Original Paper

Herbicide Mixture-Driven Neurotoxicity in the Maternal Brain: Combined Exposure Produces Greater-Than-Individual Effects at Regulatory Relevant Doses

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ABSTRACT: Background: Glyphosate is the most widely used herbicide worldwide and is frequently applied with auxinic herbicides such as dicamba and 2,4-dichlorophenoxyacetic acid (2,4-D). Regulatory limits are largely based on single-compound toxicity but real-life exposure occurs as mixtures. Objective: To evaluate whether chronic exposure to regulatory-relevant doses of glyphosate alone or combined with dicamba and 2,4-D during pregnancy and lactation induces neurobehavioral and histopathological alterations in maternal brain tissue. Methods: Pregnant Wistar rat dams were exposed from gestational day-6 to postnatal day-21 to: 0mg/kg/day glyphosate (control group), 0.5mg/kg/day glyphosate (European Union ADI-Gly group), 50mg/kg/day glyphosate (European Union NOAEL-Gly group), or a mixture of glyphosate, dicamba, and 2,4-D at their respective European Union ADI levels (Combination group). Neurobehavioral assessment included open field, elevated plus maze, and forced swim tests. Brain tissues were examined using hematoxylin and eosin staining with semi-quantitative scoring. Statistical analysis employed one-way ANOVA with Dunnett post-hoc comparisons. Results: Grooming behavior increased significantly in the NOAEL-Gly group and more markedly in the Combination group, consistent with anxiety-like alterations, while locomotor and depressive-like behaviors were unchanged. Histopathology revealed neuronal degeneration, perineuronal edema, and vascular congestion, most pronounced in the hippocampus of mixture-exposed dams. Structural alterations paralleled behavioural findings. Conclusions: Chronic exposure to regulatory relevant doses of glyphosate induces maternal neurotoxicity that is amplified by co-exposure to dicamba and 2,4-D, suggesting additive effects and underscoring the need to incorporate mixture toxicity into pesticide risk assessment.

KEYWORDS: *Mixtures, neurotoxicity, pesticides, glyphosate, dicamba, 2,4-D.*

Introduction

Glyphosate is currently the most widely used herbicide worldwide [1], and its extensive agricultural application has resulted in persistent environmental contamination and chronic low-level exposure in both wildlife and human populations [2-5].

With the widescale appearance of glyphosate resistant weeds, especially in North America [6,7], contemporary crop management increasingly relies on application of combined herbicide formulations, particularly mixtures containing glyphosate, dicamba, and 2,4-dichlorophenoxyacetic acid (2,4-D), which has been exacerbated by the launch of glyphosate plus dicamba and glyphosate plus 2,4-D tolerant genetically modified crop varieties [8].

Growing toxicological evidence indicates that such combined exposures may enhance biological effects through additive or synergistic mechanisms, even when individual compounds are present at doses considered safe [9,10].

Although glyphosate has traditionally been regarded as having relatively low acute toxicity in mammals, accumulating experimental data indicate that chronic exposure-even at low or environmentally relevant doses-can induce liver

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toxicity [11] and neurobiological alterations [12].

Glyphosate and glyphosate-based herbicides have been shown to disrupt neuronal homeostasis through mechanisms involving oxidative stress, mitochondrial dysfunction, excitotoxicity, and neuroinflammatory signaling, ultimately contributing to neuronal injury and behavioral impairment [13].

Experimental studies further demonstrate that developmental exposure to glyphosate can impair hippocampal neurogenesis, increase apoptotic signaling, and alter synaptic plasticity pathways, supporting the concept of persistent neurodevelopmental toxicity [14].

The developing brain is particularly vulnerable to environmental toxicants during gestation and early postnatal life, when neurogenesis, neuronal migration, synaptogenesis, and myelination occur in tightly regulated sequences.

Even minimal perturbations during these critical windows may produce long-lasting structural and functional consequences.

Experimental evidence indicates that glyphosate exposure during early life induces oxidative damage in the hippocampus, disrupts neuronal differentiation, and interferes with neurotransmitter regulation [15].

Additional studies have shown that glyphosate exposure may exacerbate neuroinflammatory processes and impair cognitive performance after prolonged exposure, highlighting its potential role as a developmental neurotoxicant [16].

In parallel, the widespread use of auxinic herbicides such as dicamba and 2,4-D has increased the likelihood of simultaneous exposure [17].

Experimental data indicate that 2,4-D can cross the blood-brain barrier, alter serotonergic signaling, and induce behavioral changes including reduced locomotor activity and increased immobility in rodents [18].

Chronic exposure to 2,4-D has also been associated with deficits in neurobehavioral performance, neuronal necrosis, and cortical structural alterations [19].

Moreover, studies evaluating herbicide combinations report that mixtures of auxinic herbicides may produce synergistic toxic effects compared with individual compounds, reinforcing concerns regarding mixture toxicity under realistic exposure conditions [20].

Assessment of developmental neurotoxicity requires integration of functional and structural endpoints.

Behavioral tests such as the open field test, elevated plus maze, rotarod, and forced swim test are widely validated tools for evaluating locomotor activity, anxiety-like behavior, motor coordination, and depressive-like responses following chronic toxicant exposure [21].

When correlated with histopathological examination, these tests allow detection of subtle neurofunctional impairments associated with neuronal degeneration, vascular alterations, and hippocampal vulnerability-brain regions known to be particularly sensitive to herbicide-induced toxicity [22].

Objective

Therefore, the present study aimed to investigate the neurotoxic effects on dams of exposure to low doses of glyphosate alone and in combination with dicamba and 2,4-D administered during gestation, and lactation in a rat model.

By integrating behavioral assessment (open field, elevated plus maze, and forced swim tests) with detailed histopathological evaluation of cortical and hippocampal structures, this study tests the hypothesis that chronic developmental exposure to low-dose herbicide mixtures induces persistent neurobehavioral alterations and structural neuronal damage, thereby contributing to improved risk assessment of herbicide mixtures under real-world exposure scenarios.

Methods

Animal experiment

The animal experiment has been presented in detail previously [23,24].

Briefly, 20 pregnant 3-month-old Wistar rats were randomly assigned into one of the four experimental groups (n=5 animals per group) and exposed to the respective treatment from gestation day (GD) 6 till weaning (post-natal (PN) day 21).

At the end of the exposure period, the neurobehavioral tests were performed and then the animals were sacrificed by exsanguination after anesthesia with xylazine and ketamine as previously described [24] to collect the brain.

Approval for the animal study was granted by the Ethical Committee of UMF Craiova, Romania (No. 120/19.11.2020).

