to the hilus ($F_{(1,\ 16)}$ = 383.16, p < 0.0001). There was no significant main effect of treatment on the number of proliferative cells ($F_{(2,\ 16)}$ = 2.7492, p = 0.0941).

Hippocampal neurogenesis (DCX). There was no effect of the treatment on total number of immature DCX-ir neurons in the dorsal ($F_{(2,15)}$ = 1.0597, p = 0.3711, Figure 4A) or ventral GCL/SVZ ($F_{(2,15)}$ = 2.0139, p = 0.1680, Figure 4B).

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DCX cell morphology. There were no significant differences between groups in the number of DCX-ir cells quantified in terms of morphology in the dorsal or ventral hippocampus (*p's* > 0.16). Repeated measures ANOVAs on the morphology of DCX-ir cells in the dorsal GCL/SGZ revealed a significant interaction between the treatment and the maturation of the cells ($F_{(4,30)}$ = 3.0955, p = 0.0302, Figure 4C) with a significantly higher proportion in post-mitotic neurons in Roundup-treated dams compared to the Control dams (p = 0.0016). There was also a significant difference in the maturation stages in the dorsal GCL/SGZ ($F_{(2,30)}$ = 16.7734, p < 0.0001, Figure 4C) with significant differences between the number of cells at each stage of development (p's < 0.04). There was no significant main effect of the treatment on the morphology of the new neurons in the dorsal GCL ($F_{(2,15)}$ = 1.7504, p = 0.2074).

For the ventral hippocampus, there was also no significant main effect of the treatment on DCX cell morphologies ($F_{(2,15)}$ = 2.014, p = 0.1680) nor an interaction effect ($F_{(4,30)}$ = 1.567, p = 0.2086, Figure 4D). Similarly to the dorsal GCL, there was a significant difference in the distribution of the morphologies ($F_{(2,30)}$ = 1515.907, p < 0.0001, Figure 4D) with significant differences between the number of cells at each stage of development (p's < 0.001).

Synaptophysin. There was a significant main effect of the treatment on synaptophysin OD in the dorsal region of the dentate gyrus ($F_{(2,16)}$ = 9.807, p = 0.0017, Figure 5A) with a significantly greater density of synaptophysin in Glyphosate and Roundup-treated dams compared to the Controls (p < 0.02). There was also a significant main effect of treatment on synaptophysin density in the ventral dentate gyrus ($F_{(2,16)}$ = 7.064, p = 0.0063, Figure 5B) with Glyphosate-treated dams having significantly greater synaptophysin density compared to both Control and Roundup treated dams (respectively p = 0.0025 and 0.0121). In the CA3

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region, there was also a significant main effect of treatment on synaptophysin density ($F_{(2,16)}$ = 5.817, p = 0.0126, Figure 5C) with Glyphosate dams having a significantly greater synaptophysin density compared to Control and Roundup groups (p's < 0.02).

When looking at synaptophysin density in the cingulate gyrus there was also a significant main effect of treatment ($F_{(2,15)}$ = 4.908, p = 0.0229, Figure 5D) with synaptophysin density being reduced in both Glyphosate and Roundup groups compared to Controls (p's < 0.03).

PSD95. There was no significant effects of the treatment on PSD95 density in the dorsal ($F_{(2,16)}$ = 0.4293, p = 0.6582, Table 4) or ventral ($F_{(2,16)}$ = 0.9545, p = 0.4059, Table 4) dentate gyrus or the CA3 region ($F_{(2,16)}$ = 1.701, p = 0.2140, Table 4).

Microbiome

We also evaluated the impact of the Glyphosate and Roundup on the gut microbiota. Composition of the gut microbiota did not display significant differences between groups at GD9, i.e before treatment (data not shown). At PND22, we found several significant differences at phylum, family and genus level between groups (Figure 6). We found a significant effect of the treatment on Bacteroidetes (p = 0.009) and Firmicutes (p = 0.006), with respectively an increase and a decrease in Roundup group compared to the Control and Glyphosate (p's < 0.04; Figure 6B). We further analyzed the gut microbiota variation at the family level and showed a significant effect of the treatment on *Lachnospiraceae* (Phylum: Firmicutes, p = 0.033) which were less abundant in Roundup compared to Glyphosate dams (p = 0.013), with a trend in Roundup group compared to Control (p = 0.065; Figure 6C and D). We also found a significant effect of the treatment on *Erysipelotrichaceae* (Phylum: Firmicutes, p = 0.039), which were conversely more abundant in Roundup-treated dams

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compared to Glyphosate ($p = 0.017$), and with a strong tendency in Roundup dams compared to Controls ($p = 0.056$; Figure 6C and D). At the genus level, there was a significant main effect of the treatment on *Alloprevotella* (Family: *Prevotellaceae*, Phylum: Bacteroidetes), *Butyricicoccus* (Family: *Clostridiaceae*, Phylum: Firmicutes), *Ruminococcaceae* UCG-013 (Family: *Ruminococcaceae*, Phylum: Firmicutes) and *Turicibacter* (Family: *Erysipelotrichaceae*, Phylum: Firmicutes) abundancy (respectively $p = 0.03$, $p = 0.021$, $p = 0.04$ and $p = 0.039$). *Alloprevotella* were more represented in the Roundup compared to Control and Glyphosate groups ($p's < 0.05$; Figure 6E). *Turicibacter* were more abundant in Roundup-treated dams compared to Glyphosate ($p = 0.017$), and there was strong tendency in Roundup compared to Control group ($p = 0.056$). In contrast *Butyricicoccus* genus decreased in Glyphosate group comparatively to Control and Roundup groups ($p's < 0.04$; Figure 6E). Moreover, *Ruminococcaceae* UCG-013 genus were significantly decreased in both Glyphosate and Roundup groups compared to Control ($p's < 0.04$; Figure 6E).

