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Prenatal exposure to low doses of fungicides corrupts neurogenesis in neonates

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ABSTRACT

Neurogenesis plays a crucial role during neurodevelopment and its dysfunction can lead to neurodevelopmental disorders. A recent hypothesis stipulates that exogenous factors could corrupt this process and predispose to neurodegenerative disorders later in life. The presence of pesticide residues in the diet represents a threat of which we have recently become aware of. Indeed, they could corrupt neurogenesis, especially during gestation, potentially leading to impaired neuronal and synaptic functions. Since the effects of this low-noise contamination have not yet been evaluated on the neurodevelopment, we investigated the impact of fungicide residues on WT mice exposed throughout gestation. Thus, mice were exposed to fungicides, cyprodinil, mepanipyrin and pyrimethanil, alone at 0.1 µg/L during gestation until P3. Besides, another group was exposed to a cocktail of these three fungicides (0.1 µg/L each) for the same time. Exposure was performed through drinking water at the regulatory limit dose of the European countries (0.1 µg/L). No general toxicity was observed in neonates on body and brain weight upon fungicide exposure. However, results showed that gestational exposure to fungicide residues substantially promoted an increase of neural precursor cells at P3. This corrupted neurogenesis was linked to increased levels of β-catenin, likely through the crosstalk of the PI3K/Akt and Wnt/β-catenin pathways, both involved in cell proliferation. Fungicide exposure also altered protein expression of PSD95 and NMDA receptors in P3 neonates, two targets of the β-catenin signaling pathway. Adult neural stem cell extractions from mice treated with the fungicide cocktail, showed an increase proliferation and differentiation combined with a reduction of their migration properties. In addition, *in vitro* studies on hippocampal primary cell cultures treated with various concentrations of fungicides showed neurotoxic effects. To conclude, corruption of neurogenesis by this chemical assault could be a fertile ground for the development of neurological diseases later in life.

1. Introduction

Neurogenesis is the process where multipotent neural stem cells (NSC) proliferate and then differentiate into neurons. This process takes place in mouse brain during embryonic development until early

postnatal days (Götz and Huttner, 2005; Paridaen and Huttner, 2014), and few neurogenic areas still remain active in adult brain (Kozareva et al., 2019; Ming and Song, 2011). However, impairments of the proliferative properties of NSC can dramatically alter neurogenesis during embryonic stages and the consecutive migration of neurons, potentially

Abbreviations: Cypro, cyprodinil; Mepa, mepanipyrin; Pyri, pyrimethanil; EFSA, European Food Safety Agency; GFAP, Glial Fibrillary Acidic Protein; IBA1, Ionized calcium Binding Adaptor molecule 1; NMDA, receptor: N-methyl-D-aspartic acid receptor; AMPA receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; P3, post-natal day 3; G0, generation 0; G1, generation 1.

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leading to neurodevelopmental disorders (Kohl et al., 2007; Nuber et al., 2008; Rodríguez et al., 2008). Recently, Arendt and colleagues suggested that during embryonic neurogenesis, there is a time window where endogenous and exogenous factors can impact this process leading to mitotic failures (aneuploidy, chromosome copy variations, somatic mutations, ...). Affected cells have been proposed to be more sensitive to brain assaults and could be a key feature of neuronal cell death in Alzheimer's disease (Arendt et al., 2017). In line with this, our hypothesis is that exogenous factors such as environmental pollutants may affect neurogenesis, especially during gestation, potentially leading to impaired neuronal and synaptic functions, later in life.

Among pollutants, the role of pesticides as potential actors in neurogenesis corruption has gained importance. Their massive use and their low biodegradability have led to their release in all environmental media (water, soil, air, diet). This pernicious contamination represents a potential threat for human health and is currently subject to much debate and concern among populations. Indeed, Rauh and colleagues showed that children exposed to high levels of chlorpyrifos (>4.39 pg/g umbilical cord-blood) exhibited enlargement of the cerebral surface, associated to frontal and parietal cortical thinning (Rauh et al., 2012). Another study also revealed that a prenatal exposure to chlorpyrifos was associated with altered brain activation and decreased neuronal activity during tasks of executive functions (Sagiv et al., 2019). Besides, an exposure to DDE, a metabolite of DDT, during the first trimester of pregnancy, was associated with a reduction of the psychomotor development index. Every doubled increase of DDE levels, reduced this index of 0.5, during the first postnatal year ($n = 244$) (Torres-Sánchez et al., 2007). Experimental studies have confirmed this evidence by using rodent models. Indeed, oral treatment of rats with chlorpyrifos, carbaryl, endosulfan or cypermethrin, from gestational day 7 (E7) to postnatal day 21 (P21), impaired motor activity and coordination of these rats (Gómez-Giménez et al., 2018) mainly due to several impacts on GABAergic and glutamatergic systems (Gómez-Giménez et al., 2018).

In this study, we focused on three widely used fungicides belonging to the anilinopyrimidine class: cyprodinil, mepanipyrim and pyrimethanil. These fungicides are widely used to preserve crops, as well as for the long-term storage of fruits and vegetables. Many reports have established the presence of residues of cyprodinil, mepanipyrim and pyrimethanil in our environment and in many foodstuffs (ANSES, 2010; EFSA, 2018, 2019; Générations Futures, 2016, 2017; Herrero-Hernández et al., 2016; Nougadère et al., 2020; PAN Europe, 2008) (Table S1). Moreover, according to the total diet study 2, these 3 fungicides are among the top 5 pesticide residues found in the French diet (ANSES, 2011), as well as in baby food jars (Nougadère et al., 2020). Besides, a study conducted on cortical primary neurons treated with mepanipyrim (0.1–100 μ M) for 8 days exhibited neurotoxicity with a LC_{50} of 26 μ M, suggesting a potential harmful effect for brain (Regueiro et al., 2015). Therefore, there is still a need to further evaluate this neurotoxic effect *in vivo*. In a previous study, we evaluated the impact of a cocktail of three fungicides (cyprodinil, mepanipyrim and pyrimethanil) at low doses in a mouse model of Alzheimer's disease (AD), during 9 months (Lafon et al., 2020). The results showed an aggravation of the main AD markers, such as inflammation, amyloid load, as well as increased hippocampal neuronal loss. As the cocktail of anilinopyrimidine fungicides is detrimental to the brain, we were interested in evaluating their impact on neurodevelopment since it has not been reported yet.

We investigated the impact of fungicide residues on WT mice exposed during all the gestational stage and its consequences on fetal neurodevelopment. Mice were exposed to cyprodinil, mepanipyrim and pyrimethanil either alone (0.1 μ g/L) or in cocktail (0.1 μ g/L each) through drinking water, at the regulatory limit dose of the European countries (Council of the European Union, 1998) until postnatal day 3 (P3). In parallel, some animals were exposed to the fungicides up to the age of 4-months to extract their adult neural stem cells (aNSC) for further analyses of their properties. Altogether, our study raises the

question whether gestational exposure to anilinopyrimidine fungicides, at the regulatory limit dose fixed by the E.U in water, can have an impact on brain development and neurogenesis processes of the offspring.

2. Materials and methods

2.1. Ethics statement

This project follows the specific French national guidelines on animal experimentation and well-being and was approved by the French National Ethic Committee for Animal Experimentation (APAFIS no. 7357-20161,025-18,055,782). C57BL/6 J wild type (WT) mice were used for this study. Animals were housed under 12 h light/12 h dark cycle, at 23 ± 2 °C and $53 \pm 10\%$ of relative humidity. Animals had free access to water and food and were fed under a standard chow diet (A03) (SAFE Diets, France).

2.2. Animals' treatment with fungicides

Fungicides used in this study, cyprodinil, mepanipyrim and pyrimethanil (Fig. 1A) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Fungicides stock solutions were prepared as previously described in Lafon et al. (2020). Briefly, stock solutions were performed in glass vials (Wheaton) at 1 mg/mL in pure DMSO according to the supplier's recommendations. These solutions were diluted 1000 times in distilled water to reach a final concentration of 1 mg/L. Solutions were then filtered (0.5 μ m) and stored in glass bottles at 4 °C until use. For animal treatment, stock solutions at 1 mg/L were further diluted 10,000 times in tap water to reach a final concentration of 0.1 μ g/L. Animals were treated either with each fungicide alone at 0.1 μ g/L, or with a cocktail of the three fungicides at 0.1 μ g/L each (cyprodinil: 444 pM, mepanipyrim: 448 pM, and pyrimethanil: 502 pM). This concentration corresponds to the regulatory maximal limit dose allowed in drinking water (Council of the European Union, 1998). According to the E.U. regulations, the total concentration of several pesticide residues in drinking water cannot exceed 0.5 μ g/L (Council of the European Union, 1998). In parallel, a solution of pure DMSO (100%) was diluted 1000 times in distilled water to a final concentration of 0.1%, filtered (0.5 μ m) and stored in glass bottles at 4 °C until use. To treat control vehicle animals, the stock solution of DMSO at 0.1% was further diluted 10,000 times in tap water ($1 \times 10^{-5}\%$ corresponding to the equivalent volume of 1 fungicide). Thus, for an equivalent volume for 3 fungicides, the final percentage of DMSO used is $3 \times 10^{-5}\%$. Mice water bottles were changed once a week for all groups and prepared from stock solutions as described above.

One-month-old C57BL/6 J WT mice were treated with fungicides either alone or in cocktail, or with an equivalent volume of DMSO for 2 months (Fig. 1B). For each group (Control, cocktail, cyprodinil, mepanipyrim, pyrimethanil), 2 males and 6 females were used (named generation G0). After 2 months, and for each group, 1 male was housed in a cage containing 3 females for mating. Treatment with fungicides was pursued throughout mating, gestation and suckling. Neonates were then sacrificed at 3 days post-natal (P3) and named as generation G1 (Fig. 1B – step 1). For each study, animals were weighted at P3. In parallel, 4 male (CTR $n = 2$ and Treated $n = 2$) and 4 female (CTR $n = 2$ and Treated $n = 2$) mice from G1, were breed and treated with DMSO or fungicide cocktail until 4 months old (Fig. 1B – step 2). They were then sacrificed for hippocampal neural stem cell dissociation and isolation.