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Treatment

The control group (5 dams) received free access to filtered tap water and special rodent food.

The ADI-Gly group (5 dams) received from GD6 till PND21 0.5 mg glyphosate/kg bw/day [25] equivalent to the European Union (EU) acceptable daily intake (ADI) value.

The NOAEL-Gly group (5 dams) received from GD6 till PND21 50mg glyphosate/kg bw/day [25] equivalent to the EU no observed adverse effect level (NOAEL) value.

The Combination group (5 dams) received from GD6 till PND21 the combination of 0.5mg glyphosate/kg bw/day equivalent [25], 0.02mg/kg bw/day 2,4-D [26] and 0.3mg dicamba/kg bw/day [27], equivalent to the EU ADI value for each individual herbicide.

Dose Selection

The exposure doses were calculated as fractions of the established ADI and NOAEL values for glyphosate, dicamba, and 2,4-D, in order to simulate regulatory relevant chronic exposure conditions.

Behavioral tests

Open field test

Exploratory behavior, locomotor activity and anxiety-related responses were evaluated using the open field test, which is a validated method of detecting neurobehavioral alterations in rodents [28].

The apparatus consisted of a 100×100cm square arena divided into 25 equal squares, with a defined central zone (nine inner squares) and peripheral zone (16 outer squares).

Each rat was placed individually in the center of the arena and allowed to explore freely for 5min.

Behavior was recorded using an overhead video camera.

The arena was cleaned and dried between trials to prevent olfactory interference.

Videos were analyzed independently by two observers (AMC and AOD) blinded to treatment.

Exploratory drive was quantified as the number of rearing movements in consecutive 1-minute intervals over a 5-minute observation period, reflecting vertical activity and environmental investigation.

Locomotor function was assessed by the number of squares crossed over the central zone (cross internal) in consecutive 1-minute intervals over a 5-minute and the number of squares crossed over the peripheral zone (cross external) in consecutive 1-minute intervals over a 5-minute observation period, and a cumulative score for number of squares crossed over the central zone/peripheral zone across the 5-minute test.

Anxiety-like and stress-related responses were evaluated by the number of grooming episodes in consecutive 1-minute intervals over a 5-minute period and cumulative grooming events recorded across the 5-minute test and the total number of fecal boluses during the 5-minute test, widely used indices of emotional reactivity in toxicological studies.

Discrepancies between observers were resolved by joint review to reach consensus.

Elevated Plus Maze Test

Anxiety-like behavior and exploratory activity were assessed using the Elevated Plus Maze (EPM), a validated technique sensitive to limbic system dysfunction and behavioral alterations induced by neurotoxicants [29].

The apparatus consisted of a cross-shaped platform elevated 50cm above the floor, comprising two opposing open arms and two opposing closed arms connected by a central platform.

Each arm extended 90cm from the center.

Rats were individually placed in the central platform facing an open arm and allowed to explore freely for 5min.

Behavior was recorded using an overhead video camera.

The apparatus was cleaned and dried between trials to eliminate olfactory cues.

Video recordings were analyzed independently by two observers (AMC and AOD) blinded to treatment group.

Anxiety-like behavior was quantified by time spent in the closed arms and number of grooming episodes, parameters reflecting avoidance behavior and stress reactivity.

Exploratory activity was evaluated by time spent in the open arms and central platform, number of rearing movements, and number of head-dipping/bending behaviors over the edges of the open arms, which are sensitive indicators of anxiety modulation and emotional state.

Discrepancies between observers were resolved by joint review to reach consensus.

Force Swim Test

Depressive-like behavior was evaluated using the Forced Swim Test (FST), which is a widely used test that is sensitive to alterations in mood and changes in monoaminergic function induced by neurotoxicants [30].

Rats were placed individually in a transparent cylindrical tank (height ~50cm, diameter ~20-25cm) containing water at a temperature of 23-25°C.

The water level was set at a depth sufficient to prevent tail support while still allowing free swimming without the option of escaping.

Each 5-minute session was recorded using a video camera positioned to capture lateral movements.

The water was changed regularly and the animals were dried and returned to their home cages after testing.

Video recordings were analyzed independently by two observers (RM and AB) who were blinded to the treatment allocation.

Behavioral parameters included immobility time, defined as floating with the minimal movements necessary to keep the head above water.

This reflects passive coping behavior.

Immobility is considered an index of behavioral despair or reduced stress-coping capacity, and it is sensitive to alterations in serotonergic and noradrenergic signaling.

Any discrepancies between the observers were resolved through a joint review in order to establish a consensus score.

Brain tissue processing and histopathological evaluation

At sacrifice the brain was excised, gently rinsed in physiological saline solution to remove blood, dried on a filter paper, weighed and immediately immersed in 10% neutral-buffered formalin for 24h at room temperature to ensure optimal preservation of tissue morphology and cellular integrity.

Following fixation, the tissues were washed in phosphate-buffered saline (PBS) before being embedded in paraffin.

Tissue processing-including graded ethanol dehydration, xylene clearing and paraffin embedding-was performed according to routine internationally accepted standard histopathological procedures [31].

Paraffin-embedded blocks were sectioned at a thickness of 4μm using a Leica RM 2125 rotary microtome, and sections were mounted on glass slides.

Sections were stained with hematoxylin and eosin (H&E) using standardized staining protocols for general morphological assessment and identification of cellular and structural alterations [32].

Microscopic evaluation was performed using a Panthera L light microscope (Motic Europe, S.L.U., Spain) at multiple magnifications appropriate for lesion detection and characterization.

For each section, five spatially distinct, non-overlapping high-power fields (HPFs; ×400 magnification) were selected.

Histopathological lesions were identified and described using standardized diagnostic terminology in accordance with the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) guidelines for rodent brain pathology [33].

Whole-slide examination was performed independently by two experienced pathologists (LC and MNA) who were blinded to experimental group allocation.

In instances of interpretation disagreement, slides were jointly reviewed and a consensus interpretation was established.

Histopathological alterations were graded using a semiquantitative severity scoring system based on both the intensity of the lesion and the extent of tissue involvement, as commonly applied in experimental toxicology studies [34].

Lesions were classified as follows:

(-) no detectable histopathological alteration;

(+) mild, characterized by minimal structural changes confined to limited focal areas;

(++) moderate, defined by clearly evident alterations affecting a broader but not extensive portion of the tissue;

(+++) severe, indicating marked and widespread structural disruption with pronounced morphological abnormalities.

Statistics

All data are presented as mean±standard deviation (SD, n=5).