DISCUSSION

Main findings of the present study show that peripartum exposure to a low dose of Glyphosate or Roundup affect maternal behavior, neuroplasticity and induce dysbiosis in dam rats. More specifically, we showed that perinatal exposure to Glyphosate or Roundup significantly altered maternal licking behavior without affecting other characteristics of the maternal behavior. In addition to the behavior, we also showed that Glyphosate alone or in formulation affected aspects of neurogenesis in the maternal brain postpartum. Indeed, there were more post-mitotic immature neurons in the dorsal dentate gyrus of Roundup dams, while cell proliferation and the total number of immature neurons were not affected. Moreover, synaptophysin was modified following Glyphosate and Roundup perinatal exposure in dorsal and ventral dentate gyrus and CA3 of the hippocampus, as well as in the

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cingulate gyrus, albeit with different profiles depending on the region of interest. It is important to highlight here that the behavioral and physiological outcomes following exposure to the active compound alone (Glyphosate) or to the formulation (Roundup) were also significantly different, confirming previous observations that the effects of formulations could be linked to adjuvants within these formulations [41,42].

Absence of general toxicity.

In the present study, Glyphosate and Roundup had no significant effect on dam weight throughout pregnancy and the postpartum period. Similarly, the litter characteristics such as length, weight, sex ratio were not altered by the treatment. This observation is in agreement with recent studies that investigated dams weight during pregnancy [18,27] and litter characteristics [18,38,68] following exposure to relatively low dose of GBH. Only higher doses were found to reduce weight in juvenile or in pregnant rat (500 and 1000 mg/kg BW/d) peripartum exposure in dams [68]. We also confirm the absence of visible toxicity at the biochemical level, reflected by the low plasma levels of AST and ALT, two hepatic transaminases released in the vascular system upon liver aggression [60]. These results are in line with those obtained in adult male rats exposed for 5 weeks to 5, 50 and 500 mg/kg BW/day of Glyphosate, where only the highest dose led to a significant increase in AST and ALT circulating levels [69].

Glyphosate and maternal behaviors.

In the present study, Control dams tended to spend more time licking their pups on the day following parturition, compared to Glyphosate or Roundup-treated animals. Additional maternal behaviors were not different between groups and time spent licking in these untreated Control dams was significantly reduced the following days. It is important to

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highlight here that on the day following parturition the offspring were handled and separated from their mother for up to 10 minutes to record offspring characteristics and cull the litter. Previous studies show that pup handling or isolation after parturition disrupts basal maternal behaviors, with most changes being associated with an increase in licking and grooming behaviors following the reintroduction of the pups with their mother [70–75]. Our observations, therefore, suggest that Glyphosate- and Roundup-treated dams do not present this normal increase in licking behavior upon reuniting with the pups. While a large number of studies have focused on the short- and long-term consequences of pup isolation on the offspring [76,77], little is known about the outcome on the mother and the underlying physiology. It is, however, likely that separation causes some form of anxiety, not only for the pup, but also for the mother. Interestingly, previous work suggests that early or adult exposure to Glyphosate-based herbicide increases anxiety and depression-like behavior in rodents [24,27,78]. Therefore, we can speculate that the altered interpretation of the social environment associated with anxiety or depressive-like behavior will result in modification in maternal care.

Rather surprisingly, the maternal behavior was later affected in the Roundup-treated animal between PD2 to 6, with an increase in licking behavior compared to dams from Control and Glyphosate groups. It could be that one of the adjuvant, such as polyethoxylated tallowamine, present in the Roundup formulation might be responsible for the effect as suggested by Mesnage and colleagues [41,42,79], but this hypothesis remains to be tested.

It is also interesting to note that the offspring influences the amount of parental care given by the mother through direct tactile contact, olfactory cues, and ultrasonic vocalizations. For example in rodents, the male is more likely to initiate contact with the mother and in turn, licking and grooming from the dam toward their male offspring is usually more important [80–82]. A misinterpretation of these cues by the mother exposed to Glyphosate or Roundup could result in different amounts of licking as observed in the present study. It

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should be kept in mind that *in utero* exposure of the offspring could have affected the sensory cues emitted by the offspring [83]. For example, perinatal exposure to organochloride pesticides such as chlorpyryfos or lindane significantly affected the ultrasonic vocalization emitted by pups [84,85]. The modification of the pup behavior following *in utero* exposure to Glyphosate would alter the care provided by the mother but this hypothesis remains to be investigated.

While most studies, including the present one, investigated only one part of the mother/offspring dyad (the mother), the behavior results from the interaction and constant feedback between the mother and the pups. Indeed, the polychlorinated biphenyl congener PCB 77 affected maternal behavior when both the mother and offspring were exposed, as confirmed by cross-fostering experiments [86]. This is an important reminder that the effects of many substances on offspring outcomes may be due, in part, to their effects on maternal care-giving behaviors. Future research needs to acknowledge the importance of maternal care-giving on offspring outcomes and investigate both parts of the mother/offspring dyad.

Glyphosate and plasticity in the maternal brain

Neurogenesis in the dentate gyrus of the hippocampus.

Two brain regions, the sub-ventricular zone of the lateral ventricles and the sub-granular zone in the dentate gyrus of the hippocampus are known to produce a large number of new neurons throughout adulthood [87–90]. Importantly, hippocampal neurogenesis is strongly modulated during pregnancy and the postpartum period in rodents [33,91–95]. Research has also shown that neuroplasticity and neurogenesis in the hippocampus is strongly affected by the chemical environment [96–99] including medication [66,100,101]. In the present work we found a significant increase in the number of post-mitotic (more mature) doublecortin-positive neurons in the dorsal granule cell layer of Roundup-treated dams compared to Controls. The effect of Glyphosate alone on this parameter was not statistically significant

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but a trend toward an increase was also observed. No treatment effects were evident in the ventral hippocampus or in measures of cell proliferation. The upregulation of the number of post-mitotic cells in the dorsal hippocampus with Roundup treatment is likely a result of a combination of several mechanisms related to a faster maturation of these neurons or a longer period cells of this stage survive. The consequences of this increase are not known but it is suggested that adult neurogenesis is not needed for learning per se but rather for the modulation and plasticity of spatiotemporal contextualization of new information. Indeed, the new neurons in the dentate gyrus of the hippocampus contribute to synaptic plasticity to the dentate gyrus [102–104] while other surrounding neurons are usually inhibited by local GABAergic interneurons. The present increase in postmitotic doublecortin neurons might therefore leads to stronger plasticity within this region and the behavioral consequences remain to be investigated.