2.3. Tissue collection and preparation

P3 mice were sacrificed and brains were collected and weighted. Due to the small size of the heart of P3 mice, intracardiac perfusion with PBS has not been performed. For each group, 50% of the brains collected were immediately frozen in liquid nitrogen for biochemical analyses and stored at -80 °C. Other brains were fixed in a commercial

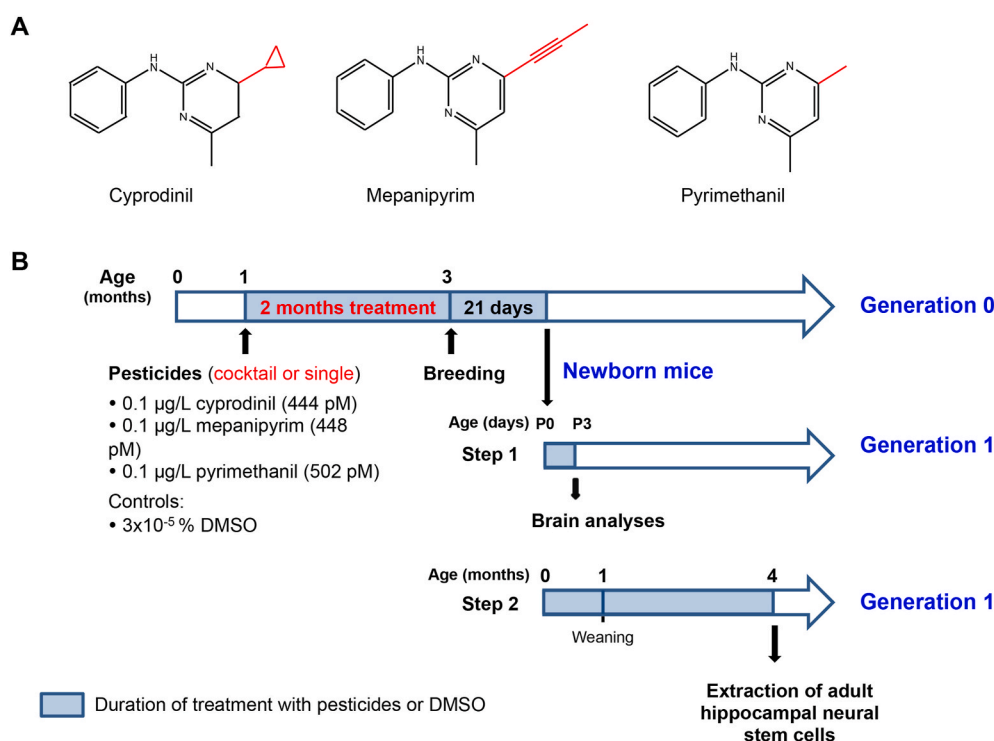


Fig. 1. Schema of the exposure of WT mice to fungicide residues during gestation. **A.** Chemical structures of cyprodinil, mepanipyrim and pyrimethanil, three fungicides belonging to the class of anilino-pyrimidines. **B.** *In vivo* study design of the treatment with fungicides. One-month old WT male and female mice (Generation 0, G0) were treated with either 0.1 µg/L of cyprodinil, or mepanipyrim, or pyrimethanil or with a cocktail of the three fungicides (0.1 µg/L of each compound), through tap water for 2 months. WT male and female mice treated with an equivalent volume of DMSO (3 × 10⁻⁵%) were used as controls. Then, 3 females and 1 male, receiving the same treatment, were mated in the same cage (breeding). After 21 days of gestation, mimicking a vertical contamination with fungicides, the offspring (Generation 1, G1) were sacrificed at 3 days postnatal (P3) for brain histological and biochemical analyses (step 1). In parallel, some animals from Generation 1 were kept under fungicide cocktail treatment, until culling at 4 months (Step 2). Then, hippocampi were collected in order to extract adult neural stem cells (aNSC) for analyses of their properties.

paraformaldehyde solution (Antigenfix, Diapath) for 24 h, at 4 °C. Brains were then rinsed in PBS, cryoprotected in a 30% sucrose solution for 4 days at 4 °C and included in OCT (Tissue-Tek, Sakura Finetek). Brains embedded in OCT were quickly frozen in acetone chilled on dry ice and conserved at -80 °C until use. Brains embedded in OCT were then processed in a cryostat (Leica) to perform 20 µm frontal sections that were directly put on Superfrost Plus glass slides (Micom France).

2.4. Immunohistochemistry and quantifications

Prior to labeling, the 20 µm frontal brain tissue sections were rinsed with PBS to remove OCT, blocked for 1 h at room temperature (RT) (3% goat serum, 0.3% Triton X-100 in PBS). Sections were then incubated overnight at 4 °C, with primary antibodies (Table S2, column IHC). Sections were washed with PBS and incubated with the appropriated secondary antibody, either a goat anti-mouse Cy3 (1:500, Jackson ImmunoResearch) or a goat anti-rabbit Alexa488 (1:1000, Invitrogen) for 1.5 h at RT. All sections were counterstained with DAPI (1:50,000 in PBS; Molecular Probes) to stain nuclei. Images were taken using a Zeiss Axioimager (Zeiss) and quantifications were performed using the Fiji software (version 2.0, National Institutes of Health, Bethesda, MD) on 4 distinct areas of the hippocampus: dentate gyrus (DG), CA1, CA2 and CA3. Astroglisis was evaluated by quantifying the number of GFAP⁺ astrocytes in the different areas analyzed. The total number of microglial cells and the activated subset were quantified as described previously in Watson et al. (2012). To decipher the subset of activated microglial cells from the quiescent ones, quantifications were performed based on their cell body diameter (Batchelor et al., 1999). Microglial cells whose cell body diameters is less than 7 µm were quantified as quiescent cells and are characterized by long ramified processes. On the contrary, microglial cells with cell body diameters over 7 µm were quantified as activated cells (amoeboid) possessing short processes and decreased branching. All the results are presented as mean ± SEM of cells/mm².

2.5. Interleukins and cytokines assays by TR-FRET (HTRF)

Brain homogenates were obtained as described previously (Lafon

et al., 2020). Briefly, frozen brains from P3 newborns were homogenized in 20% weight/volume (w/v) in a homogenization buffer (140 mM KCl, 10 mM Na₂HPO₄, KH₂PO₄, 1 mM EDTA) containing proteases inhibitors (Complete Ultra, Roche) and phosphatases inhibitors (PhosStop, Sigma). Homogenization was performed using microbead-containing tubes and a ribolysor apparatus (Biorad). Samples were then aliquoted and immediately frozen at -80 °C until use. IL1β, IL6 and TNFα levels were determined using TR-FRET (Time Resolved - Fluorescence Resonance Energy Transfer) also called HTRF assay (Cisbio) as described in Lafon et al. (2020). Briefly, 2% of SDS was added to brain homogenates which were then centrifuged at 14,000 rpm, for 20 min at 4 °C. Then, 16 µL of supernatant were mixed with 2 µL donor antibody coupled to Eu³⁺-cryptate and 2 µL of acceptor antibody coupled to d2, both antibodies recognizing IL1β, IL6 or TNFα. The mix was incubated for 1 h (IL6 and TNFα) or overnight (IL1β) at 25 °C. Standard curves of each kit were performed using the homogenization buffer used for samples and containing SDS. FRET signal was measured using the d2 acceptor emission (665 nm) and the Eu³⁺-cryptate donor emission (620 nm) with a 100 µs lag time and a 400 µs integration time after excitation at 337 nm on a Spark 20 M (Tecan). HTRF ratio (signal 665 nm/620 nm × 10⁴) was calculated to normalize the different wells. Results were then normalized according to their protein concentration determined by BCA (bicinchoninic acid method) (Pierce Biotechnology).

2.6. Immunoblotting

Brain homogenates were centrifuged at 14,000 rpm, 15 min at 4 °C, to remove membrane debris. Protein concentration was measured on supernatants using a BCA assay kit. Samples normalized to the same concentration and final volume, were diluted (1:4) in 4X loading buffer (0.5 M DTT, 3% SDS, 40% glycerol, 0.8 M Tris-HCl pH 6.8, bromophenol blue). Samples were boiled 10 min at 90 °C and loaded either on 12% or 15% SDS-PAGE gel. After protein transfer, membranes were blocked in a 5% non-fat milk solution and were incubated with the primary antibodies listed in Table S2 (column WB), overnight at 4 °C. Membranes were then incubated with the corresponding secondary antibodies: peroxidase-conjugated anti-mouse antibody (1:20,000; Jackson

ImmunoResearch) or anti-rabbit (1:20,000; Jackson ImmunoResearch) for 2 h at RT. Protein loading controls were performed using a mouse anti- β -actin antibody (1:2000, Sigma-Aldrich). Membranes were revealed using ChemiDoc MP Imager (Biorad). Western blot signals were quantified using ImageLab software (Biorad) and are presented as mean \pm SEM of the signal of the protein of interest normalized to the signal of β -actin.

2.7. Primary culture of hippocampal neurons and fungicide treatment

Pregnant Swiss mice were purchased from Janvier Labs. They were sacrificed by cerebral dislocation. E17.5 embryos were extracted, and hippocampi were collected using sterile surgical tools. Cerebral tissues were dissociated according to the protocols described by STEMCELL Technologies®. Briefly, hippocampi were collected in ice-cold NeuroCult™ Tissue Collection Buffer. Tissues were enzymatically dissociated with 3 mL of 0.25% Trypsin/EDTA for 10 min at 37 °C. Reaction was stopped by adding 7 mL of DMEM complete medium (DMEM high glucose medium with 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin (P/S)). Dissociated tissues were then centrifuged at 200 g for 1 min. The supernatant was discarded and the pellet was washed 2 times with DMEM complete medium and resuspended in 1 mL Complete Plating Medium (9.8 mL NeuroCult™ Basal Medium, 0.2 mL NeuroCult™ SM1 Neuronal Supplement, 25 μ L L-Glutamine at 200 mM, 18.5 μ L L-Glutamic acid at 2 mg/mL and 1X P/S). The pellet was mechanically dissociated by gently pipetting up and down 4–5 times with a 1 mL pipette. The volume was then adjusted to 10 mL and cell suspension was centrifuged at 600 g for 1 min. Cellular pellet was resuspended with 1 mL of Complete Plating Medium. Then, cell suspension was filtered on a 40 μ m cell strainer placed on a top of a 50 mL corning tube. Cell number was counted using a cell counter apparatus (Biorad). 1.5×10^4 cells were seeded on 12 mm coverslips pre-coated with 20 μ g/mL of poly-D-lysine (PDL) in standard 24 wells plate (Nunc). After 5 days, half of the complete medium was replaced by the Complete Maturation Medium (9.8 mL BrainPhys™ neuronal medium, 0.2 mL NeuroCult™ SM1 Neuronal Supplement and 1X P/S) (STEMCELL Technologies®). Fungicides or an equivalent volume of DMSO were then added to cells at the same time. Cells were further cultivated during 5 days in the maturation medium and then fixed with Antigenfix solution at RT for 15 min. Coverslips were washed with cold PBS and conserved at 4 °C until use. Stock solutions of fungicides used for this experiment were prepared at 10 mM, 20 mM and 50 mM in pure DMSO. The percentage of neuronal cell survival was evaluated by performing the ratio of the pycnotic nuclei out of the total number of nuclei of BIII-tubulin + cells, using the DAPI staining. Cell survival after fungicide treatment was quantified using Fiji software on 10 images for each coverslip, and 3–7 coverslips were used per condition.