Statistical analyses were performed using standard parametric methods appropriate for normally distributed data with the help of STATA (STATACORP, USA).

Statistical analyses were performed using one-way analysis of variance (ANOVA) to assess overall differences among the experimental groups at each individual time point.

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Dunnett's post-hoc test was applied to compare each exposure group directly with the control group.

The parameters evaluated for open field were: latency to start from the center (latency), the number of crossing over internal squares (cross internal) at minut 1, 2, 3, 4, 5 and total for all 5 minutes, the numbers of crossing over external squares (cross external) at minute 1, 2, 3, 4, 5 and total for all 5 minutes, the number of rearings (rearings) at minute 1, 2, 3, 4, 5 and total for all 5 minutes, the number of grooming (grooming) at minute 1, 2, 3, 4, 5 and total for all 5 minutes and the number of boluses.

To evaluate sustained rearing, grooming, and locomotor activity, a cumulative grooming, rearing and cross internal and cross external scores were calculated for each animal as the sum of respective parameters recorded across the 5-minute test.

Because cumulative scores were derived from individual animal data, standard deviations reflect true inter-individual variability.

The parameters evaluated for elevated plus-maze were the number of bending (bending), the number of rearings (rearings), the number of grooming (grooming), the time spend in the open arms (open), the time spend in the center (center), the time spend in the dark area (closed).

The parameter evaluated for the force swim was the time of immobility.

No statistics were performed for the scoring system for the histopathological alterations as the analysis was semiquantitative.

Results

Neurobehavioral Tests

Open Field test

A minute-by-minute analysis revealed that grooming behavior differed significantly between groups only at the two-minute interval.

A significant overall effect of treatment was identified by one-way ANOVA.

Subsequent Dunnett post hoc comparisons revealed elevated values in the NOAEL-Gly group ($p<0.05$) and a more pronounced increase in the herbicide Combination group ($p<0.01$) relative to the Control animals.

The ADI-Gly group did not differ statistically from controls, and no meaningful differences were observed at the remaining observation intervals (1, 3, 4, and 5min) (Table 1).

When grooming activity was evaluated cumulatively across the entire 5-minute observation period, a significant overall group effect was detected (one-way ANOVA, $F(3,16)=3.55$, $p=0.038$).

Post hoc comparisons revealed that cumulative grooming was significantly increased in Combination-exposed animals compared with Controls ($p<0.01$), while the NOAEL-Gly group showed a non-significant trend towards increased grooming.

No significant difference was observed between the ADI-Gly and control groups (Table 1).

No other significant changes were observed for any of the other evaluated parameters at any time point.

Table 1. Open field test evaluation at the end of the herbicide exposure period.

	Control	ADI-Gly	NOAEL-Gly	Combination
Variable Definition	Mean±Std. Dev.	Mean±Std. Dev.	Mean±Std. Dev.	Mean±Std. Dev.
Latency (s)	1.60±0.89	3.00± 3.08	2.00±1.87	1.60±2.51
Cross internal 1	3.00±1.58	5.20±3.27	5.20±3.56	4.60±2.07
Cross internal 2	2.40±1.52	2.20±2.49	2.80± 3.11	4.80±3.27
Cross internal 3	4.40±1.52	2.00±2.00	3.60±2.61	4.20±2.95
Cross internal 4	2.20±2.49	3.80±5.85	2.00±2.83	1.60±1.82
Cross internal 5	1.40±1.52	1.00±1.00	2.40± 3.36	2.80±2.77
Cross internal total	13.40±5.32	14.00±12.73	16.00±12.39	18.00±5.15
Cross external 1	26.60±2.70	22.60±6.54	33.80± 6.06	32.40± 6.15
Cross external 2	23.00±2.65	20.60±8.05	26.00±13.44	26.80±11.08
Cross external 3	20.00±2.35	20.00±8.86	18.60±11.52	20.60± 9.84
Cross external 4	14.00±8.49	16.20±5.50	16.80±10.45	26.20±19.94
Cross external 5	14.80±7.40	15.80±	14.00±10.12	22.20±20.75
Cross external total	98.40±18.84	95.00±24.72	109.2±44.65	128.2±57.13
Rearing 1	9.00±1.00	6.80±2.59	7.80±2.59	11.40±5.32
Rearing 2	9.00±2.83	6.80±4.60	8.60±5.94	10.00±3.54
Rearing 3	5.20±1.64	8.60±3.36	6.40±4.83	7.80±4.87
Rearing 4	4.00±2.65	6.60± 2.88	5.00±4.53	7.40±3.65

Rearing 5	3.20±2.28	3.80±1.79	5.40±4.62	6.40±3.91
Rearing total	31.40±7.27	32.40±13.16	33.20±21.11	43.00±15.20
Grooming 1	0.20±0.45	0.60±0.55	0.80±0.45	0.80±1.10
Grooming 2	0.60±0.89	0.80±0.84	**2.00±1.58***	**3.00±0.71****
Grooming 3	1.20±0.84	1.20±0.84	1.60±1.34	1.80±0.84
Grooming 4	0.60±0.89	1.60±1.34	1.6±1.52	1.60±1.14
Grooming 5	0.6±0.55	1.20±1.64	1.6±1.82	2.00±0.71
Grooming total	3.2±0.84	5.4±3.36	**7.6±4.77***	**9.2±1.92****
Boluses	3.00±1.22	0.80±1.10	1.60±2.61	4.60±1.82

*p<0.05; **p<0.01; ***p<0.001 compared with controls (n=5 dams per group) (Dunnett's test).

Elevated Plus-Maze test

Grooming behavior assessed during the elevated plus maze test showed increased values in all exposed groups compared with the control group (see Table 2).

One-way ANOVA revealed a trend towards a treatment effect that did not reach statistical significance ($F(3, 16)=2.63$, $p=0.086$).

However, a post-hoc Dunnett's test comparing each exposure group with the control group revealed a significant increase in grooming behavior in the NOAEL-Gly group ($p<0.05$), as well as a highly significant increase in the Combination group ($p<0.01$).

No statistically significant difference was observed between the ADI-Gly group and the control group (see Table 2).

No other significant changes were observed for any of the other evaluated parameters at any time point.

Table 2. Elevated plus-maze test evaluation at the end of the herbicide exposure period.