Synaptic plasticity in the hippocampus

Findings of the present study also show that peripartum exposure to Glyphosate and Roundup increased synaptophysin expression in the dorsal region of the dentate gyrus and interestingly, decreased synaptophysin expression in the cingulate gyrus, an area of the medial prefrontal cortex. Glyphosate alone increased synaptophysin expression in both ventral dentate gyrus and CA3 regions of the hippocampus. This suggests region specific effects of Glyphosate and Roundup on synaptic proteins throughout the brain.

Synaptophysin is an membrane protein expressed at the surface of synaptic vesicles and can be found in most neurons within the central nervous system [105,106]. To our knowledge, the precise function of this protein is still not clear but studies suggest that synaptophysin is involved in calcium binding [107], chanel formation [108,109], exocytosis [110,111], as well as synaptic vesicle recycling [112]. The change in synaptophysin expression following

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peripartum exposure to Glyphosate is perhaps not surprising as several studies have demonstrated a change in neurotransmitter release and metabolism in various regions of the rat brain after exposure to Glyphosate or Glyphosate-based herbicides [21–23,26,27]. For example, high doses of Glyphosate lead to an increase in serotonin release and turnover in the striatum and hypothalamus and in dopamine turnover in prefrontal cortex and hippocampus [22]. The change in dopamine is likely to be related to the reduction of tyrosine hydroxylase expression [24], the key enzyme responsible for dopamine synthesis. The modulation of dopamine levels can also be associated to the reduction in D1-dopamine specific receptor binding in the nucleus accumbens in adult male Sprague-Dawley rats exposed to Glyphosate [23]. It should be noted that the nucleus accumbens is a fundamental brain region involved in the motivation aspect of maternal behavior and modulation of activity within this brain region only can alter proper maternal care toward the offspring [113–115]. In addition to monoamines, acetylcholine esterase, the enzyme responsible for the breakdown of acetylcholine in the synaptic cleft to terminate synaptic transmission, is inhibited in the prefrontal cortex, striatum and hippocampus of male and female adult offspring after a perinatal exposure to Glyphosate [26,38]. Extracellular glutamate concentration and the reuptake of amino acids are also hindered in the hippocampus following exposure to Glyphosate-based herbicide [21,26].

Whether or not the effects of glyphosate on the maternal brain reported in this study are unique to motherhood remain to be determined. Previous research shows that pregnancy and the postpartum period both affect plasticity in the hippocampus [33]. How exposure to this herbicide interacts with the normative changes in the female brain during pregnancy and the postpartum period is needed to understand the complexities of these relationships and how they may affect behavioral outcomes.

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Molecular target of Glyphosate

As introduced earlier, the molecular target of Glyphosate is the protein EPSPS, an enzyme found in plants and bacteria. This mode of action suggests that vertebrates are relatively safe if exposed to Glyphosate-based herbicides. However, the increased number of reports pointing to effects of Glyphosate on animal physiology open the rarely asked question about the molecular target of the Glyphosate in vertebrates. Only a handful of studies have suggested direct molecular target for Glyphosate.

First, as mentioned in the introduction, the inhibition of the enzyme EPSPS not only impacts the growth and survival of plants, but also that of bacteria. Multicellular organisms are colonized with hundreds of species of bacteria, collectively known as microbiota and they exert a profound influence on key physiological processes. More precisely, microbiota present in the digestive system is known to exert major influence on neuroinflammation, activation of the stress axes, neurotransmission, and neurogenesis to name a few, resulting in the modulation complex behaviors, such as sociability and anxiety [116–120]. Gut microbiota influences the central nervous system through its ability to modulate the immune system, to provide bioactive metabolites such as fatty acids and synthesize neurotransmitters including GABA, noradrenaline, and dopamine [121,122]. Any dysregulation of the gut microbiome, or dysbiosis, is known to significantly affect the central nervous system and the resulting behavior, leading to increased susceptibility to psychiatric disorders [120,123]. Our results clearly demonstrate that gut microbiota is affected by glyphosate and glyphosate-based herbicide, albeit in formulation-specific manner. Indeed, we showed an increase in the phylum Bacteroidetes and a reduction of Firmicutes following Roundup exposure, the Glyphosate alone having no impact on the 2 phyla. The effect of glyphosate or glyphosate based herbicide on bacterial population is not a complete surprise. A few studies, mostly on soil bacteria[124], but also in animal species, from insects[48,125] to reptiles[126] and mammals[127,128],

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investigated the effect of the pesticide on microorganism ecology. While the precise role of each of these bacteria phyla for brain physiology is, to our knowledge, far from understood, a few studies have suggested that a dysregulation of Bacteroidetes or Firmicutes populations is associated to mental health disorders, including autism [129–131] and Alzheimer's disease [132,133]. To support our findings, other studies, in rats but also in bees, report similar changes in Bacteroidetes and/or Firmicutes following Glyphosate or Glyphosate-based herbicide exposure [40,48,127,128]. Similarly, the population of the genera Alloprevotella is increased in our study, while studies have found an association between Parkinson and Alzheimer's disease and reduced population of the family of Prevotellaceae [134–137]. It is obviously important to note that these studies are only correlational and the precise consequences of the impact of Glyphosate or Glyphosate-based herbicide on various species of bacteria, as well as on the complex microbiota ecosystem, deserve to be investigated in more detail to understand how Glyphosate, alone or in combination with adjuvants, might impact the organisms. Indeed, it is interesting to note that the most pronounced effects on the microbiome in the present study are linked to Roundup treatment. One hypothesis is the presence of multiple, yet mostly undefined, adjuvants added to the formulation to increase the penetration of glyphosate within the target organisms[138,139]. A low penetration of glyphosate in the absence of adjuvant could result in less consequences for the potential targeted organisms. Alternatively, these adjuvants by themselves could be affecting the microbiota population, as suggested Mesnage and collaborators for vertebrate toxicity [41,42,79]. A very few studies, on soil bacteria suggest that this might be the case[124]. This differential effect should be investigated.

