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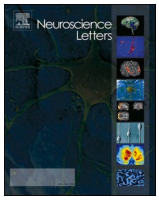

Research article

Glyphosate-based herbicide exposure affects cognitive flexibility and social cognition in adult mice

Yassine Ait bali [a,b,*], Fatiya Alfari Madougou [b], Saadia Ba-M'hamed [b], Maurizio Giustetto [c,*], Mohamed Bennis [b]

[a] Lumbricidae, Improving Soil Productivity and Environment Unit, Higher Normal School, Mohammed V University in Rabat, Rabat, Morocco
[b] Laboratory of Pharmacology, Neurobiology, Anthropobiology and Environment, Cadi Ayyad University, Marrakech, Morocco
[c] Department of Neuroscience Rita Levi-Montalcini, University of Turin, Turin, Italy

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ABSTRACT

Glyphosate (Gly) is the active ingredient of several widely used herbicide formulations. Studies on Gly and glyphosate-based herbicide (GBH) exposure in different experimental models have suggested that the nervous system represented a key target for its toxicity, especially the prefrontal cortex (PFC). However, it is still unknown whether exposure to GBH affects higher brain functions dependent on PFC circuitry. The present work aimed to examine the effects of subtoxic doses of GBH on social cognition and cognitive flexibility as two functions belonging to higher brain function in mice. To do so, adult male mice were exposed daily to GBH by gavage at doses of 250 or 500 mg/kg for a sub-chronic period lasting 6 weeks. Then, mice were subjected to behavioral testing using the three-chamber and the Barnes maze paradigms. Our results indicate that GBH did not affect sociability. However, we found that GBH affects social cognition expressed by a lower discrimination index in the three-chamber test. Moreover, spatial memories evaluated during the probe trial, and cognitive flexibility evaluated during the reversal probe, were affected in mice exposed to GBH. Based on these results, exposure to subtoxic doses of GBH led to neurobehavioral alterations affecting the integrity of social cognition and cognitive flexibility functions. Finally, these data urge a thorough investigation of the cellular and molecular mechanisms underlying these alterations.

1. Introduction

The widespread use of agrochemicals, particularly the prevalent pesticide glyphosate (Gly), has led to their continuous release into the environment [1], becoming pervasive pollutants affecting humans and other organisms. Gly is detected in water and human urine samples, indicating significant exposure and an increasing trend over the years [2]. Gly was considered one of the herbicides least toxic to humans and animals [3]. However, studies have shown that chronic exposure to Gly formulations was correlated to diseases such as cancer, endocrine effects, and also neurodegenerative disorders [4–6]. In this sense, Gly was reclassified by The International Agency for Research on Cancer as Category 2a (probable carcinogen) despite the continued debate about this compound [7]. Furthermore, a large body of evidence has shown that exposure to GBH induces toxicity in different animal tissues [8,9]. Notably, early or late exposure to GBH has been shown to cause a decrease in locomotor activity, an increase in anxiety levels, and depression-like behavior of the animals. Furthermore, cognitive functioning is affected by the action of these compounds. In this regard, it was shown that exposure to GBH or Gly caused an impairment in learning and different forms of memory [10,11]. Even though the precise mechanism by which GBH exerts its toxic effect remains poorly understood, it is well-documented that exposure to GBH induces changes in different areas of the brain. In this context, the PFC, a brain structure strongly associated with cognitive function, seems to be especially susceptible. Specifically, among others, neuroinflammation, loss of neurotransmitters, and oxidative stress, as well as neuronal hyperactivity, were observed in the PFC of rodents exposed to this herbicide [12–14]. However, it is still unknown whether exposure to GBH affects higher brain functions (e.g. cognitive flexibility and social cognition) dependent on PFC circuitry. Therefore, we hypothesized that GBH, by interfering with PFC integrity, may result in long-lasting

* Corresponding authors at: Lumbricidae, Improving Soil Productivity and Environment Unit, Higher Normal School, Mohammed V University in Rabat, Rabat, Morocco (Y. Ait bali). Department of Neuroscience Rita Levi-Montalcini, University of Turin, Turin, Italy (M. Giustetto).
E-mail addresses: yassine.aitbali@gmail.com (Y. Ait bali), maurizio.giustetto@unito.it (M. Giustetto).

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alterations of higher-order function. Thus, in the present work, we investigated the effects of subchronic exposure to GBH on social cognition and cognitive flexibility in adult mice.

2. Materials and methods

2.1. Animals

Male Swiss mice (2-month-old) were housed under standard conditions as described by [14]. All procedures were conducted in accordance to European Council Directive: EU2010/63. The study was approved by the Council Committee of Research Laboratories of the Faculty of Sciences, Cadi Ayyad University, Marrakech.

2.2. Pesticide

Roundup herbicide (glyphosate concentration 360 g/l in the form of glyphosate isopropylamine salt 486 g/l) was used in the liquid commercial form supplied by Monsanto Company (St. Louis, MO, USA).

2.3. Doses and protocol of exposure

Healthy mice were assigned equally to three experimental groups, which were group-housed, exposed daily to a single dose of GBH for a sub-chronic period lasting 6 weeks through oral force-feeding at doses of 0 (control group, n = 6 animals), 250 or 500 mg/kg (treated groups, n = 6 animals). These doses were selected based on Roundup's subchronic no-observed adverse effect level (NOAEL) of 500 mg/kg/day [15].

2.4. Behavioral tests

On the last day of the exposure period, all animals were tested between 9:00 h and 12:00 h during the light cycle. All behaviors were recorded and analyzed using the Ethovision XT Noldus 8.5 video tracking program (Noldus Information Technology b.v., Wageningen, The Netherlands) connected to a video camera (JVC, Yokohama, Japan).