	Control	ADI-Gly	NOAEL-Gly	Combination
Variable Definition	Mean±Std. Dev.	Mean±Std. Dev.	Mean±Std. Dev.	Mean±Std. Dev.
bending	7.80±1.10	4.80±7.09	3.60±3.29	4.80±2.95
rearing	9.60±1.67	7.80±4.21	10.20±6.87	11.20 ±3.63
grooming	3.40±1.34	5.60±3.65	**6.80±0.45***	**9.40±5.68****
open	54.40±2.88	24.00±29.57	21.60±27.92	39.00±20.46
center	20.40±5.03	14.00±13.45	10.60±11.04	17.60±4.16
closed	225.2±7.46	262.0±41.32	267.8±38.93	243.4±23.66

*p<0.05; **p<0.01 compared with controls (n=5 dams per group) (Dunnett's test).

Force Swim test

No significant changes between control and herbicide exposure groups was detected in the time of immobility recorded during the forced swim test (see Table 3).

Table 3. Force swim test evaluation at the end of the herbicide exposure period.

	Control	ADI-Gly	NOAEL-Gly	Combination
Variable Definition	Mean±Std. Dev.	Mean±Std. Dev.	Mean±Std. Dev.	Mean±Std. Dev.
Time of imobility	27.6±9.58	49.8±24.6	55.6±21.66	35.20±18.66

Histopathological evaluation

No differences in brain weight were identified between the control and treatment groups (data not shown).

In the Control group, the morphology and arrangement of the brain tissue were normal.

There were no signs of pathological alterations, and tissue integrity was preserved. Choroid plexuses with normal histological features were observed across all animals (Figure 1A, Table 4).

In the NOAEL-Gly group, changes were observed in the histological aspects of both the cerebral cortex and the hippocampus.

The cerebral cortex showed signs of neuronal cell degeneration, with shrunken neurons and increased eosinophilic cytoplasm.

Changes to vascular aspects were also evident, with signs of vascular congestion.

The hippocampal region revealed even more pronounced findings, including loss of neurons, degenerative changes and vacuolization (Figure 1B, Table 4).

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The cerebral cortex of the ADI-Gly group showed changes, but these were less intense and more localized than in the NOAEL-Gly group.

Neuronal degeneration was present, albeit in a mild form with slightly eosinophilic cytoplasm.

Alongside vascular congestion, enlarged vessels or hyalinized walls were present.

Similar changes were observed in the hippocampus, but with reduced intensity and more limited distribution (Figure 1C and Table 4).

The rat brain tissue from the Combination group displayed more pronounced and diffuse histopathological alterations compared to the NOAEL-Gly and ADI-Gly groups.

In the cerebral cortex, neuronal degeneration was observed, often accompanied by dense eosinophilic cytoplasm and perineuronal oedema.

Vascular hyperemia was maintained and accompanied by hyaline walls.

The hippocampus of this group showed numerous degenerating cells interspersed with pink deposits (Figure 1D and Table 4).

We evaluated all the histopathological aspects encountered in the brain tissue using a scoring system based on the intensity of the lesions and the number of specimens involved.

Intensity was classified as absent, mild, moderate or intense (Table 4).

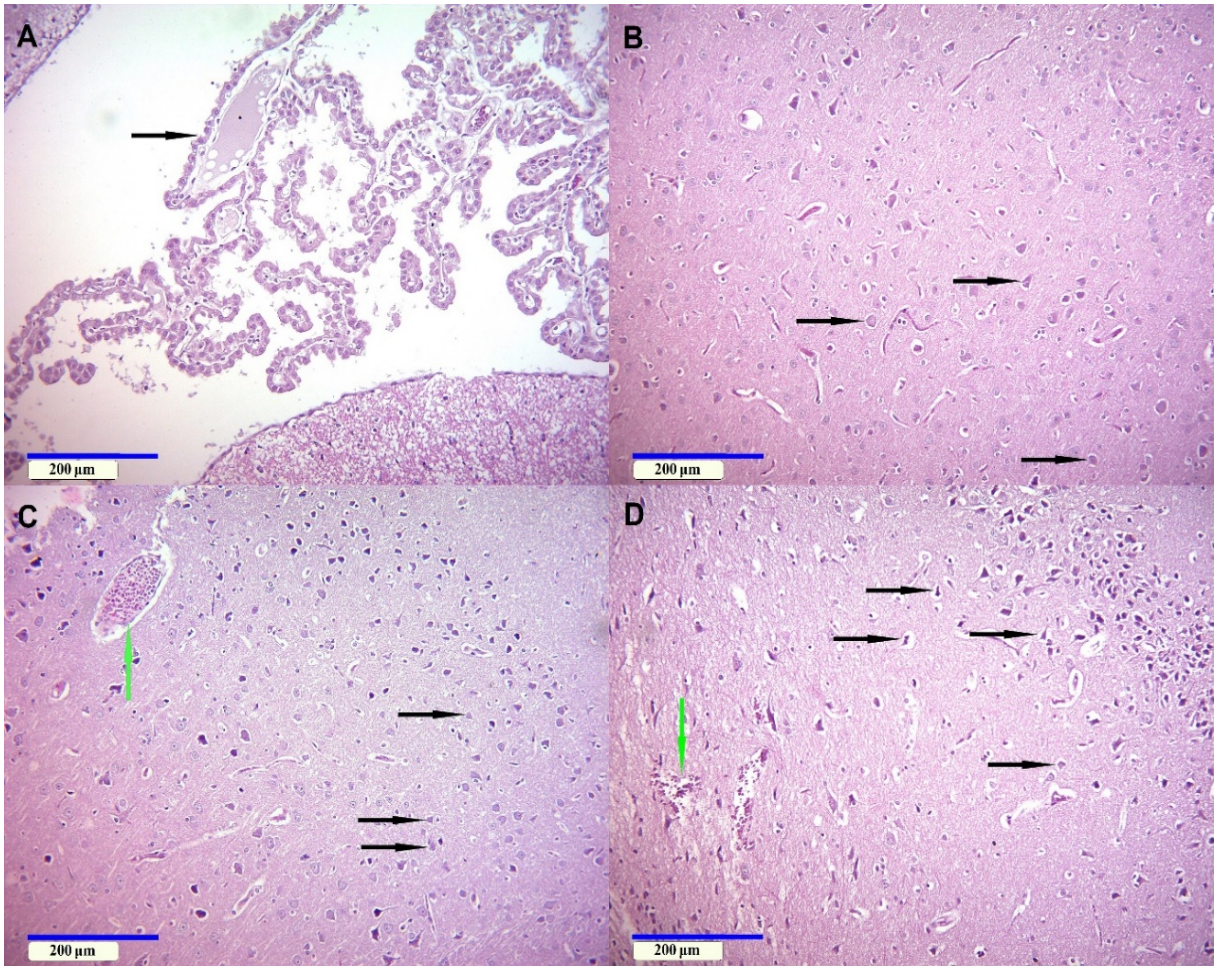

Figure 1. H&E staining of rat brain tissue sections from animals exposed to glyphosate alone or in combination with 2,4-D and dicamba.
A: Control group: normal architecture, choroid plexuses-black arrow;
B-NOAEL-Gly group: neuronal degeneration-black arrow;
C-ADI-Gly group: neuronal degeneration -black arrow, enlarged vessel with hyperemia -green arrow;
D-Combination group: neuronal degeneration and perineuronal edema-black arrow,
hyperemic vessels-green arrow. Magnification: X200.