A second molecular target of Glyphosate is the direct activation of glutamatergic NMDA receptor, suggested by Cattani and Colleagues[26]. Indeed, their molecular dynamic simulation demonstrates that Glyphosate may bind to glutamate and glycine cavities of NMDA receptors, with lower stability of Glyphosate binding at glycine binding pocked

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compared with glutamate one. This interaction would likely increase the neuronal Ca^{2+} entry, triggering mitochondrial dysfunction and oxidative damage [26]. This direct interaction remains to be tested experimentally. It should be pointed out that oxidative stress, independently of NMDA receptor activation, was also observed in other tissues, such as the liver and the kidney following exposure to Glyphosate or various Glyphosate-based herbicides [140–143].

A third hypothesis for molecular target is the chelating role of Glyphosate [50,144–146], particularly with divalent cations. The presence of divalent cations, including Ca^{2+}, Fe^{2+} or Mg^{2+} is of fundamental importance for the organisms as they participate in various physiological functions, including bone formation and cellular communication (Ca^{2+}), oxygen transport (Fe^{2+}) and enzyme cofactors (Ca^{2+}, Mg^{2+}) to name a few. An alteration of these elements would therefore lead to major adverse effects for the organism. In the present study plasma levels of Ca^{2+}, Fe^{2+}, Mn^{2+} and Mg^{2+} were quantified but not affected by treatments. To our knowledge, only one study investigated the effect of Glyphosate on ion concentrations in vertebrate. Tang and colleagues revealed that low (5 mg/kg), medium (50 mg/kg) or high doses (500 mg/kg) of Glyphosate altered levels of Zn^{2+} and Fe^{2+} in liver, spleen and kidney in a dose dependent-way in adult male rat [69]. In addition, they found a significant increase of Mg^{2+} in the brain following exposure for 35 days to 50 and 500 mg/kg Glyphosate. There was no information about the plasma levels. It is clear that additional studies should investigate this potential effect of Glyphosate on divalent cations and define whether the observed changes could lead to alter physiology in the organism.

Future Directions

This is the first study to investigate how glyphosate may affect the maternal brain and behavior. However, we acknowledge the much research is needed to determine the extent of these effects on the mother and offspring. Of particular importance in the present study would be to investigate how changes in the maternal brain are causally related to changes in the

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maternal care-giving behaviors. In the present study we focused on hippocampal plasticity at the time of weaning, but future work would benefit from investigating brain changes in areas playing a key role in maternal care-giving behaviors (i. e. MPOA) at the time the care-giving is at its peak, early in the postpartum period.

The present study used a relatively low dose compared to most other studies (usually ≥ 50 mg/kg/day) but high compared to authorized daily intake (ADI) in the USA (1.75 mg/kg/bw/day or in the European Union (0.5 mg/kg/bw/day). Further research investigating a range of doses on both the mother and offspring would be valuable to determine the extent of this exposure on neurobehavioral outcomes.

It remains to be determined whether Glyphosate or Roundup alters peripheral and central actions of gonadal and adrenal hormones in the mother. Previous research on human cell lines, human breast cancer cells and rodents have shown that glyphosate-based herbicides in formulation can disrupt steroid secretion[147–149], estrogen receptors[150] and aromatase activity[151] albeit the exact mechanisms remains to determined. The physiological and behavioral effects of glyphosate observed in the present could be linked to endocrine disruption, and future work will investigate this possibility.

Conclusion

Our work adds to a growing body of research highlighting that low doses of Glyphosate and Roundup can impact vertebrate physiology. In the present study we show that Glyphosate alone or in formulation (Roundup), at a tenth of the currently accepted NOAEL, alters maternal behavior, neuronal plasticity and induces gut dysbiosis in the mother. Importantly, we also show that exposure to the active compound of Glyphosate or to the formulation can have different outcomes, potentially due to a direct action of adjuvants or to the interaction of the adjuvants and Glyphosate. Taken together, this work points to the important role that environmental chemicals can have on mother. Most experimental

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paradigms have been developed to investigate toxicity of various compounds on offspring development and, rather surprisingly, potential effects on the dam's brain and behavior are almost never investigated. Further research in this area is needed so that regulatory agencies can better evaluate the outcomes following exposure to Glyphosate and Glyphosate-based herbicides.

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FIGURE LEGENDS

Figure 1. Timeline of the present study. Dams were treated from GD10-PD22. Maternal behavior was observed on PD1-PD6 and brains were collected on PD 22. GD=gestational day. PD=postpartum day.

Figure 2. Mean (+SEM) percentage of total time spent in maternal behavior (%) A: during postpartum day (PD) 1 and B: during PD2 to 6. Untransformed data are presented * denotes significance ($p < 0.05$).
ABN = Arched-back nursing, NB = Nest building.

Figure 3. Representative photomicrographs of A: Ki67-ir cells in the granule cell layer and subgranular zone (GCL/SGZ). Mean (+SEM) of number of proliferative (Ki67-ir cells) in the B: dorsal and C: ventral dentate gyrus and hilus of the hippocampus. There was a significant effect of the regions with GCL/SGZ having higher number of proliferative cells and a trend on the interaction effect between treatment and area in the ventral hippocampus.
GCL = granular cell layer, SGZ = subgranular zone. Scale bar is 20 µm. Arrows shown the Ki67-ir cells.

Figure 4. Representative photomicrographs of A: doublecortin-ir cells in the GCL with B: proliferative, C: intermediate and D: post-mitotic stages at 40x objective. Mean (+SEM) of E: total number (X10) doublecortin-ir cells in the dorsal granule cell layer (GCL), F: total number (X10) doublecortin-ir cells in the ventral GCL, G: percentage of doublecortin-ir cells morphology in the dorsal GCL and H: percentage of doublecortin-ir cells morphology in the

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ventral GCL. Groups sharing the different letters are significantly different. There was a significant difference on the proportion of the morphologies for both dorsal and ventral GCL. There was a significant interaction between the cell morphology and the treatment in the dorsal GCL. * denotes significance ($p < 0.05$). Scale bar is 20 µm.

Figure 5. Representative photomicrographs of A: synaptophysin-ir in the GCL/SGZ. Mean (+SEM) synaptophysin density (OD) in B: the dentate gyrus (DG) of the dorsal hippocampus; C: the dentate gyrus of the ventral hippocampus; D: the CA3 region of the dorsal hippocampus and E: the cingulate gyrus (CG). * denotes significance ($p < 0.05$). mPFC = medial prefrontal cortex.