2.8. Adult neural stem cells extraction and neurospheres amplification

Hippocampi from 4 months old C57BL/6 J WT mice treated with a cocktail of cyprodinil, mepanipyrin and pyrimethanil at 0,1 μ g/L each, or with an equivalent volume of DMSO ($3 \times 10^{-5}\%$) were collected to extract aNSC. Hippocampal tissues were dissociated according to the protocol described by STEMCELL Technologies®. Briefly, hippocampi were collected on ice-cold Tissue Collection Buffer and cut into small pieces. Then tissues were enzymatically dissociated with 1 mL of Dissociation Medium (NeuroCult™ dissociation kit for adult CNS tissue) for 7 min at 37 °C. Reaction was stopped by adding 1 mL of the inhibition solution. Dissociated tissues were then centrifuged at 100 g for 7 min. The supernatant was discarded, and the pellet was washed in 150 μ L of sterile PBS and resuspended by gently pipetting up and down 4–5 times with a 200 μ L pipette. Then 1 mL PBS was added, and the cellular mix was gently pipetted up and down 4–5 times with a 1 mL pipette. The volume was then adjusted to 5 mL and centrifuged at 100 g for 7 min. The resulting pellet was resuspended with 1 mL of Complete Adult

Neurocult™ Proliferation Medium (45 mL NeuroCult™ basal medium, 5 mL NeuroCult™ Proliferation Supplement and 500 μ L 1X P/S) (STEMCELL Technologies®) to which 20 μ g/mL of EGF and 10 μ g/mL of bFGF were added. Then, cell suspension was filtered on a 40 μ m cell strainer placed on a top of a 50 mL corning tube. The filter was rinsed with 1 mL Complete Adult Neurocult™ Proliferation Medium and cell suspension was cultured in a low adhesion 6 well plate. Medium was changed every 2 days and 20 μ g/mL of EGF and 10 μ g/mL of bFGF were added every day for aNSC amplification until formation of neurospheres (between 7 and 10 days).

2.9. Edu proliferation assay

Neurospheres were dissociated with 0.05% Trypsin/EDTA at 37 °C for 3 min, the digestion was stopped with DMEM/F12 medium with 10% fetal bovine serum. Suspension was then centrifuge at 100 g for 5 min and pellets were washed with Complete Adult Neurocult™ Proliferation medium. Cells were then centrifuged 150 g for 5 min and pellets were resuspended in Complete Adult Neurocult™ Proliferation medium by gently pipetting up and down 4–5 times using 200 μ L pipette, and then the volume was adjusted to 5 mL. Cell number was determined using a cell counter apparatus (Biorad), and 1×10^5 cells was distributed in low adhesion 6 well plate. aNSC were amplified in the Complete Adult Neurocult™ Proliferation Medium for 2 days to form neurospheres. Neurospheres were then transferred into 12 well plate containing 18 mm coverslips coated with PDL. Ten micromolars of Edu was added to the medium for 24 h. Then, neurospheres were fixed with Antigenfix at RT for 15 min, washed 2 times with PBS, and 2 times with 3% BSA in PBS. Neurospheres were permeabilized with 0.5% Triton X-100 in PBS at RT for 20 min, washed with 3% BSA in PBS, and incubated with 500 μ L Click-iT reaction cocktail (430 μ L 1X Click-it reaction buffer, 20 μ L CuSO₄, 1.2 μ L AlexaFluor, 50 μ L reaction buffer additive) at RT for 30 min. Then, wells were washed 2 times with 1 mL 3% BSA in PBS and rinsed once with PBS. Nuclei were stained with Hoechst 33,342 (ThermoFisher) (1:2000 in PBS) at RT for 30 min. Images were acquired using a Zeiss Axioimager. Quantifications were performed using Fiji software.

2.10. Differentiation of adult neural stem cells

Neurospheres were dissociated with 0.05% Trypsin/EDTA at 37 °C for 3 min, as described above in the Edu proliferation assay. 1×10^5 cells were distributed in 24 well plate (Nunc) containing 12 mm coverslips pre-coated with 20 μ g/mL of PDL. After 6 h, when the aNSC were attached to the coverslip, Complete Adult Neurocult™ Proliferation Medium was replaced by the Complete Neurocult™ Differentiation medium from STEMCELL Technologies® (9.5 mL NeuroCult™ basal medium, 1 mL NeuroCult™ differentiation supplement and 1X P/S). Cells were cultivated 7 days in the differentiation medium and then fixed with Antigenfix solution at RT for 15 min, washed in cold PBS and conserved at 4 °C until use.

2.11. Immunocytofluorescence on primary culture and differentiated aNSC

Fixed cells were blocked for 1 h at RT with PBS containing 2% BSA and 0.1% Triton X-100. Then, cells were incubated with the appropriate primary antibodies (Table S2, column IF), overnight at 4 °C. Then, cells were washed with PBS and incubated with the appropriate secondary antibody: either a goat anti-mouse Cy3 antibody or a goat anti rabbit Alexa488 antibody (1:500) at RT for 1.5 h. Nuclei were stained using DAPI and images were acquired using a Zeiss Axioimager. Quantifications were performed using Fiji software on 10 images for each coverslip, and 3–7 coverslips were used per condition.

3.12. Software and statistical analyses

Before any statistical analysis, all data were analyzed using Shapiro-Wilk's normality test. For parametric statistical analyses, Student's T-test or one-way ANOVA, followed by Holm-Sidak's *post-hoc* analysis, were used and * referred to statistical significance. For non-parametric analyses, Kruskal & Wallis test followed by Dunn's *post-hoc* analysis was used when appropriate and referred with #. Statistical differences were measured using GraphPad Prism 7.2 software (GraphPad Software, La Jolla, CA, USA). For all analyzes, a probability of 0.05 has been defined as a significant difference.

3. Results

3.1. Gestational exposure to very low doses of fungicides did not affect body and brain weight of P3 neonates

The aim of this study was to evaluate the impact of a prenatal exposure to three widely detected fungicide residues on brain development. Prior to histological and biochemical analyses of brain tissues, we measured the body weight (Fig. 2A) and of the brain weight (Fig. 2B) of P3 neonates exposed during gestation to fungicides. Results did not reveal any differences compared to control mice exposed to an equivalent volume of DMSO (CTR) (Kruskal-Wallis tests followed by Dunn's *post-hoc* analysis, p -values > 0.05), suggesting no general toxic effects upon exposure.

3.2. Gestational exposure to fungicides enhanced microgliosis and astrogliosis in the CA1 and CA3 areas of the hippocampus in P3 neonates

To study the microglial-mediated inflammation we used the IBA1 (ionized calcium binding adaptor molecule 1) constitutive marker of microglial cells (Fig. 3A). IBA1 labelling in the DG, CA1, CA2 and CA3 areas of the hippocampus allowed us to quantify the total number of IBA1⁺ cells (Fig. 3B), as well as the number of activated microglial cells (Fig. 3C). Analyses revealed that treatment with the cocktail of the three fungicides significantly increased the total number of microglial cells in the CA1 (Figs. 3B and 129 ± 9.4 microglial cells/mm² for CTR vs 184.8 ± 11.4 for cocktail, p -value > 0.001) and in the CA3 areas (Figs. 3B and 160.1 ± 11.8 for CTR vs 229 ± 21.7 for cocktail, p -value = 0.029) in P3 neonates' brain compared to DMSO-exposed mice (CTR). However, single fungicide exposure during gestation did not modify the total number of IBA1⁺ cells in the different areas analyzed (p -values > 0.005) (Fig. 3B). In addition, gestational exposure to the cocktail of fungicides also increased the number of activated microglial cells in P3 neonates both in the CA1 (Figs. 3C and 37 ± 4.2 activated microglial cells/mm² for CTR vs 65.5 ± 8.1 for cocktail, p -value = 0.004) and the CA3 areas (Figs. 3C and 44.9 ± 6.4 for CTR vs 78.5 ± 12 for cocktail, p -value = 0.039) compared to DMSO-exposed mice. No impact on the number of activated microglial cells was observed in P3 neonates, upon exposure to

a single fungicide (p -values > 0.05) (Fig. 3C).

In parallel, astrocytes activation was evaluated by using an anti-GFAP antibody and the total number of GFAP⁺ astrocytes was quantified in different regions of the hippocampus: dentate gyrus (DG), CA1, CA2 and CA3 areas (Figs. S1A–B). *In utero* exposure to the cocktail of fungicides and to pyrimethanil significantly increased the total number of astrocytes in the CA1 area of the hippocampus (Fig. S1B, 300.4 ± 19.4 astrocytes/mm² for CTR vs 429.3 ± 26.3 for cocktail, p -value < 0.001 and vs 397.2 ± 18.3 for pyri, p -value = 0.029). Astroglia was exacerbated in the CA3 area of P3 neonates when exposed *in utero* to mepanipyrim compared to CTR mice (Fig. S1B, 469.1 ± 28.2 astrocytes/mm² for CTR vs 616.4 ± 33 for mepa, p -value = 0.015).

As fungicides exposure during gestation exacerbated astrogliosis and microgliosis in P3 neonates, we next measured interleukin 1 β (IL1 β), interleukin 6 (IL6) and tumor necrosis factor α (TNF α) levels in brain homogenates by TR-FRET (Fig. S2). Regarding IL1 β , no levels were detectable in brains excepted for cocktail and pyrimethanil (Fig. S2A). Regarding, IL6 and TNF α levels, results showed that there are no statistical differences in the brain of P3 neonates, exposed to fungicides, either alone or in cocktail, compared to CTR mice (Figs. S2B–C, one-way ANOVA - Holm-Sidak's *post-hoc* analysis, p -values > 0.05). However, we noticed a statistically significant difference between cyprodinil and pyrimethanil regarding IL6 (Fig. S2B, 3.92 ± 1.54 pg/mg for cypro vs 15.82 ± 2.94 for pyri, p -value = 0.0077) and TNF α levels (Fig. S2C, 4.03 ± 0.86 pg/mg for cypro vs 8.88 ± 1.26 for pyri, p -value = 0.0053). Results showed that pyrimethanil has a rather more pro-inflammatory action, whereas cyprodinil has an opposite effect, likely both effects are counterbalanced in the cocktail treatment.

Altogether, these results showed that if exposure to fungicides, either alone or in cocktail, during gestation triggered astrogliosis and microgliosis (CA1 and CA3 areas) in P3 newborn mice (Fig. 3 and Fig. S1), those were not sufficient to promote a significant increased secretion of IL6 and TNF α , compared to CTR mice.

3.3. Gestational exposure to fungicides substantially raised NSCs and impaired mature neurons, in P3 neonates

Since herbicides as glufosinate-ammonium inhibits neurogenesis in the subventricular zone in newborn mice (Feat-Vetel et al., 2018) and paraquat inhibits hippocampal neurogenesis in adult mice (Li et al., 2017), we were interested to study the effect of a gestational exposure to fungicides on neurogenesis. First, we studied by immunohistochemistry and western blotting two different markers: nestin, which is expressed by neural progenitor cells during the development of the central nervous system (Lendahl et al., 1990) and doublecortin (DCX), expressed by neuroblasts and immature neurons (des Portes et al., 1998) (Fig. 4). Analysis of hippocampi on brain tissue sections of P3 neonates exposed to the cocktail of the three fungicides and labeled with anti-nestin or anti-DCX antibodies revealed a very strong increase in the fluorescence intensity compared to the DMSO-exposed mice (CTR) (Fig. 4A). As we

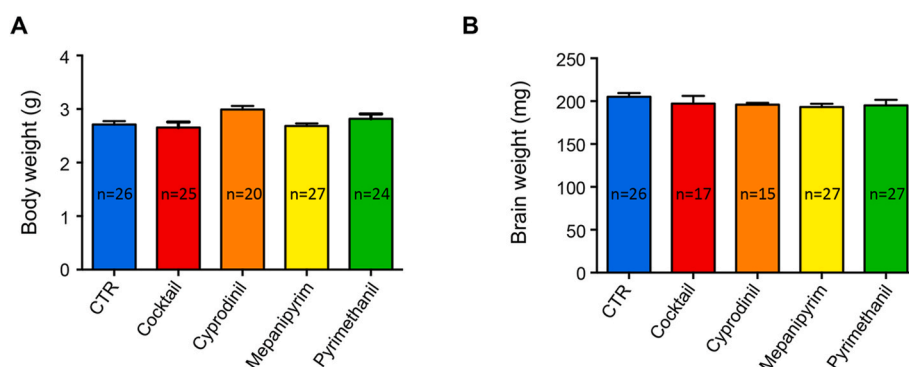


Fig. 2. Treatment of WT mice with fungicides, alone or in cocktail, during gestation, did not impact body and brain weights. Body weight (A) and brain weight (B) of P3 newborn mice (generation 1) exposed during gestation to 0.1 μ g/L of cyprodinil, or mepanipyrim, or pyrimethanil or with a cocktail of the three fungicides (0.1 μ g/L of each compound). Mice exposed to an equivalent volume of DMSO ($3 \times 10^{-5}\%$) were used as controls (CTR). Data are expressed as mean \pm SEM and statistical differences were analyzed using a Kruskal-Wallis test followed by a Dunn's *post-hoc* analysis (# p -value < 0.05). (n = 15–27 animals per group).