Table 4. Severity grading and incidence of brain histopathological alterations across exposure and control groups based on microscopic examination following H&E staining.

Parameters	Experimental groups							
	Control		ADI-Gly		NOAEL-Gly		Combination	
	Intensity	Specimens Number	Intensity	Specimens Number	Intensity	Specimens Number	Intensity	Specimens Number
cerebral cortex and the hippocampus neuronal cell degeneration	-	0/5	+	2/5	+	2/5	+	4/5
perineuronal edema	-	0/5	-	0/5	+	1/5	++	3/5
vascular congestion	-	0/5	+	3/5	+	4/5	++	4/5

(-) none, (+) mild, (++) moderate, (+++) intense

Discussion

The present study demonstrates that chronic exposure of rat dams to regulatory relevant doses of glyphosate produces detectable neurobehavioral and structural brain alterations, while co-exposure with dicamba and 2,4-D markedly amplifies these effects.

Importantly, the observed changes occurred at exposure levels corresponding to regulatory ADI and NOAEL thresholds, suggesting that these limits may not fully protect the maternal nervous system during sensitive life stages.

Behavioral effects: early neurobehavioral alterations at the NOAEL level

The most consistent behavioral alteration was the increase in grooming behavior.

Notably, grooming was already significantly elevated in the NOAEL-Gly group and became markedly more pronounced in the herbicide Combination group.

The ADI-Gly group did not significantly differ from controls.

This progressive pattern indicates a dose-interaction relationship rather than random variability.

In rodents, grooming represents a sensitive index of emotional status and limbic activation rather than locomotor stimulation.

Increased grooming reflects altered corticostriatal and hippocampal circuit activity and is commonly interpreted as an anxiety-like response following exposure to neuroactive toxicants [35].

The absence of significant locomotor impairment and the lack of changes in forced swim immobility indicate selective disturbance of anxiety-related pathways rather than general neurotoxicity or depressive-like behavior.

Glyphosate is known to alter glutamatergic signaling, impair mitochondrial respiration and induce oxidative stress in the hippocampus, mechanisms strongly associated with anxiety-like phenotypes [12,14].

Therefore, the detection of behavioral alterations at the NOAEL-Gly dose suggests that subtle neurobehavioral endpoints are more sensitive indicators of toxicity than apical endpoints traditionally used for regulatory dose derivation.

Additive interaction with 2,4-D and dicamba

The magnitude of grooming behavior was greatest in the herbicide Combination exposed group, indicating that dicamba and 2,4-D enhance glyphosate neurotoxicity through additive mechanisms.

Auxinic herbicides such as 2,4-D cross the blood-brain barrier and alter monoaminergic neurotransmission, affecting serotonergic and dopaminergic pathways involved in emotional regulation [36-38].

Behavioral studies have shown that 2,4-D exposure induces neurobehavioral alterations consistent with increased stress reactivity [18] whilst dicamba has been associated with oxidative neuronal injury and membrane destabilization [39].

Glyphosate primarily disrupts mitochondrial function and redox homeostasis [40], whereas auxinic herbicides interfere with neurotransmission [41].

Simultaneous impairment of metabolic and signaling pathways produces cumulative functional disturbance.

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Mixture toxicology research consistently shows additive or greater-than-additive effects when pesticides act through complementary mechanisms [42,43].

Thus, the progressive increase in grooming from control through NOAEL-Gly and Combination exposure supports a biologically coherent additive neurotoxicity model.

Histopathological correlates of behavioral changes

The behavioral alterations were accompanied by structural brain injury, particularly neuronal degeneration, perineuronal edema and vascular congestion, which was most pronounced in the herbicide Combination exposure group.

The hippocampus showed neuronal loss and vacuolization, regions critically involved in anxiety regulation [44].

Hippocampal injury is strongly linked to increased emotional reactivity and stress-related behaviors [45].

Glyphosate has been shown to impair neurogenesis and synaptic plasticity in hippocampal neurons [14], consistent with the behavioral phenotype observed.

The presence of vascular congestion and hyalinized vessels suggests impairment of the neurovascular unit.

Cerebrovascular dysfunction contributes to neuronal injury by disrupting blood-brain barrier integrity and oxygen delivery [46].

Auxinic herbicides may potentiate this process through membrane destabilization and inflammatory signaling [38], explaining the more pronounced lesions in the Combination group.

Together, the concordance between anxiety-like behavior and hippocampal degeneration indicates that grooming reflects true neurobiological injury rather than adaptive behavioral modulation.

Vulnerability of pregnancy and lactation

Pregnancy and lactation represent a uniquely sensitive biological window for the maternal nervous system because the brain undergoes profound, tightly regulated neuroendocrine and structural remodeling to support maternal behaviors, stress adaptation, and metabolic demands [47].

Converging evidence shows that the peripartum period is characterized by marked plasticity across the hippocampus, prefrontal cortex, and amygdala, including changes in adult neurogenesis, synaptic remodeling, and dendritic architecture [48].

These adaptations are driven by dramatic fluctuations in steroid and peptide hormones and by altered stress-axis (hypothalamic-pituitary-adrenal) set-points, which can reshape anxiety-related behaviors and may lower the threshold for toxicant-induced perturbation of limbic circuitry [49,50].

For example, prolactin-dependent stimulation of neurogenesis during pregnancy is considered critical for normal postpartum behavioral responses, illustrating how physiological neuroplasticity during this stage can be both essential and vulnerable to disruption [51,52].

From a toxicological perspective, pregnancy has also been recognized as a “heightened susceptibility” period for environmental chemicals because rapid physiological changes (including immune modulation, vascular adaptations, and altered metabolism) may amplify biological responses to exposures and increase maternal health risks [53].

In parallel, the perinatal window is also broadly perceived as an extremely vulnerable period for neurotoxicant effects because neuroendocrine and neuroimmune pathways are highly dynamic; contaminants can act through oxidative stress, endocrine disruption, and immune signaling, producing durable alterations in brain function and behavior [54].