Figure 6. Analysis of the effects on gut microbiota by 16S rRNA gene high-throughput sequencing. A : composition of abundant bacterial phyla identified in the microbiota of the three different groups. B : Phyla significantly affected by Glyphosate or Roundup (OTU abundance). C and D : C, percentage of 16S rRNA gene sequences and D, OTU abundance of the different bacterial families in each sample among the Control, Glyphosate and Roundup groups. E : OTU of the various bacterial genera in each sample among each group of rats. Data are presented as box plots. Significant difference (* $p < 0.05$ and ** $p < 0.01$, respectively). OTU=Operational Taxonomic Units.

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TABLES

Marker (primary antibody)	Blocking	Primary antibody				Secondary antibody			
		Primary antibody	Company and catalog number	Dilution	Incubation	Secondary antibody	Company	Dilution	Incubation
ki67 (Pawluski *et al.*, 2011; Pawluski *et al*., 2015)	NDS, Sigma 5%	rabbit anti-ki67	Vector Laboratories VP-RM04	1/250	48 h	Donkey anti-rabbit biotinylated antibody	Jackson ImmunoResearch	1/1000	2 h
DCX (Pawluski *et al*., 2015; Gemmel *et al*., 2017)	NDS, Sigma 5%	goat anti-DCX	Sant Cruz sc-8066	1/300	12 h	Donkey anti-goat biotinylated antibody	Jackson ImmunoResearch	1/500	2 h
Synaptophysin *Hippocampus* (Gemmel *et al*., 2017, Pawluski *et al*., 2014)	NGS, Sigma 5%	mouse anti-synaptophysin	sigma S5768	1/500	12 h	Goat anti-mouse biotinylated antibody	DAKO	1/500 for hippocampus 1/400 for CG	2 h
PSD95 (Gemmel *et al*., 2017)	NGS, Sigma 2%	rabbit anti-PSD95	abcam ab18258	1/1000	12 h	Goat anti-rabbit biotinylated antibody	DAKO	1/200	2 h

Table 1. Summary of immunohistochemistry processing.
NDS = Normal Donkey Serum, NGS = Normal Goat Serum, CG = cingulate gyrus.

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	Control	Glyphosate	Roundup	F statistics	p value
Weight (g) dams at GD10	297.71 ± 8.77	294.14 ± 7.50	286.29 ± 9.61	$F_{(2,18)} = 0.454$	0.6417
Weight (g) dams at PD1	304.67 ± 11.76	309.50 ± 13.89	291.43 ± 11.52	$F_{(2, 16)}=0.595$	0.5633
Weight (g) dams at PD22	343.20 ± 9.76	305.30 ± 14.61	321.90 ± 12.59	$F_{(2,16)} = 2.171$	0.1465
AST (UI/L)	47.83 ± 5.62	64.00 ± 11.82	45.86 ± 1.97	$F_{(2,16)} = 1.857$	0.1883
ALT (UI/L)	40.17 ± 2.02	41.33 ± 3.91	46.00 ± 1.99	$F_{(2,16)} = 1.347$	0.2881
Ca^{2+} (nmol/L)	2.77 ± 0.08	2.58 ± 0.06	2.70 ± 0.04	$F_{(2,16)} = 2.216$	0.1414
Fe^{2+} (µmol/L)	83.57 ± 4.38	90.13 ± 10.53	88.24 ± 9.30	$F_{(2,16)} = 0.146$	0.8649
Mg^{2+} (mmol/L)	1.07 ± 0.02	0.92 ± 0.06	1.06 ± 0.04	$F_{(2,16)} = 3.499$	0.0549
Mn^{2+} (nmol/L)	105.6 ± 5.38	109.2 ± 12.77	118.9 ± 10.21	$F_{(2,16)} = 0.489$	0.6219
Interleukin-6 (pg/mL)	2.66 ± 2.48	3.44 ± 3.44	9.10 ± 6.31	$F_{(2,16)} = 0.591$	0.5656
Litter weight (g) at PD1	93.67 ± 4.49	90.50 ± 5.93	95.57 ± 4.26	$F_{(2,16)} = 0.277$	0.7616
Number of male pups in a litter	6.00 ± 0.26	5.17 ± 0.95	7.43 ± 0.53		
Number of female pups in a litter	6.00 ± 0.68	6.33 ± 0.56	5.14 ± 0.70		
Sex ratio	1.07 ± 0.13	0.89 ± 0.20	1.58 ± 0.22	$F_{(2,16)} = 3.559$	0.0526

Table 2. Mean (±SEM) of measures of toxicity (AST, ALT), weight and litter characteristics and serum analysis.
GD= gestation day, PD = postpartum day, AST = alanine transaminase, AST= aspartate transaminase, Ca^{2+} = calcium, Fe^{2+} = iron, Mg^{2+} = magnesium and Mn^{2+} = manganese.

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	Control	Glyphosate	Roundup	F statistics	p value
Hippocampal volume (mm^3)	11.38 ± 0.61	11.10 ± 0.54	11.60 ± 0.36	$F_{(2,16)}$ = 0.263	0.7718
PSD95 Dorsal DG	0.01028 ± 0.0046	0.0095 ± 0.0030	0.0142 ± 0.0040	$F_{(2,16)}$ = 0.429	0.6582
PSD95 Ventral DG	0.0244 ± 0.0035	0.0309 ± 0.0052	0.0335 ± 0.0053	$F_{(2,16)}$ = 0.954	0.4059
PSD95 CA3	0.00922 ± 0.0036	0.0105 ± 0.0055	0.0206 ± 0.0052	$F_{(2,16)}$ = 1.701	0.2140

Table3. Mean (±SEM) of hippocampal volume and PSD95 optical density (OD) in the dentate gyrus (DG) and CA3 hippocampal regions.