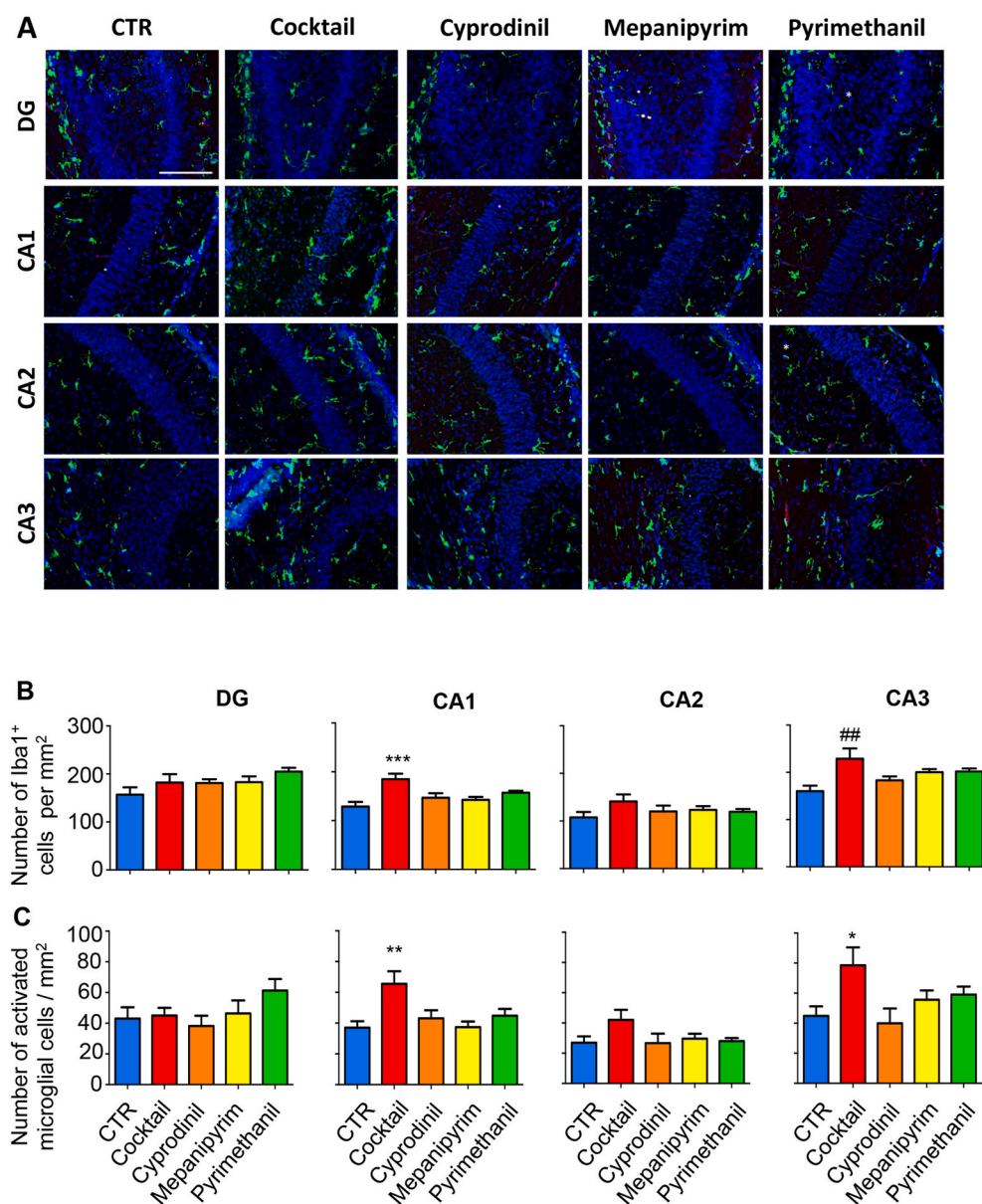


Fig. 3. *In utero* exposure to the cocktail of fungicide residues exacerbates microglial inflammation in the hippocampus of P3 neonates. **A.** Representative images of brain tissue sections of neonates at P3, exposed during gestation either to 0.1 $\mu\text{g/L}$ of cyprodinil, or mepanipyrim, or pyrimethanil or to a cocktail of the three fungicides (0.1 $\mu\text{g/L}$ of each compound), and labeled with an anti-IBA1 antibody. Mice exposed to an equivalent volume of DMSO ($3 \times 10^{-5}\%$) were used as controls (CTR). Scale bar: 100 μm . **B.** Quantification in the different areas of hippocampus (DG, CA1, CA2, CA3) of the total number of microglial cells per mm^2 . **C.** The number of activated microglial cells per mm^2 . For all immunohistochemistry analyses, 4–9 animals per group were used (CTR: $n = 9$; Cocktail: $n = 8$; Cyprodinil: $n = 4$; Mepanipyrim: $n = 6$ and Pyrimethanil: $n = 6$) and 6 to 13 tissue sections containing hippocampi per animal were analyzed. Results are presented as mean \pm SEM. A one-way ANOVA followed by a Holm-Sidak's *post-hoc* analysis (* p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001) or a Kruskal-Wallis test followed by a Dunn's *post-hoc* analysis (## p -value < 0.01) were used to evaluate statistical differences ($\alpha = 0.05$), depending on the distribution of the data sets analyzed with the Shapiro-Wilk's normality test.

cannot quantify rigorously this fluorescence difference on tissue sections, we analyzed their protein expression by western blotting. Nestin expression from brain homogenates of P3 neonates showed a significant increase, of more than 4-folds, in mice exposed to single fungicide treatment compared to CTR mice (Figs. 4B and 0.232 ± 0.12 A.U. for CTR vs 1.05 ± 0.04 for cypro, p -value < 0.001 ; vs 1.15 ± 0.06 for mepa, p -value < 0.001 ; vs 0.89 ± 0.08 for pyri, p -value < 0.001). In addition, nestin expression was also increased (~ 5 -folds) in P3 neonates exposed to the fungicide cocktail compared to CTR mice (Figs. 4B and 0.22 ± 0.10 A.U. for CTR vs 1.09 ± 0.14 for cocktail, p -value < 0.001), confirming the results obtained by immunohistochemistry (Fig. 4A). DCX protein levels were also significantly increased in P3 neonates (5 to 9-folds) exposed to fungicides during gestation, either alone (Figs. 4C and 0.11 ± 0.04 A.U. for CTR vs 1.02 ± 0.09 for cypro, p -value = 0.005; vs 0.63 ± 0.14 for mepa, p -value = 0.038; vs 0.86 ± 0.22 for pyri, p -value = 0.014), or in cocktail (Figs. 4C and 0.24 ± 0.02 A.U. for CTR vs 1.14 ± 0.17 for cocktail, p -value < 0.001), compared to the CTR mice. In addition, results showed a significant 2-folds decrease of NeuN protein levels, a marker of mature neurons, in the brains of P3 neonates exposed to the fungicide cocktail (Figs. 4D and 0.83 ± 0.07 A.U. for CTR vs 0.43

± 0.06 for cocktail, p -value = 0.002), compared to CTR. However, no significant modifications of NeuN expression were observed in P3 neonates exposed to single fungicides (Fig. 4D, one-way ANOVA, Holm-Sidak's *post-hoc* analysis, p -values > 0.05). This decrease of NeuN expression, observed in cocktail-exposed mice, suggested a potential neurotoxic effect of these fungicides. To better study this effect, we performed primary cell cultures of hippocampal neurons extracted from Swiss mouse embryos at E17.5 (Fig. S3A). After 5 days of culture, neurons were treated with 50 μM of cyprodinil, or mepanipyrim, or pyrimethanil, or with a cocktail of the three fungicides at different concentrations (10 μM , 20 μM and 50 μM), for 5 days (Figs. S3A–C). Cells without any treatment (NT) or treated with an equivalent volume of DMSO were used as controls. The impact of the fungicides' treatment on neuronal cell survival was quantified by counting the pycnotic nuclei among the β III-tubulin⁺ cells (Fig. S3B) and revealed a significant decrease upon treatment with 50 μM of fungicides, either alone or in cocktail (Fig. S3C - left panel, Table S3). Treatment of cells with various concentrations of the cocktail (10, 20 and 50 μM) showed a dose-dependent decrease of the neuronal cell survival (Fig. S3C - right panel, Table S4). These results showed that gestational exposure to