This framework is relevant to pesticide mixtures because combined exposures may simultaneously disrupt mitochondrial/redox homeostasis and neurotransmission, thereby interfering with the normal peripartum adaptations that shape maternal stress reactivity and emotional regulation.

In this context, the present findings-showing anxiety-related behavioral shifts (grooming) and hippocampal/cortical injury in exposed dams, with stronger effects in the herbicide Combination group-are consistent with the concept that the maternal brain during pregnancy and lactation is a sensitive target for low-dose neurotoxicant and mixture effects.

Limitations

Several limitations should be acknowledged.

The relatively small sample size, typical for mechanistic animal toxicology studies, may limit statistical power and the detection of subtle behavioral differences.

The investigation focused exclusively on maternal animals, and therefore potential

neurodevelopmental consequences in offspring were not evaluated.

In addition, molecular endpoints such as oxidative stress markers, inflammatory mediators, or neurotransmitter alterations were not assessed, which restricts direct mechanistic interpretation of the observed structural and behavioral changes.

The study employed active substances rather than commercial herbicide formulations that contain adjuvants capable of modifying toxicity, and the behavioral battery was limited to classical paradigms without inclusion of higher cognitive testing.

Nevertheless, the concordance between behavioral and histopathological findings supports the biological relevance of the observed effects.

Future perspectives

Future research should further elucidate the mechanistic basis of the observed neurotoxicity by integrating molecular and biochemical endpoints, including markers of oxidative stress, mitochondrial dysfunction, neurotransmitter imbalance, and neuroinflammation.

Long-term studies evaluating maternal cognitive performance and memory are warranted in order to determine whether the structural alterations translate into persistent functional impairment.

Because maternal exposure may have consequences beyond the exposed individual, transgenerational and offspring neurodevelopmental assessments should also be incorporated.

Additional investigations comparing active substances with full commercial formulations and environmentally realistic mixtures are necessary to better approximate real-world exposure scenarios.

Finally, the integration of transcriptomic and metabolomic approaches could provide pathway-level insight and support the refinement of cumulative risk assessment strategies in regulatory toxicology.

Conclusions

Chronic exposure to regulatory relevant doses of glyphosate produced measurable neurotoxicity in rat dams, and co-exposure with dicamba and 2,4-D markedly amplified these effects.

Behavioral alterations emerged with the NOAEL-Gly dose and were consistent with the structural findings of neuronal degeneration and hippocampal injury, indicating biologically meaningful neurotoxic damage rather than adaptive behavioral variability.

The progressive increase in grooming from glyphosate alone to herbicide Combination exposure supports an additive interaction between herbicides acting through complementary mechanisms.

This observation may have particular health implication in the USA where exposure to a combination of glyphosate, 2,4-D and dicamba has been escalating in recent years [17].

These findings suggest that regulatory thresholds based on single-compound testing may underestimate risk during sensitive physiological periods such as pregnancy and lactation, and they highlight the maternal brain as a relevant target for pesticide risk assessment.

Overall, the results support the need for incorporating mixture toxicity and vulnerable life stages into future neurotoxicity evaluation frameworks.

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Author Contributions

Conceptualization, A.M.C., A.O.D., R.Mes., M.N.A., A.O.D. and B.C..; Methodology, A.M.C., L.C., R.Mitr., M.A., D.A., A.O.D. and B.C.; Investigation, A.M.C, L.C., R.Mitr., A.B. and A.O.D.; Data analysis, A.M.C, L.C., R.Mitr., A.B., R.Mes., A.O.D; Manuscript writing and initial draft preparation, A.M.C, L.C., R.Mitr., A.B., R.Mes. and A.O.D.; Manuscript review and editing, M.N.A. D.C. and B.C.; Supervision, M.N.A. D.C. and B.C.

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Conflicts of interest

RM and MNA have served as consultants on glyphosate risk assessment issues as part of litigation in the US over glyphosate-based herbicide health effects.

The other authors declare no competing interests.

Institutional Review Board

The protocols utilized therein were approved by the Ethical Committee of UMF Craiova, Romania, No. 120/19.11.2020.

Consent Statement

Not applicable.

Data availability

All data presented in the manuscript are available from the authors upon request.