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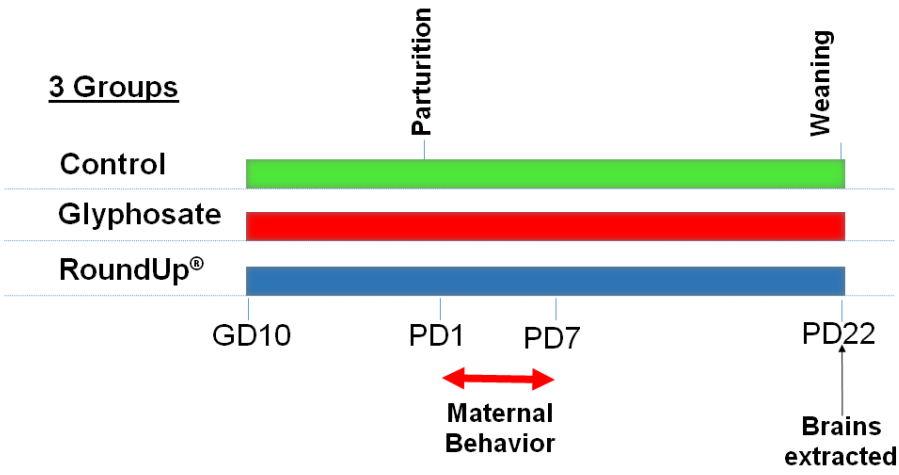

FIGURE 1 Timeline of the present study. Dams were treated from gestational day (GD)10 to postpartum day (PD)22. Maternal behaviour was observed on PD1-PD6 and brains were collected on PD 22

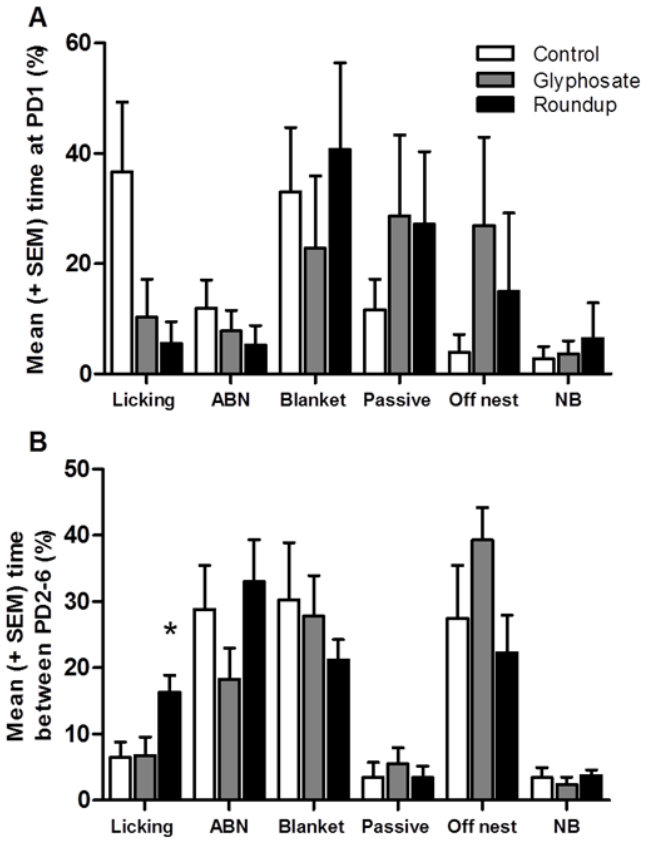

FIGURE 2 Mean ± SEM percentage of total time spent in maternal behaviour (%) A, during postpartum day (PD)1 and B, during PD2 to 6. Untransformed data are presented. *Significance ($P < 0.05$). ABN, arched-back nursing; NB, nest building