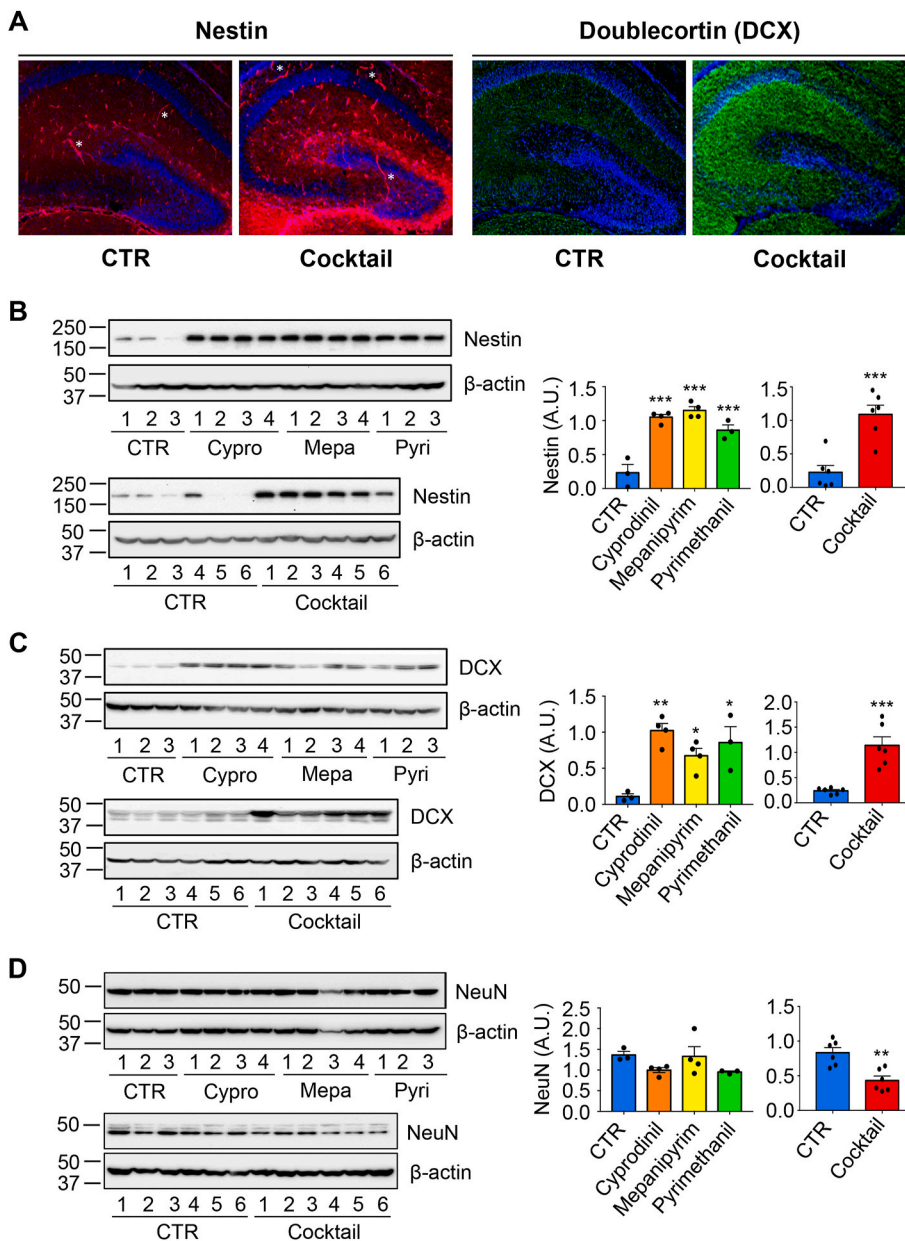


Fig. 4. *In utero* exposure to fungicides exacerbates the proliferation of neural precursor cells and induces a decrease of mature neurons in P3 neonates. **A.** Representative images of hippocampi illustrating the labelling with anti-nestin (left panel) or anti-doublecortin (DCX, right panel) antibodies of P3 neonates exposed during gestation to an equivalent volume of DMSO (CTR) or to a cocktail of the three fungicides (0.1 µg/L of each compound). Scale bar: 100 µm. White stars on the images show non-specific labelling of GFAP linked to the blood vessels. Protein levels of nestin (**B**), DCX (**C**) and neuronal nuclear antigen (NeuN, **D**) were analyzed from brain homogenates of P3 mice exposed to 0.1 µg/L of fungicides, either alone or in cocktail. Mice treated with an equivalent volume of DMSO ($3 \times 10^{-5}\%$) were used as controls (CTR). Protein loading was performed using an anti-β-actin antibody. Membranes were revealed with a Chemidoc MP imager and signals were quantified on Fiji software. All signals quantified were normalized using β-actin signals and results are displayed as mean ± SEM. Statistical differences were analyzed using a one-way ANOVA followed by a Holm-Sidak's *post-hoc* analysis (single treatment vs CTR) or a student T-test (CTR vs Cocktail) ($\alpha = 0.05$, * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001). Black dots on graphics represent the distribution of each value obtained per animal. Numbers (1–6) correspond to brain homogenates from different animals analyzed ($n = 3$ –6 animals per group). Cypro: cyprodinil, Mepa: mepanipyrim and Pyri: pyrimethanil.

fungicides, either alone or in cocktail, substantially increased neuronal precursors (nestin⁺ and DCX⁺) in P3 neonates but decreased mature neurons (NeuN⁺) in mice exposed to the fungicide cocktail, likely due to a neurotoxic effect.

3.4. Increased proliferation of neuronal precursors is dependent on PI3K/Akt and Wnt/β-catenin pathways

β-Catenin has been shown to regulate the self-renewal of neural progenitor cells (Adachi et al., 2007) and neuronal differentiation (Lie et al., 2005) in adult neurogenesis. In addition, it is well established that PI3K/Akt and Wnt/β-catenin signaling pathways are key regulators of cell proliferation and differentiation, both pathways sharing GSK3β as a crosstalk protein (Marchetti, 2018; Sharma et al., 2002). Since we observed variations in nestin, DCX and NeuN in P3 neonates exposed to fungicides, we wanted to determine whether β-catenin could be involved in this process. Analyses of β-catenin protein levels by immunoblotting showed a 2-fold increase in brains of P3 mice exposed to fungicides alone (Fig. 5A, 0.52 ± 0.12 A.U. for CTR vs 1.09 ± 0.09 for cypro,

p-value = 0.024; vs 1.01 ± 0.09 for mepa, *p*-value = 0.044; vs 1.29 ± 0.14 for pyri, *p*-value = 0.006) and a 2-fold increase upon cocktail exposure (Figs. 5A and 0.42 ± 0.04 A.U. for CTR vs 0.95 ± 0.13 for cocktail, *p*-value = 0.003), compared to CTR mice. PI3K/Akt signaling pathway is known to regulate phosphorylation of β-catenin by inhibiting the activity of GSK3β proteins (Sharma et al., 2002). To this purpose, we studied the phosphorylated form on serine 473 (Ser 473) of Akt in P3 neonates' brain, known to activate Akt proteins (Alessi et al., 1996). Analysis on P3 neonate's brain homogenates showed increased levels of P-Akt (S473), of 2.5–3.5 folds, upon cyprodinil and mepanipyrim gestational exposure (Figs. 5B and 0.39 ± 0.08 A.U. for CTR vs 1.08 ± 0.15 for cypro, *p*-value = 0.045; vs 1.37 ± 0.24 for mepa, *p*-value = 0.011; vs 0.63 ± 0.06 for pyri, *p*-value = 0.39). In addition, gestational exposure to the cocktail of the three fungicides revealed a 1.5-fold increase of P-Akt levels (Figs. 5B and 0.74 ± 0.07 A.U. for CTR vs 1.23 ± 0.04 for cocktail, *p*-value < 0.001), compared to CTR mice. Activated Akt kinase, can phosphorylate GSK3β proteins on their serine 9 (Ser9), thus inhibiting GSK3β activity (Cross et al., 1995; Manning and Cantley, 2007; Manning and Toker, 2017). GSK3β can also be inhibited by the

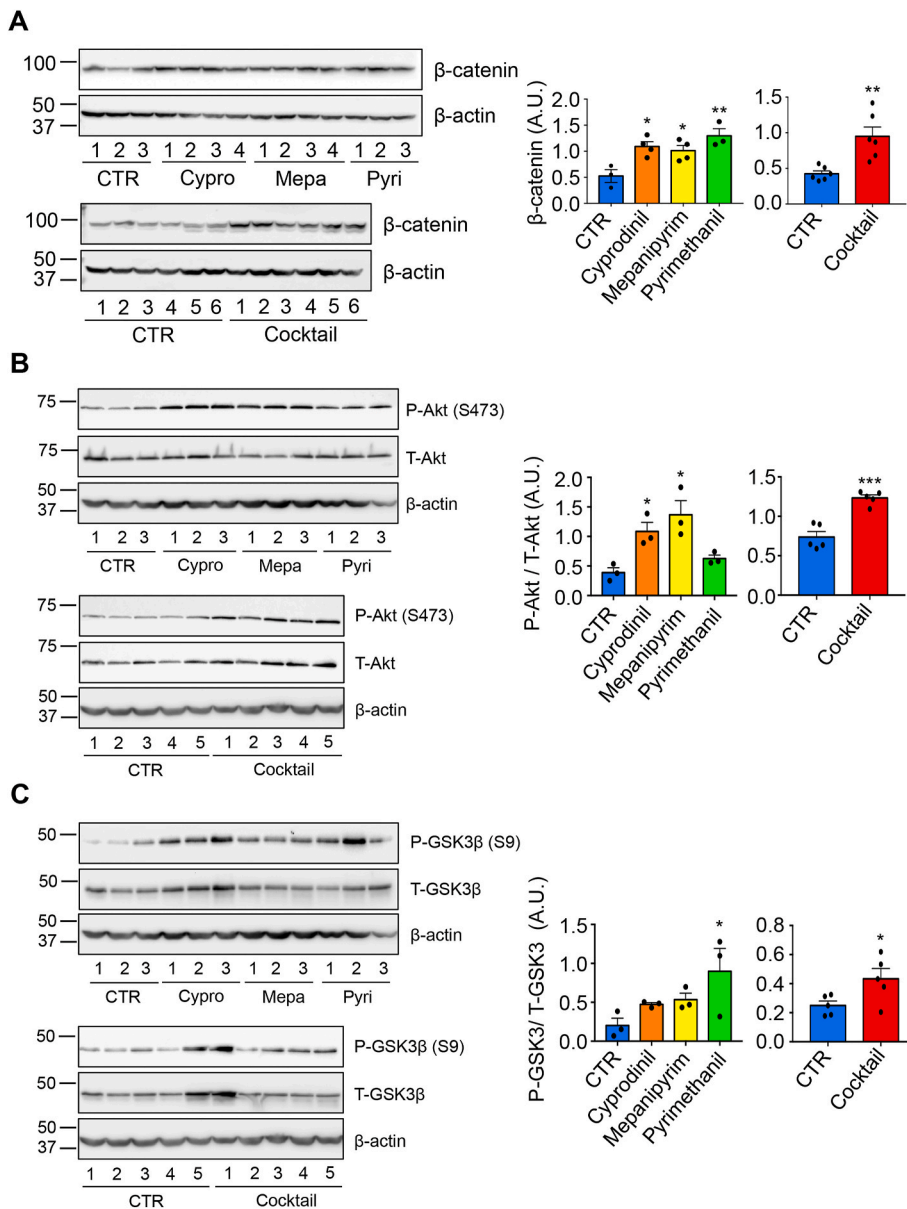


Fig. 5. *In utero* exposure to fungicide residues increases β -catenin and P-Akt expression in P3 neonates' brain. Protein expression of β -catenin (A), total and phospho-Akt (S473) (B) and total and phosphorylated GSK3 β (S9) (C) were analyzed on brain homogenates of P3 neonates exposed during gestation to either 0.1 μ g/L of cyprodinil, or mepanipyrim, or pyrimethanil, or with a cocktail of the three fungicides (0.1 μ g/L of each compound). Mice exposed with an equivalent volume of DMSO ($3 \times 10^{-5}\%$) were used as controls (CTR). Protein loading was performed using β -actin. Membranes were revealed with a Chemidoc MP imager and signals were quantified on Fiji software. Protein signals were normalized using β -actin signals and results are displayed as mean \pm SEM. Statistical differences were analyzed using a one-way ANOVA followed by a Holm-Sidak's *post-hoc* analysis (single treatment versus CTR) or a student T-test (CTR versus Cocktail) ($\alpha = 0.05$, * p -value < 0.05, ** p -value < 0.01, *** p -value < 0.001). Black dots on graphics represent the distribution of each value obtained per animal. Numbers (1–6) correspond to brain homogenates from different animals analyzed ($n = 3$ –6 animals per group). Cypro: cyprodinil, Mepa: mepanipyrim and Pyri: pyrimethanil.