References

1. Benbrook CM. Trends in glyphosate herbicide use in the United States and globally. Environ Sci Eur, 2016, 28(1):3.
2. Gillezeau C, Lieberman-Cribbin W, Taioli E. Update on human exposure to glyphosate, with a complete review of exposure in children. Environmental Health, 2020, 19(1):115.
3. Klátyik S, Simon G, Oláh M, Mesnage R, Antoniou MN, Zaller JG, Székács A. Terrestrial ecotoxicity of glyphosate, its formulations, and co-formulants: evidence from 2010-2023. Environmental Sciences Europe, 2023, 35(1):51.
4. Klátyik S, Simon G, Oláh M, Takács E, Mesnage R, Antoniou MN, Zaller JG, Székács A. Aquatic ecotoxicity of glyphosate, its formulations, and co-formulants: evidence from 2010 to 2023. Environmental Sciences Europe, 2024, 36(1):22.
5. Klátyik S, Simon G, Takács E, Oláh M, Zaller JG, Antoniou MN, Benbrook C, Mesnage R, Székács A. Toxicological concerns regarding glyphosate, its formulations, and co-formulants as environmental pollutants: a review of published studies from 2010 to 2025. Arch Toxicol, 2025, 99(8):3169-3203.
6. Baek Y, Bobadilla LK, Giacomini DA, Montgomery JS, Murphy BP, Tranel PJ. Evolution of Glyphosate-Resistant Weeds. Rev Environ Contam Toxicol, 2021, 255:93-128.
7. Heap I, Duke SO. Overview of glyphosate-resistant weeds worldwide. Pest Manag Sci, 2018, 74(5):1040-1049.
8. Mesnage R, Antoniou M. Mammalian toxicity of herbicides used in intensive GM crop farming. In: Mesnage R, Zaller JG (Eds): Herbicides, Elsevier, 2021, Amsterdam, 143-180.
9. Mesnage R, Teixeira M, Mandrioli D, Falcioni L, Ducarmon QR, Zwittink RD, Mazzacuva F, Caldwell A, Halket J, Amiel C, Panoff JM, Belpoggi F, Antoniou MN. Use of Shotgun Metagenomics and Metabolomics to Evaluate the Impact of Glyphosate or Roundup MON 52276 on the Gut Microbiota and Serum Metabolome of Sprague-Dawley Rats. Environ Health Perspect, 2021, 129(1):17005.
10. Docea AO, Cirstea AE, Cercelaru L, Drocas AI, Dinca V, Mesnage R, Marginean C, Radu A, Popa DG, Rogoveanu O, Mitrut R, Antoniou MN, Tsatsakis A, Hernández AF, Calina D. Effect of perinatal exposure to glyphosate and its mixture with 2,4-D and dicamba on rat dam kidney and thyroid function and offspring's health. Environ Res, 2023, 237(Pt 1):116908.
11. Mesnage R, Renney G, Séralini GE, Ward M, Antoniou MN. Multiomics reveal non-alcoholic fatty liver disease in rats following chronic exposure to an ultra-low dose of Roundup herbicide. Sci Rep, 2017, 7:39328.
12. Costas-Ferreira C, Durán R, Faro LRF. Toxic Effects of Glyphosate on the Nervous System: A Systematic Review. Int J Mol Sci, 2022, 23(9): 4605
13. Chávez-Reyes J, Gutiérrez-Reyes CD, Hernández-Cuellar E, Marichal-Cancino BA. Neurotoxicity of glyphosate: Focus on molecular mechanisms probably associated with alterations in cognition and behavior. Environmental Toxicology and Pharmacology, 2024, 106:104381.
14. Luna S, Neila LP, Vena R, Borgatello C, Rosso SB. Glyphosate exposure induces synaptic impairment in hippocampal neurons and cognitive deficits in developing rats. Arch Toxicol, 2021, 95(6):2137-2150.
15. Ojiro R, Okano H, Takahashi Y, Takashima K, Tang Q, Ozawa S, Zou X, Woo GH, Shibutani M. Comparison of the effect of glyphosate and glyphosate-based herbicide on hippocampal neurogenesis after developmental exposure in rats. Toxicology, 2023, 483:153369.
16. Bartholomew SK, Winslow W, Sharma R, Pathak KV, Tallino S, Judd JM, Leon H, Turk J, Pirrotte P, Velazquez R. Glyphosate exposure exacerbates neuroinflammation and Alzheimer's disease-like pathology despite a 6-month recovery period in mice. J Neuroinflammation, 2024, 21(1):316.
17. Daggy JK, Haas DM, Yu Y, Monahan PO, Guise D, Gaudreau É, Larose J, Benbrook CM. Dicamba and 2,4-D in the Urine of Pregnant Women in the Midwest: Comparison of Two Cohorts (2010-2012 vs. 2020-2022). Agrochemicals, 2024, 3(1):42-56.
18. Oliveira GH, Palermo-Neto J. Effects of 2,4-dichlorophenoxyacetic acid (2,4-D) on open-field behaviour and neurochemical parameters of rats. Pharmacol Toxicol, 1993, 73(2):79-85.
19. Conejo-Bolaños LD, Mora AM, Hernández-Bonilla D, Cano JC, Menezes-Filho JA, Eskenazi B, Lindh CH, van Wendel de Joode B. Prenatal current-use pesticide exposure and children's neurodevelopment at one year of age in the Infants' Environmental Health (ISA) birth cohort, Costa Rica. Environmental Research, 2024, 249:118222.
20. Ruiz de Arcaute C, Larramendy ML, Soloneski S. Genotoxicity by long-term exposure to the auxinic herbicides 2,4-dichlorophenoxyacetic acid and dicamba on Cnesterodon decemmaculatus (Pisces: Poeciliidae). Environmental Pollution, 2018, 243:670-678.