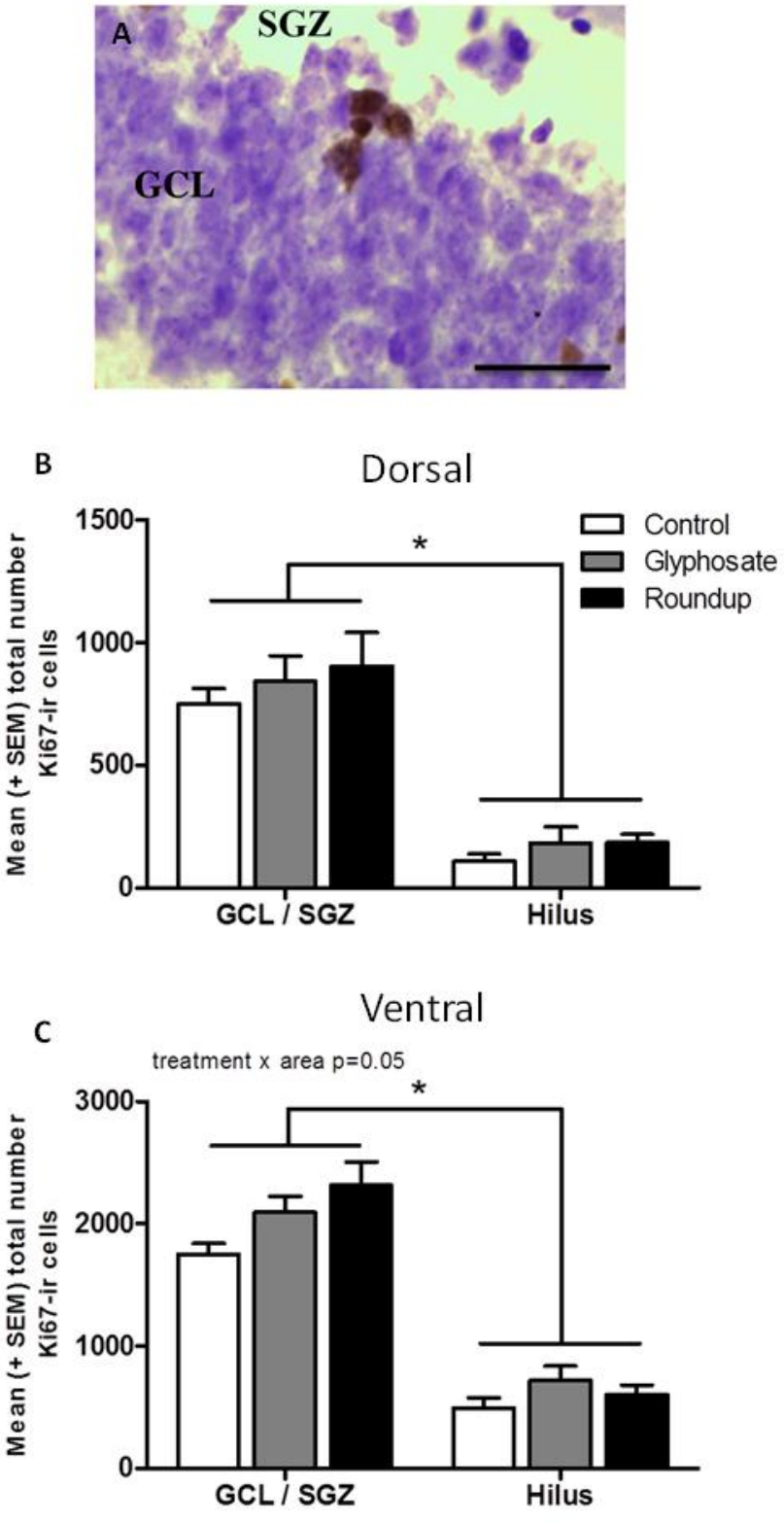

FIGURE 3 Representative photomicrographs of A, Ki67-immunoreactive (-IR) cells in the granule cell layer and subgranular zone (GCL/SGZ). Mean ± SEM number of proliferative (Ki67-IR cells) in the (B) dorsal and (C) ventral dentate gyrus and hilus of the hippocampus. There was a significant effect of the regions with GCL/SGZ having a higher number of proliferative cells and a trend on the interaction effect between treatment and area in the ventral hippocampus. *Represents statistically significant difference between GCL/SGZ and Hilus ($P < 0.05$) Scale bar = 20 μm. Arrows show the Ki67-IR cells

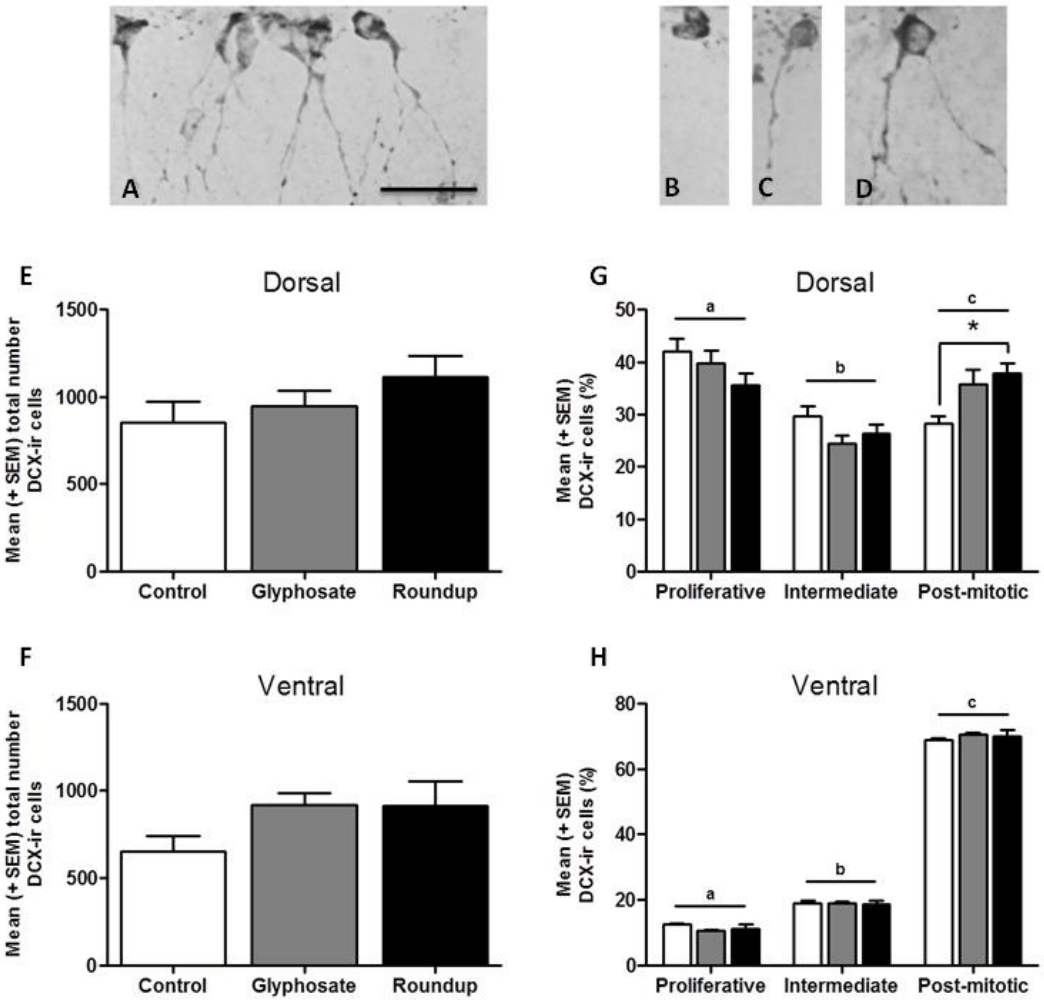

FIGURE 4 Representative photomicrographs of (A) doublecortin-immunoreactive (-IR) cells in the granule cell layer (GCL) with (B) proliferative, (C) intermediate and (D) post-mitotic stages shown at 40×. Mean ± SEM of (E) total number (×10) doublecortin-IR cells in the dorsal GCL, (F) total number (×10) doublecortin-IR cells in the ventral GCL, (G) percentage of doublecortin-IR cells morphology in the dorsal GCL and (H) percentage of doublecortin-IR cells morphology in the ventral GCL. Groups sharing different lowercase letters are significantly different. There was a significant difference on the proportion of the morphologies for both dorsal and ventral GCL. There was a significant interaction between the cell morphology and the treatment in the dorsal GCL. *Significance ($P < 0.05$). Scale bar = 20 μm