Wnt signaling pathway (Noort et al., 2002). Inhibited GSK3 β is unable to phosphorylate β -catenin which is then stabilized and therefore accumulates in cells, and is not anymore degraded by the ubiquitin/proteasome system (Noort et al., 2002; Sharma et al., 2002), thus sustaining NSCs proliferation. Analysis by western blotting of GSK3 β protein levels (Ser9) on P3 neonate's brain homogenates revealed a tendency of increase of its levels upon cyprodinil and mepanipyrim exposure and a significant increase (~ 2 -folds) when mice were exposed to pyrimethanil (Figs. 5C and 0.20 ± 0.09 A.U. for CTR vs 0.47 ± 0.02 for cypro, p -value = 0.33; vs 0.53 ± 0.08 for mepa, p -value = 0.33; vs 0.89 ± 0.29 for pyri, p -value = 0.046), compared to CTR mice. In addition, significant modifications of P-GSK3 β (Ser9) levels were observed in P3 neonates exposed to the cocktail of fungicides (Figs. 5C and 0.25 ± 0.03 for CTR vs 0.43 ± 0.07 for cocktail, p -value = 0.044) compared to CTR. These data suggested that the increased nestin⁺ and DCX⁺ precursors are linked to β -catenin accumulation, via the cross-talk of PI3K/Akt and Wnt/ β -catenin signaling pathways, both inhibiting GSK3 β activity.

3.5. Gestational exposure to fungicides modified postsynaptic proteins and altered NMDA/AMPA ratio

During neurogenesis, synapse formation is a very important process. Besides, β -catenin overexpression has been shown to increase dendritic arborization in hippocampal neurons (X. Yu and Malenka, 2003). Since activation of the β -catenin signaling pathway has been shown to target postsynaptic proteins such as PSD95 and NMDA receptors (Ramos-Fernández et al., 2019), we wondered if the increased levels of β -catenin in P3 neonates upon gestational exposure to fungicides could impact PSD95 levels and its associated receptors (NMDA and AMPA). Immunoblotting revealed an increase of PSD95 levels by more than 6-folds in P3 mice exposed to fungicides alone (Figs. 6A and 0.15 ± 0.05 A.U. for CTR vs 0.92 ± 0.09 for cypro, p -value = 0.0055; vs 0.98 ± 0.17 for mepa, p -value = 0.0039; vs 0.98 ± 0.07 for pyri, p -value = 0.0055). In addition, mice exposed to the fungicide cocktail exhibited a 2-fold increased levels of PSD95 levels (Figs. 6A and 0.53 ± 0.18 A.U. for CTR vs 1.17 ± 0.10 for cocktail, p -value = 0.012), compared to their control littermates (CTR). As PSD95 interacts and stabilizes the ionotropic NMDA receptors (Won et al., 2016), we then wondered whether PSD95 increase could

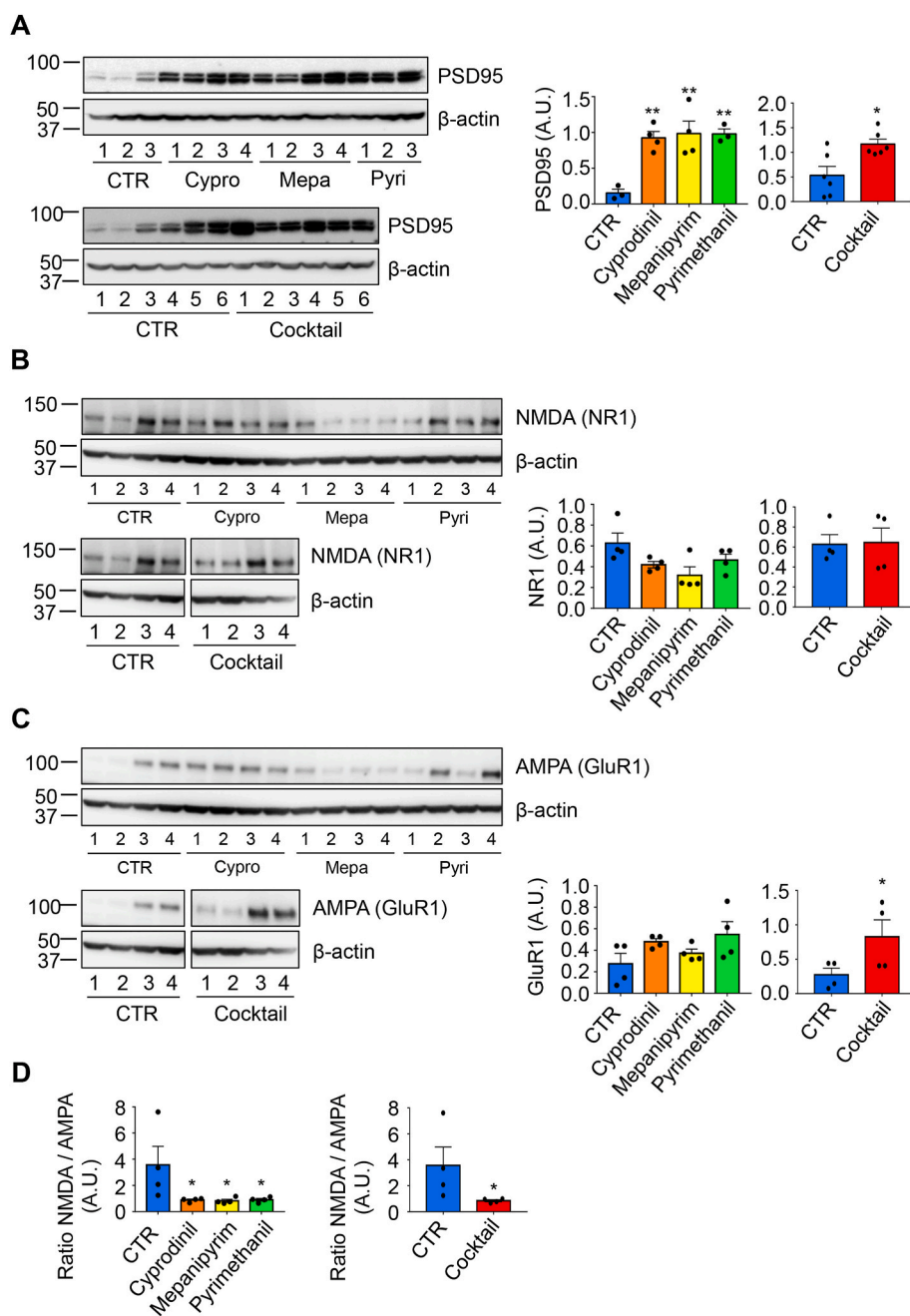


Fig. 6. Fungicide residues exposure during gestation modifies post-synaptic proteins and alters the ratio of NMDA and AMPA receptors in P3 newborn mice. Protein levels of PSD95 (A), NMDA receptor- NR1 subunit (B) and AMPA receptor- GluR1 subunit (C) were measured on brain homogenates from P3 neonates exposed *in utero* to 0.1 μ g/L of fungicides, either alone or in cocktail. Control animals (CTR) were exposed with an equivalent volume of DMSO ($3 \times 10^{-5}\%$). β -Actin was used as a protein loading control. Membranes were revealed with a Chemidoc MP imager and signals were quantified on Fiji software. Target proteins signals were normalized using their respective β -actin signals. Black dots on graphics represent the distribution of each value obtained per animal. Numbers (1–6) correspond to brain homogenates from different animals analyzed ($n = 3-6$ animals per group). The NMDA/AMPA ratio were quantified for all samples (D). Statistical differences were analyzed using a one-way ANOVA followed by a Holm-Sidak's *post-hoc* analysis (single treatment versus CTR) or a student T-test (CTR versus Cocktail) ($\alpha = 0.05$, * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001). Cypro: cyprodinil, Mepa: mepanipyrim and Pyri: pyrimethanil.

affect the levels of NMDA receptors. To address this question, we analyzed the expression of the NR1 subunit common to all NMDA receptors at P3 (Feldman, 2009). Immunoblots showed a tendency of decrease of NR1 expression upon *in utero* exposure to cyprodinil, mepanipyrim and pyrimethanil (Figs. 6B and 0.63 ± 0.09 A.U. for CTR vs 0.42 ± 0.03 for cypro, p -value = 0.31; vs 0.32 ± 0.08 for mepa, p -value = 0.06; vs 0.46 ± 0.06 for pyri, p -value = 0.796). No differences were observed upon exposure to the cocktail of the three fungicides (Fig. 6B, 0.63 ± 0.09 A.U. for CTR vs 0.64 ± 0.14 for cocktail, p -value = 0.92), compared to DMSO-exposed mice, likely due to interindividual variability. Interestingly, the study of the ionotropic AMPA receptors revealed a tendency of increase of its subunit GluR1 in mice exposed to cyprodinil, mepanipyrim and pyrimethanil (Figs. 6C and 0.27 ± 0.09 A.U. for CTR vs 0.48 ± 0.03 for cypro, p -value = 0.185; vs 0.37 ± 0.04 for mepa, p -value = 0.408; vs 0.55 ± 0.12 for pyri, p -value = 0.099). In addition, mice exposed to the cocktail of the three fungicides exhibited a

significant increase of GluR1 subunit levels (Figs. 6C and 0.63 ± 0.09 A.U. for CTR vs 0.83 ± 0.25 for cocktail, p -value = 0.037), compared to CTR mice. Original full-length blots of NR1 and GluR1 are presented in Fig. S4. The ratio NMDA/AMPA in the animals exposed to fungicides revealed a nearly 2-fold significant decrease after exposure to the single fungicides (Figs. 6D and 3.57 ± 1.42 A.U. for CTR vs 0.88 ± 0.07 for cypro, p -value = 0.033; vs 0.82 ± 0.12 for mepa, p -value = 0.033; vs 0.91 ± 0.10 for pyri, p -value = 0.033) and to the fungicide cocktail (Figs. 6D and 3.57 ± 1.42 A.U. for CTR vs 0.85 ± 0.08 for cocktail, p -value = 0.033) compared to CTR. These results showed that the vertical contamination with fungicides modified the expression of the postsynaptic protein PSD95 and impaired the NMDA/AMPA ratio.