21. Sheta R, Bérard M, Musiol D, Martínez-Drudis L, Oueslati A. Behavioral analysis of motor and non-motor impairment in rodent models of Parkinson's disease. Frontiers in Aging Neuroscience, 2024, 16:1464706.
22. Solomon J, Gutierrez-Reyes CD, Chávez-Reyes J, Onigbinde S, Marichal-Cancino BA, López-Lariz CH, Beck M, Mechref Y. Neuroglycome alterations of hippocampus and prefrontal cortex of juvenile rats chronically exposed to glyphosate-based herbicide. Frontiers in Neuroscience, 2024, 18:1442772.
23. Cirstea AE, Docea AO, Cercelaru L, Drocas AI, Mesnage R, Marginean C, Marinas C, Diaconu M, Golokhvast KS, Mitrut R, Antoniou MN, Tsatsakis A, Calina D. Changes in Rat Mammary Tissue Architecture Following Pregnancy/Lactation Exposure to Glyphosate Alone or with 2,4-D and Dicamba. Curr Health Sci J, 2024, 50(1):94-105.
24. Mesnage R, Nepka C, Ferguson S, Kouretas D, Stivaktakis PD, Calina D, Tsatsakis AM, Antoniou M, Docea AO. The GlyphoMix protocol: Multiorgan changes in rats caused by prenatal exposure to glyphosate or its mixture with 2,4-D and dicamba. Public Health and Toxicology, 2023, 3(1):1-7.
25. Authority EFS, Álvarez F, Arena M, Auteri D, Binaglia M, Castoldi AF, Chiusolo A, Crivellente F, Egsmose M, Fait G, Ferilli F, Gouliarmou V, Nogareda LH, Ippolito A, Istace F, Jarrah S, Kardassi D, Kienzler A, Lanzoni A, Lava R, Linguadoca A, Lythgo C, Mangas I, Padovani L, Panzarea M, Parra Morte JM, Rizzuto S, Romac A, Rortais A, Serafimova R, Sharp R, Szentes C, Terron A, Theobald A, Tiramani M, Vianello G, Villamar-Bouza L. Peer review of the pesticide risk assessment of the active substance glyphosate. EFSA Journal, 2023, 21(7):e08164.
26. Authority EFS. Conclusion on the peer review of the pesticide risk assessment of the active substance 2,4-D. EFSA Journal, 2014, 12(9):3812.
27. Authority EFS. Conclusion on the peer review of the pesticide risk assessment of the active substance dicamba. EFSA Journal, 2011, 9(1):1965.
28. Ruiyong W. Open Field Test. In: Kan Z (Eds): The ECPH Encyclopedia of Psychology, Springer Nature Singapore, 2024, Singapore, 1032-1033.
29. Grigor'ev NR, Batalova TA, Kirichenko EF, Sergievich AA, Cherbikova GE. Typological features in the behavior of rats. Neuroscience and Behavioral Physiology, 2008, 38(6):597-603.
30. Drayson OGG, Vozenin M-C, Limoli CL. A rigorous behavioral testing platform for the assessment of radiation-induced neurological outcomes. In: Sato A, Kraynak J, Marciscano AE, Galluzzi L (Eds): Radiation Oncology and Radiotherapy Part B, Academic Press, 2023, San Diego, 177-197.
31. Layton C, Bancroft JD, Suvarna SK. Fixation of tissues. In: Suvarna SK, Layton C, Bancroft JD (Eds): Bancroft's Theory and Practice of Histological Techniques (Eighth Edition), Elsevier, 2019, Amsterdam, 40-63.
32. Bancroft JD, Layton C. The hematoxylins and eosin. In: Suvarna SK, Layton C, Bancroft JD (Eds): Bancroft's Theory and Practice of Histological Techniques (Eighth Edition), Elsevier, 2019, Amsterdam, 126-138.
33. Bradley AE, Bolon B, Butt MT, Cramer SD, Czasch S, Garman RH, George C, Gröters S, Kaufmann W, Kovi RC, Krinke G, Little PB, Narama I, Rao DB, Sharma AK, Shibutani M, Sills R. Proliferative and Nonproliferative Lesions of the Rat and Mouse Central and Peripheral Nervous Systems: New and Revised INHAND Terms. Toxicologic Pathology, 2020, 48(7):827-844.
34. Meyerholz DK, Beck AP. Fundamental Concepts for Semiquantitative Tissue Scoring in Translational Research. Ilar j, 2018, 59(1):13-17.
35. Kalueff AV, Stewart AM, Song C, Berridge KC, Graybiel AM, Fentress JC. Neurobiology of rodent self-grooming and its value for translational neuroscience. Nat Rev Neurosci, 2016, 17(1):45-59.
36. Bortolozzi AA, Evangelista De Duffard AM, Duffard RO, Antonelli MC. Effects of 2,4-dichlorophenoxyacetic acid exposure on dopamine D2-like receptors in rat brain. Neurotoxicol Teratol, 2004, 26(4):599-605.
37. Yan Y, Luo X, Song Y, Gao H, Wang Y, Li Y, Yang H, Zhou J. Exploring the Mechanism of 2,4-Dichlorophenoxyacetic Acid in Causing Neurodegenerative Diseases Based on Network Toxicology and Molecular Docking. Int J Mol Sci, 2025, 26(24):11980.
38. Freitas L, Valadares LdA, Camozzi M, de Oliveira P, Ferreira Machado M, Lima F. Animal models in the neurotoxicology of 2,4-D. Human & Experimental Toxicology, 2019, 38(10):1178-1182.
39. Villagran D, Lombardero L, Crupkin A, Mendieta J, Medici S, Lavarello F, Simoniello M, Menone M. Can the Herbicide Dicamba Produce Oxidative Stress in the Native South American fish Jenynsia lineata at Environmentally Relevant Concentrations? Bulletin of Environmental Contamination and Toxicology, 2023, 111(3):41.
40. Fang Y-W, Lin H-C, Wang C, Lin C-Y. Glyphosate Exposure, Oxidative Stress, Mitochondrial Dysfunction, and Mortality Risk in US Adults: Insights from the National Health and Nutrition Examination Survey. Toxics, 2025, 13(5):373.
41. Bortolozzi A, Duffard R, Duffard A. Asymmetrical Development of the Monoamine Systems in 2,4-Dichlorophenoxyacetic Acid Treated Rats. Neurotoxicology, 2003, 24:149-157.
42. LeBlanc GA, Wang G. Chemical mixtures: greater-than-additive effects? Environ Health Perspect, 2006, 114(9):A517-518; author reply A518-519.
43. 43.Bloch D, Diel P, Epe B, Hellwig M, Lampen A, Mally A, Marko D, Villar Fernández MA, Guth S, Roth A, Marchan R, Ghallab A, Cadenas C, Nell P, Vartak N, van Thriel C, Luch A, Schmeisser S, Herzler M, Landsiedel R, Leist M, Marx-Stoelting P, Tralau T, Hengstler JG. Basic concepts of mixture toxicity and relevance for risk evaluation and regulation. Arch Toxicol, 2023, 97(11):3005-3017.

 10.12865/CHSJ.52.01.08

44. Shi HJ, Wang S, Wang XP, Zhang RX, Zhu LJ. Hippocampus: Molecular, Cellular, and Circuit Features in Anxiety. Neurosci Bull, 2023, 39(6):1009-1026.
45. Fanselow MS, Dong HW. Are the dorsal and ventral hippocampus functionally distinct structures? Neuron, 2010, 65(1):7-19.
46. Venkat P, Chopp M, Chen J. Blood-Brain Barrier Disruption, Vascular Impairment, and Ischemia/Reperfusion Damage in Diabetic Stroke. Journal of the American Heart Association, 2017, 6(6):e005819.
47. Kim P. Human Maternal Brain Plasticity: Adaptation to Parenting. New Dir Child Adolesc Dev, 2016, 2016(153):47-58.
48. Blankers SA, Go KA, Surtees DC, Splinter TFL, Galea LAM. Cognition and Neuroplasticity During Pregnancy and Postpartum. In: Brunton PJ, Grattan DR (Eds): Neuroendocrine Regulation of Mammalian Pregnancy and Lactation, Springer Cham, 2024, Cham, 253-281.
49. Barba-Müller E, Craddock S, Carmona S, Hoekzema E. Brain plasticity in pregnancy and the postpartum period: links to maternal caregiving and mental health. Arch Womens Ment Health, 2019, 22(2):289-299.
50. Duthie L, Reynolds R. Changes in the Maternal Hypothalamic-Pituitary-Adrenal Axis in Pregnancy and Postpartum: Influences on Maternal and Fetal Outcomes. Neuroendocrinology, 2013, 98(2):106-115.
51. Larsen CM, Grattan DR. Prolactin, neurogenesis, and maternal behaviors. Brain Behav Immun, 2012, 26(2):201-209.
52. Leff-Gelman P, Pellón-Díaz G, Camacho-Arroyo I, Palomera-Garfias N, Flores-Ramos M. Neuroendocrine Regulation and Neural Circuitry of Parenthood: Integrating Neuropeptides, Brain Receptors, and Maternal Behavior. International Journal of Molecular Sciences, 2025, 26(18):9007.
53. Varshavsky J, Smith A, Wang A, Hom E, Izano M, Huang H, Padula A, Woodruff TJ. Heightened susceptibility: A review of how pregnancy and chemical exposures influence maternal health. Reprod Toxicol, 2020, 92:14-56.
54. Rock KD, Patisaul HB. Environmental Mechanisms of Neurodevelopmental Toxicity. Curr Environ Health Rep, 2018, 5(1):145-157.

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