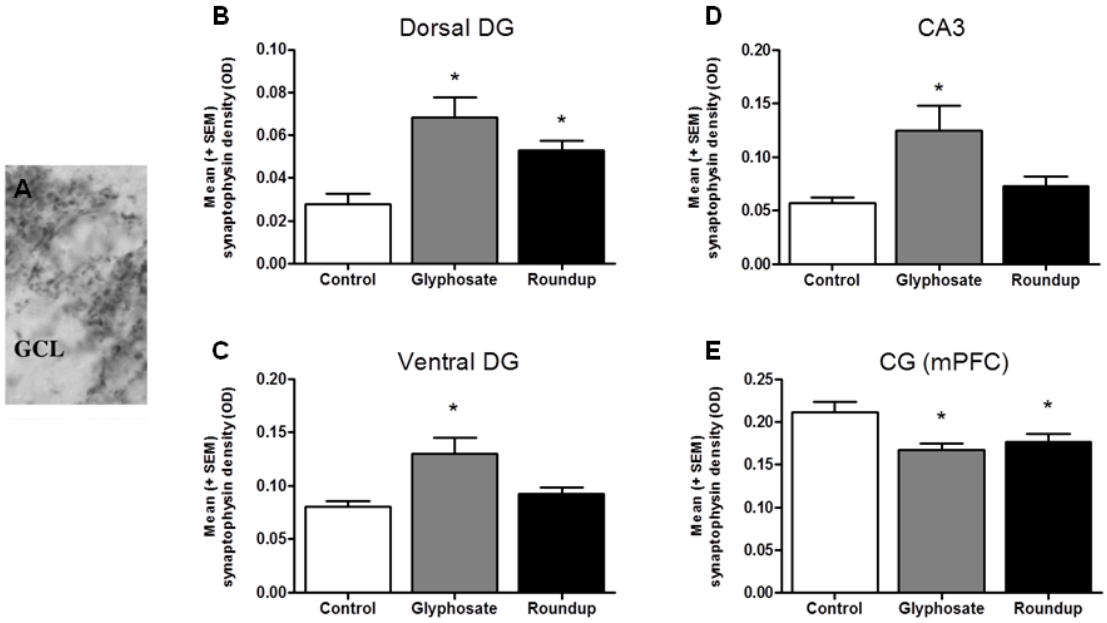

FIGURE 5 Representative photomicrographs of A, synaptophysin-immunoreactive (-IR) in the granule cell layer and subgranular zone (GCL/SGZ). Mean ± SEM synaptophysin density (OD) in (B) the dentate gyrus (DG) of the dorsal hippocampus, (C) the dentate gyrus of the ventral hippocampus, (D) the CA3 region of the dorsal hippocampus and (E) the cingulate gyrus (CG). *Significance ($P < 0.05$). mPFC, medial prefrontal cortex

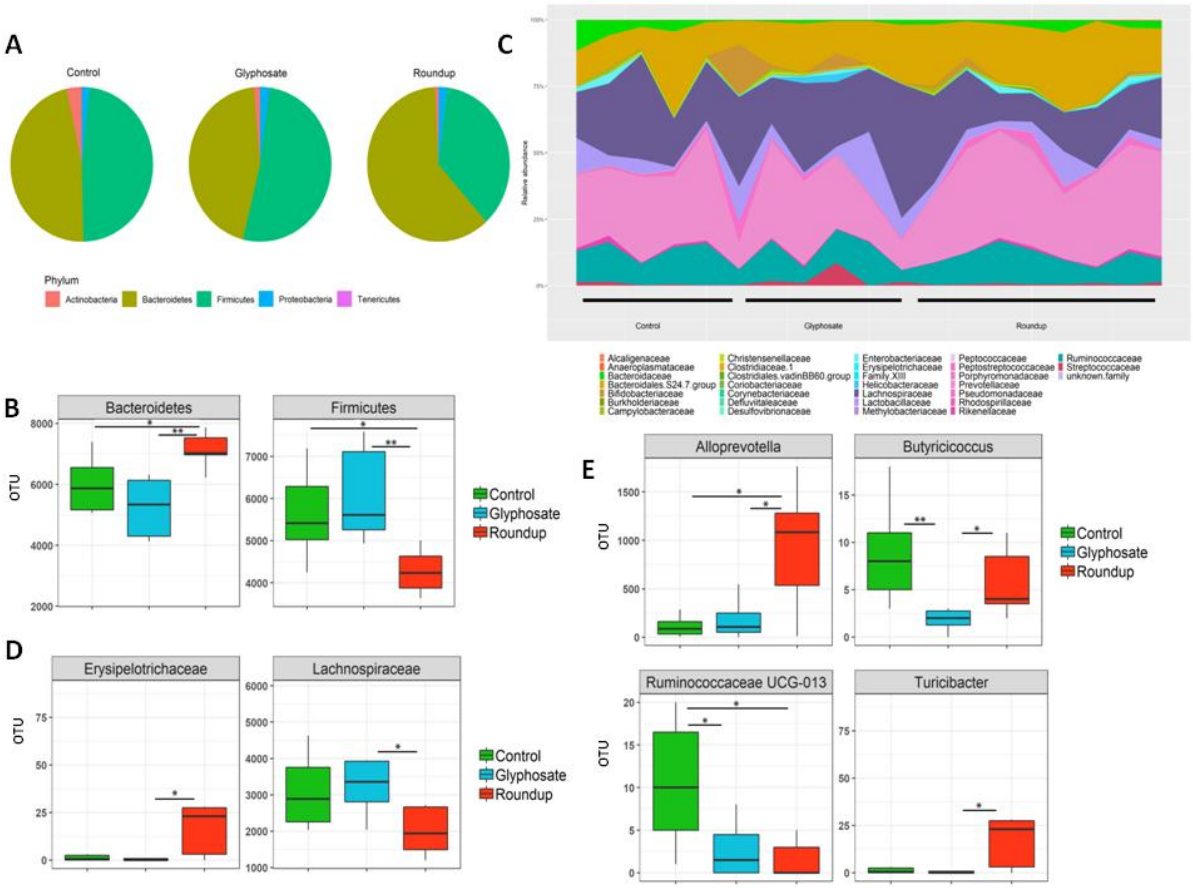

FIGURE 6 Analysis of the effects on gut microbiota by 16S rRNA gene high-throughput sequencing. A, Composition of abundant bacterial phyla identified in the microbiota of the three different groups. B, Phyla significantly affected by Glyphosate or Roundup [operational taxonomic unit (OTU) abundance]. C, percentage of 16S rRNA gene sequences. D, OTU abundance of the different bacterial families in each sample among the Control, Glyphosate and Roundup groups. E, OTU of the various bacterial genera in each sample among each group of rats. Data are presented as box plots. Significant difference (*$P < 0.05$ and **$P < 0.01$, respectively)