3.6. Gestational exposure to fungicides modified the proliferation and differentiation properties of adult neural stem cells extracted from 4-months old animals

Our results showed that gestational exposure to fungicide residues, either alone or in cocktail, increased the neuronal precursors in the hippocampus of P3 mice likely through the β -catenin pathway (Figs. 4–5). We wondered if proliferation and differentiation properties of neural stem cells, upon fungicide exposure could be altered, particularly at older stages. For this purpose, the treatment of Generation 1 mice was pursued until 4 months of age, with the cocktail of the three fungicides or with an equivalent volume of DMSO, as controls (Fig. 1B – step 2). Mice were then sacrificed, and the adult hippocampal neural stem cells were extracted (Figs. 1B and 7A–B). Collected cells were cultured in a proliferation medium containing EGF and bFGF until neurospheres formation (Fig. 7B, steps 1–2 and Fig. 7C, step 2). Neurospheres were then dissociated (Fig. 7B, step 3) and were subjected to differentiation assays on monolayer cells (Fig. 7B–C, step 4) and proliferation assays on neurospheres (Fig. 7B–C, step 5). Proliferation properties of adult neural stem cells (aNSC) were investigated by adding Edu to the proliferation medium containing neurospheres for 24 h. Edu marker incorporated in proliferating cells (Fig. 8A) revealed a smaller size for neurospheres isolated from animals exposed to fungicide cocktail than those extracted from control animals (CTR) (Figs. 8B, 163 and $468 \pm 6729 \mu\text{m}^2$ for CTR vs $124,123 \pm 6097$ for cocktail, p -value < 0.001). The study of the migration length of cells escaping from the neurospheres revealed that those exposed to fungicides migrated significantly less compared to control cells (Figs. 8C and $440.3 \pm 8.39 \mu\text{m}$ for CTR vs 381.2 ± 11.08 for cocktail, p -value < 0.001). This

phenotype was confirmed by evaluating the total number of cells outside the neurospheres which was significantly lowered in NSCs isolated from animals exposed to fungicide cocktail than those extracted from control animals (CTR) (Figs. 8D and 234.7 ± 15.34 cells/ mm^2 for CTR vs 145.1 ± 13.03 for cocktail, p -value < 0.001), as well as for the Edu⁺ cells outside the neurospheres (Figs. 8E and 119.4 ± 8.10 cells/ mm^2 for CTR vs 68.65 ± 5.99 for cocktail, p -value < 0.001). Conversely, the quantification of Edu intensity in both conditions revealed that aNSC extracted from mice exposed to the fungicide cocktail exhibited an increased intensity by more than 2-folds compared to the control condition (CTR) (Figs. 8F, 1 and $405,547 \pm 379,688$ A.U. for CTR vs $4,672,608 \pm 740,629$ for T, p -value < 0.001), reflecting an increased proliferation inside the neurospheres. In a subsequent experiment, neurospheres were dissociated and plated to study the impact of the cocktail of fungicides on cell differentiation properties (Fig. 7B, step 4). Analysis of the different markers revealed a significant increase of GFAP⁺ (Figs. S5A–B, 46.57 ± 0.85 GFAP⁺ cells (%) for CTR vs 50.63 ± 1.01 for T, p -value = 0.0026), nestin⁺ (Figs. S5A and C, 44.83 ± 0.85 nestin⁺ cells (%) for CTR vs 51.62 ± 1.11 for T, p -value < 0.001) and DCX⁺ cells (Fig. 9A–B, 6.59 ± 0.53 DCX⁺ cells (%) for CTR vs 7.12 ± 0.62 for T, p -value = 0.0451), in the cells extracted from mice exposed to the fungicide cocktail compared to those exposed to an equivalent volume of DMSO (CTR). In addition, analysis of more neuronal committed cells showed a substantial increase of the β III-tubulin⁺ cells in the fungicide-exposed condition (Fig. 9A, C, 8.20 ± 0.41 β III-tubulin⁺ cells (%) for CTR vs 12.66 ± 0.68 for T, p -value < 0.001) compared to CTR. All these results showed that aNSC isolated from 4 months old mice exposed to the cocktail of fungicides exhibited an increased proliferation property, as well as a greater ability to differentiate into neurons, compared to

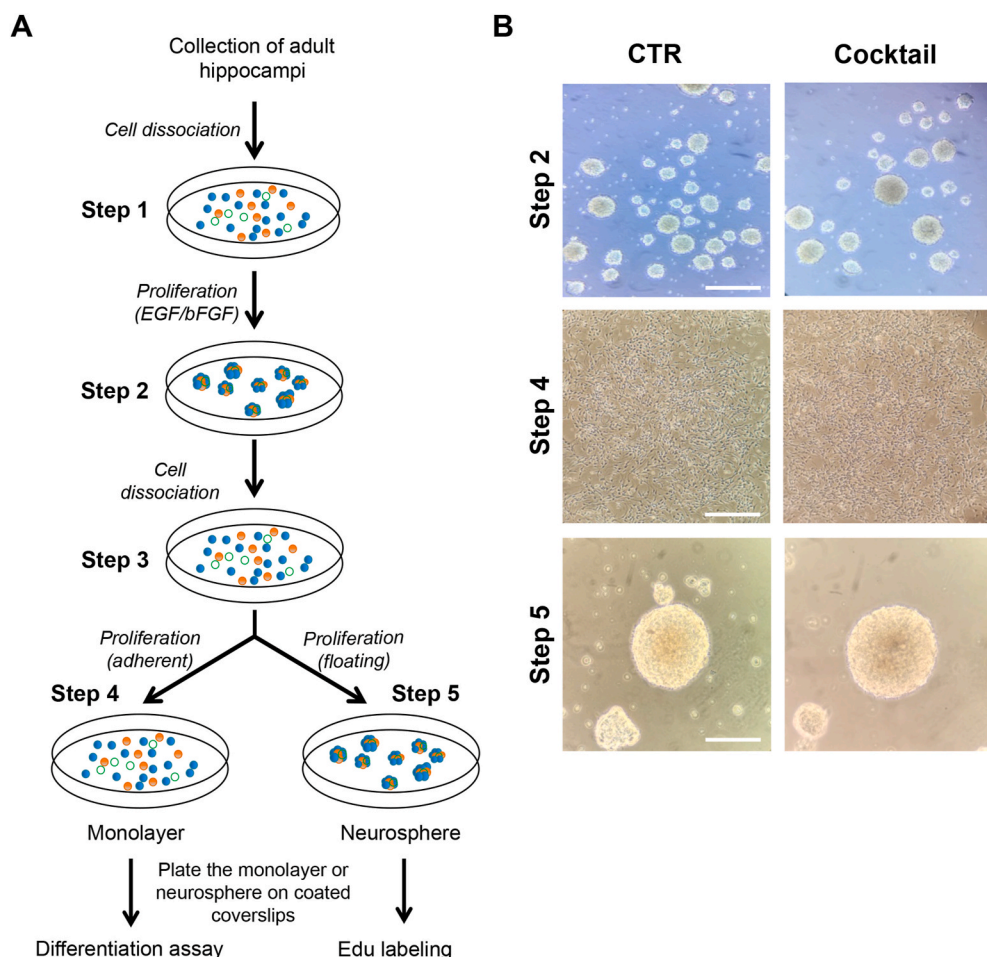


Fig. 7. Proliferation and differentiation assays of adult neural stem cells extracted from 4-months old mice exposed to a cocktail of fungicides. A. Hippocampi collected from WT mice exposed to the cocktail of fungicides or to an equivalent volume of DMSO during gestation up to 4-months old were dissociated in appropriate medium to isolate aNSC (Step 1); proliferation medium containing EGF and bFGF was used for 7–10 days until the formation of neurospheres (Step 2). Dissociation of neurospheres were done to analyze their properties using different assays (Step 3). To analyze their differentiation potential into neurons, aNSC were seeded in adherent monolayer condition (Step 4). In parallel, to analyze their proliferation properties (Edu assays), aNSC were cultivated in floating condition (Step 5). B. Representative images of aNSC isolated from CTR and fungicide cocktail exposed animals at the different steps (2, 4 and 5). Scale bars: 400 μm (Steps 2 and 4) and 200 μm (Step 5).

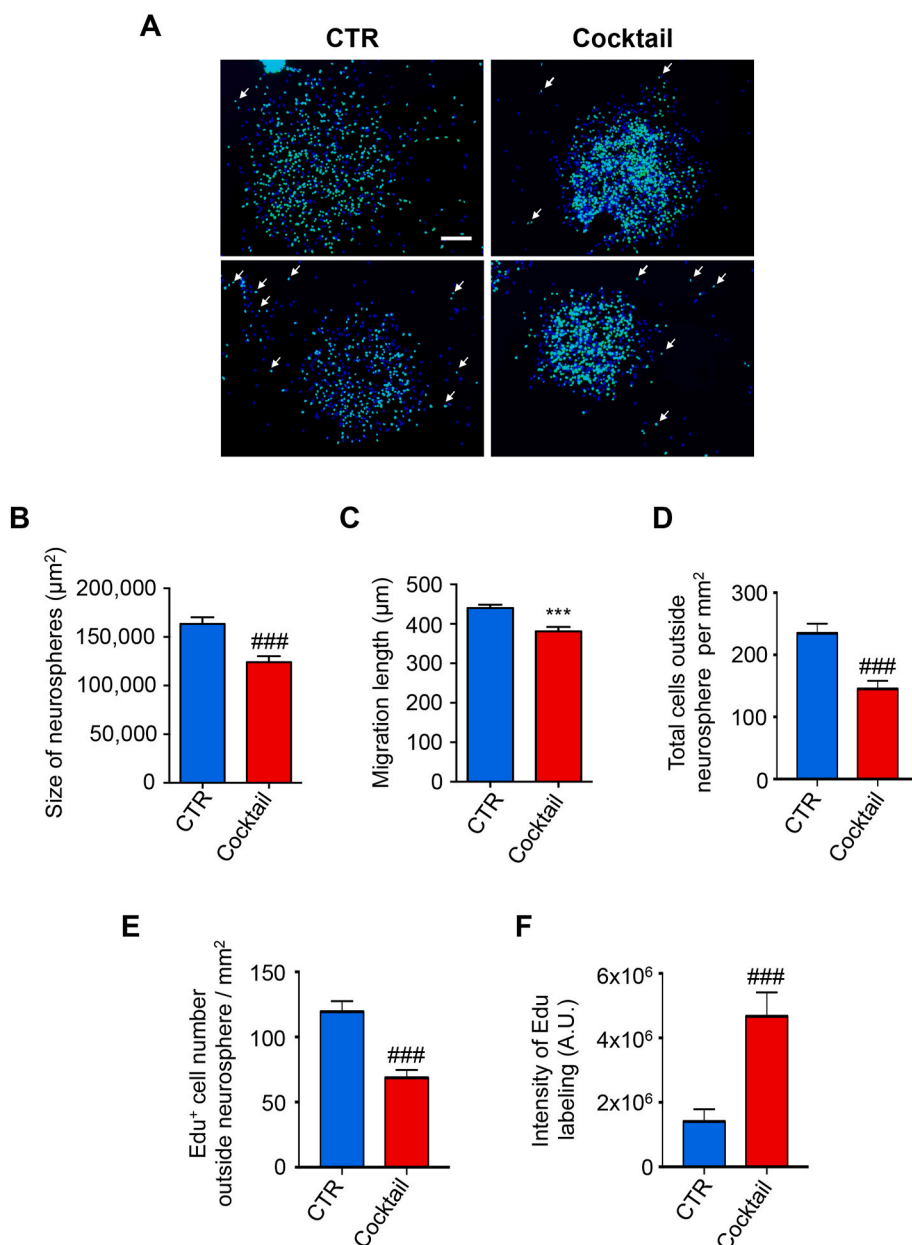


Fig. 8. Proliferation of aNSC isolated from mice exposed to fungicide cocktail during the gestation stage until 4 months-old. **A.** Representative images of neurospheres obtained from aNSC extracted from 4-months WT mice treated with a cocktail of the three fungicides (0.1 $\mu\text{g/L}$ each) or with an equivalent volume of DMSO (CTR, $3 \times 10^{-5}\%$). Cells were incubated with Edu for 24 h prior to fixation (green labelling). Scale bar: 100 μm . Quantifications of the size of the neurospheres (μm^2) (**B**), of the migration length of cells (μm) (**C**), of the total number of cells outside the neurospheres (**D**), the total number of Edu⁺ cells outside the neurospheres (**E**), and the intensity of the Edu labeling (**F**) were done. All results are presented as mean \pm SEM and statistical analyses were performed using a student T-test ($\alpha = 0.05$, *** p -value < 0.001) or a Mann-Whitney test (### p -value < 0.001). $n = 12$ coverslips per condition and 10 images per coverslip. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

control cells.

4. Discussion

The effects of chronic exposure to multiple pesticide residues on the most vulnerable persons are subject of many concerns. To protect consumers from adverse effects of pesticides, the European Union (E.U.) has fixed a limit dose for tap water, of 0.1 $\mu\text{g/L}$ for 1 pesticide and cannot exceed 0.5 $\mu\text{g/L}$ for several pesticide residues (Council of the European Union, 1998). These low doses in water have been selected to “be consumed safely on a lifelong basis and thus represent a high level of health protection” (Council of the European Union 1998). Currently, no study has yet evaluated the impact of such low doses of anilinopyrimidine fungicides on brain development. Thus, it is critical to further explore their effects during the gestational stage when the neurodevelopment takes place, as several neurodegenerative disorders are now suspected to have a developmental origin (Arendt et al., 2017).

4.1. Altered neurogenesis upon gestational exposure to fungicide residues

A recent hypothesis stipulates that during the neurogenic period, exogenous factors could impair the pool of progenitor cells and affect cortical development (Arendt et al., 2017). Altered neuronal cells could then be more sensitive to brain assaults and neuronal cell death, a key feature of Alzheimer’s disease (Arendt et al., 2017). In line with this, we evaluated if exogenous factors such as fungicides, particularly at low doses (0.1 $\mu\text{g/L}$) could affect neurogenesis after a gestational exposure (Fig. 1B). Our results showed that *in utero* exposure to fungicides, alone or in cocktail, substantially increased nestin and DCX levels in brains, reflecting an increased pool of neural precursors (Fig. 4A–C). Regarding this boost upon gestational fungicide-exposure, we hypothesize that this effect could be linked: (i) to the NeuN decreased levels (marker of mature neurons) likely reflecting neuronal death that could stimulate the proliferation of neural precursors (nestin⁺) to replace degenerated neurons (Fig. 4D) especially since we observed neurotoxicity in hippocampal primary cultures upon fungicide treatment (Fig. S3); (ii) to the increased levels of β -catenin (Fig. 5A), known to regulate the

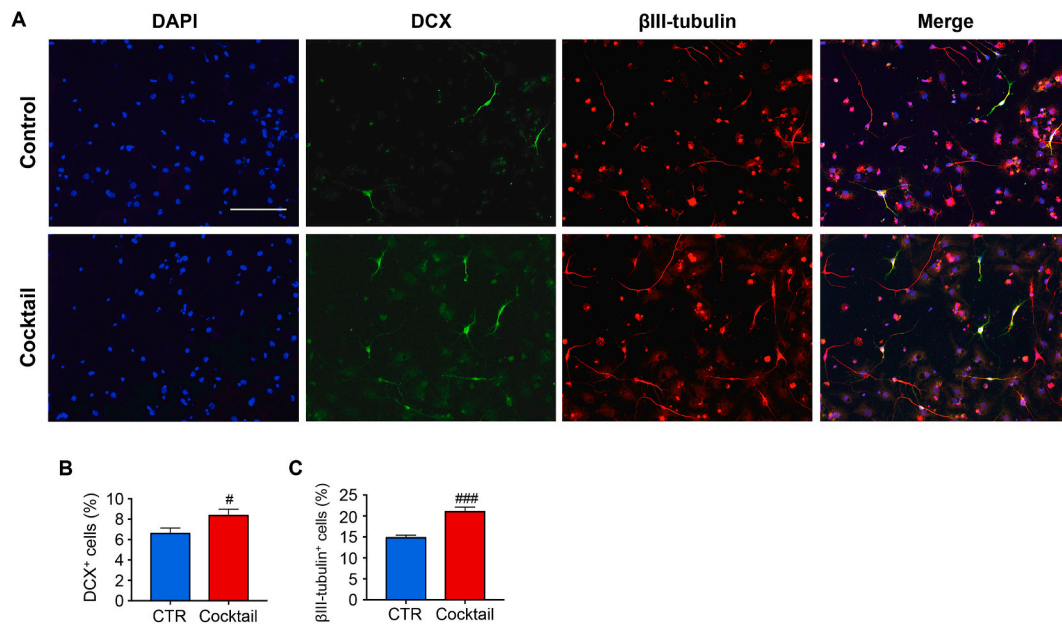


Fig. 9. Increased differentiation potential into neurons of aNSC isolated from 4 months old mice exposed to the cocktail of fungicides. **A.** Representative images of aNSC isolated from 4 months-old mice exposed to the fungicide cocktail (0.1 $\mu\text{g/L}$ each) or DMSO ($3 \times 10^{-5}\%$). Cells cultivated in adherent monolayer conditions, were labeled with anti-DCX (in green) or anti- $\beta\text{III-tubulin}$ (in red) antibodies. Quantifications of DCX⁺ (**B**) and $\beta\text{III-tubulin}^+$ (**C**) were presented as a percentage of the total cells. Results are presented as mean \pm SEM and statistical analyses were performed using a student T-test ($\alpha = 0.05$, ** p -value < 0.01 , *** p -value < 0.001) or a Mann-Whitney test (# p -value < 0.05 , ### p -value < 0.001). $n = 5$ coverslips per condition and 10 images per coverslip. Scale bar: 100 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

self-renewal of neural progenitor cells (Adachi et al., 2007) and neuronal differentiation (Lie et al., 2005), through an increased cross-talk of PI3K/Akt and Wnt/ β -catenin pathways, both inactivating GSK3 β (Fig. 5B–C); and/or (iii) to a blockage of cell differentiation. Increased DCX⁺ cells could be associated with a precocity of neural commitment of the NSC but failed to give rise to mature neurons since NeuN levels are decreased.

At first glance, this boost of proliferation might be seen as a positive mechanism to compensate neuronal loss as previously demonstrated in models of brain trauma (Sun et al., 2007; T.-S. Yu et al., 2016). A model of traumatic brain injuries demonstrated that 3 days post-injury, rats expressed twice more DCX⁺ immature granule cells in the dentate gyrus linked to an increased proliferation of neural precursor cells (NPC) (Neuberger et al., 2017). Forty-five days post-injury there was a 64% increase of mature granular cells compared to controls. However, this burst of DCX⁺ cells resulted in a later exhaustion of the stem cells proliferation properties thus accelerating the depletion of the pool of NPC. This could result in long-term cognitive deficits consecutive to this brain “physical assault” (Neuberger et al., 2017). Then, we hypothesize that the boost of precursor cells observed at P3 upon fungicide exposure could result, later in life, in an impaired proliferation and differentiation properties. To this aim, we pursued the treatment of P3 neonates with the cocktail of fungicides until 4 months of age and we isolated hippocampal adult neural stem cells (aNSC) (Fig. 1B – step 2 and Fig. 7) to evaluate their potential changes (Fig. 8 and Fig. S5). Results showed that these aNSC still exhibit proliferation potencies (Fig. 8A, F) and differentiation properties into neurons (Fig. 8 and Fig. S5), both being increased upon fungicide-exposure. However, our results showed that aNSC capacity to escape from neurospheres and to migrate were altered (Fig. 8A, C–E), suggesting that these alterations could limit the ability of the NSC to replace damaged neurons and to repair the neuronal network *in vivo*, during normal or pathological aging.

4.2. Exacerbated neuroinflammation processes and postsynaptic alterations upon *in utero* fungicide exposure

During embryogenesis and early postnatal stages, astrocytes and microglia play an important role in synaptogenesis and neurogenesis. Particularly, microglial cells have been described as key actors regulating neurogenesis during embryonic and early postnatal stages (Allen, 2013; Sato, 2015). During neuroinflammation processes both astrocytes (Eng et al., 1992) and microglial cells (Ito et al., 1998), undergo morphological changes resulting in an activated state and thus altering their functions. Neuroinflammation has been linked to several pathologies (Mottahedin et al., 2017) whose origin can start at perinatal stages (Hagberg et al., 2012; Meyer et al., 2011). Upon gestational fungicide exposure, P3 neonates exhibited astrogliosis (Figs. S1A, C, E) and microgliosis (Fig. 3A–C) in the CA1 and CA3 areas of the hippocampus. We believe that these increased inflammatory processes can alter neuronal network formation and *in fine* synaptic transmission. Moreover, activation of the β -catenin-dependent signaling pathway has been shown to target postsynaptic proteins such as PSD95 and NMDA receptors (Ramos-Fernández et al., 2019). As we observed increased levels of β -catenin (Fig. 5A), we thus evaluated if these postsynaptic proteins can be affected. We showed that gestational exposure to those fungicides exacerbated PSD95 expression levels (Fig. 6A) and impaired the expression of NMDA and AMPA receptors resulting in a collapse of the NMDA/AMPA ratio (Fig. 6B–D). β -Catenin is also involved in synaptic function, synaptogenesis and dendrite maturation (Rosso and Inestrosa, 2013; X. Yu and Malenka, 2003). Given that fungicide exposure impaired β -catenin levels and consequently affected PSD95, NMDA and AMPA receptors, we suspect an alteration of the synaptic transmission in fungicide exposed mice. In addition, a previous study showed that during early synapse formation, newly formed neurons that are in presence of an inflammatory environment exhibited increased number of dendritic spines associated to a clustering of PSD95 (Chugh et al., 2013). Such alterations have been shown to be implicated in several neurological disorders such as autism (Kotagiri et al., 2014), schizophrenia (Garey, 2010) or even Alzheimer’s disease (Rodríguez et al.,

2008), suggesting that at much older stages, mice exposed to low doses of fungicides could be more prone to develop neurological alterations, and possibly pathologies.

5. Conclusion

To our knowledge, this study is the first one to demonstrate an impact of a gestational exposure to such low doses of fungicides (0.1 µg/L) on neurodevelopment. Our results showed a modified pattern of proliferation and differentiation of NSC associated to exacerbated neuroinflammation, as well as impairments of postsynaptic proteins upon fungicide exposure. All these effects could be explained, in part, by the altered levels of β -catenin which is a key regulator involved in proliferation pathways and synaptic processes. Besides, our data supports Arendt's hypothesis regarding the effects of exogenous factors on neurodevelopment, as we showed that fungicide residues preferentially target neurogenesis whose alteration is visible in neonates as early as the P3 stage. An environment contaminated with pesticide residues does not stop its impact at the gestational stage but contaminate individuals throughout their lives. Our results showed that aNSC isolated from brains of 4 months old animals being constantly exposed to this low noise contamination, also exhibited altered proliferation, migration, colonization and differentiation properties. We can believe that these processes could be a fertile ground for the development of neurological diseases upon chemical assaults of the brain.

Credit author statement

Yunyun Wang: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. Pierre-André Lafon: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. Lucie Salvador-Prince: Conceptualization, Methodology, Validation, Formal analysis, Investigation. Aroa Relano Gines: Methodology, Investigation. Françoise Trousse: Methodology, Writing – review & editing. Joan Torrent: Writing – review & editing. Corinne Prevostel: Writing – review & editing. Carole Crozet: Methodology, Investigation, Writing – review & editing. Jianfeng Liu: Writing – review & editing, Funding acquisition. Véronique Perrier: Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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Ethic statement

This project follows the specific French national guidelines on animal experimentation and well-being and was approved by the French National Ethic Committee for Animal Experimentation (APAFIS no. 7357-20161,025-18,055,782).

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2021.110829>.

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