2.4.1. Three-chambered sociability test

The social interaction test was run in a three-chambered arena (45 cm wide × 20 cm long × 30 cm high) made of clear glass as described by [16]. In the first phase, a mouse was placed in the apparatus and allowed to explore the environment freely for 10 min for habituation. Then, the mouse was gently guided to the center chamber, and its two entrances were blocked while a stranger mouse (stranger 1) was placed in one side chamber. In the second phase (sociability), the position of stranger 1 was alternated between tests to prevent side preference. The two entrances were then opened to allow the subject mouse to explore the new environment freely for 10 min. In the third phase (social novelty), stranger 2 was placed in the other empty side chamber and the subject mouse again was allowed to freely explore all three chambers for 10 min. All stranger mice were males of the same age and were previously habituated to the plastic cage for 30 min during the previous day. The apparatus was cleaned between tests using a 70 % ethanol/ water solution. Using the Ethovision XT Noldus 8.5 video tracking program we recorded and scored the time spent in each chamber. We also calculated the difference index as described by [17].

2.4.2. Barnes maze test

Spatial memory and cognitive flexibility were assessed on a Barnes maze apparatus, a circular platform with a 92 cm diameter and 20 equally spaced escape holes (5 cm of diameter) along the perimeter, one of which leads to a "target" escape box. The apparatus was illuminated by a 75 W lamp allowing an approximate brightness of 200 lx. The assay consisted of five phases: adaptation, forward acquisition training, forward probe trials, reversal training, and reversal probe trials. For adaptation, each mouse was placed in a dark start chamber in the middle of the maze for 10 s, then uncovered and guided gently to the escape box. Forward acquisition training consisted of two trials per day for 4 days, with each mouse starting in the dark start chamber in the middle of the maze and subsequently allowed to explore the maze for 3 min. The trial ends when the mouse enters the target escape hole or after 3 min has elapsed, after which the mouse is guided gently to the escape hole. After reaching the escape hole, the mouse was allowed to remain there for 1 min. Forward probe trials were conducted on day 5, 24 h after the last training day. During the probe trial, the maze is in the same position as the training days, and the target hole is closed. Each trial lasted 90 s, during which the number of errors (pokes into non-target holes) made before reaching the target hole is quantified. Days 6–10 consisted of reversal training, conducted using a similar protocol as forward acquisition training, except that the target was a stable escape hole moved 180° from its location during forward acquisition training. Reversal probe trials were conducted on day 11 as described above for the forward probe trials. The maze is subdivided into four quadrants, each consisting of 5 holes with the target hole in the center of the target quadrant. The number of total errors, the latency to reach the escape hole, and the time spent in the target quadrant as well were measured.

2.5. Statistical analysis

The sociability and social novelty results were analyzed using one or two-way ANOVA [treatment and chamber] while the Barnes maze data were analyzed using the repeated measure two-way ANOVA [treatment and the time], followed by Holm-Sidak's post hoc for multiple comparisons.

3. Results

3.1. GBH-exposed mice showed impaired social behavior

The data analysis revealed a significant effect of the chamber (holding mouse *vs* middle *vs* empty) [$F_{(2,17)} = 88.62$; $p < 0.001$]. However, there was no significant effect of both the treatment and the interaction between the two factors [$F_{(2,17)} = 2.05$; $p = 0.09$; $F_{(2,17)} = 2.05$; $p = 0.09$, respectively]. The *post hoc* analysis showed that the control, 250 mg/kg and 500 mg/kg groups showed an increased preference for spending time in the chamber with the S1 compared to the empty one ($t = 8.16$; $p < 0.001$; $t = 4.73$; $p < 0.001$; $t = 5.99$; $p < 0.001$, respectively) (Fig. 1a).

In contrast, adverse effects on the preference for the novel stimulus were observed in the novelty phase in treated groups. The two-way ANOVA comparisons revealed a significant effect of the treatment factor ($F_{(2,17)} = 51.97$; $p < 0.001$) and a significant interaction between the two factors ($F_{(2,17)} = 17.09$; $p < 0.001$), but not of the chamber factor ($F_{(2,17)} = 1.20$; $p > 0.05$). Multiple comparisons revealed that the control group spent more time in the chamber containing S2 compared to S1 ($t = 5.90$; $p < 0.001$). In contrast, *post hoc* analysis showed that the 250 mg/kg group spent more time in the chamber containing S1 compared to the chamber containing S2 ($t = 4.73$; $p < 0.001$). On the other hand, the analysis did not reveal a significant difference in the time spent in the S1 and S2 chambers for the 500 mg/kg group ($t = 1.21$; $p > 0.05$) (Fig. 1b). Therefore, the data indicate that GBH-treated mice showed a significant deficit in social novelty.

We then calculated the sociability discrimination score. The one-way ANOVA analysis revealed no significant variation between the control and the treated groups ($F_{(2,17)} = 0.46$; $p > 0.05$) (Fig. 1c). We also calculated the discrimination score for the social novelty. The ANOVA analysis revealed a significant difference between groups ($F_{(2,17)} = 19.38$; $p < 0.05$). Indeed, *post hoc* analysis showed that both the 250 mg/kg and the 500 mg/kg groups had a lower discrimination score compared to the control group ($t = 6.08$; $p < 0.001$; $t = 3.51$; $p < 0.01$, respectively). Finally, the group treated with 250 mg/kg showed a lower discrimination index compared to the 500 mg/kg group ($t = 3.51$; $p <$

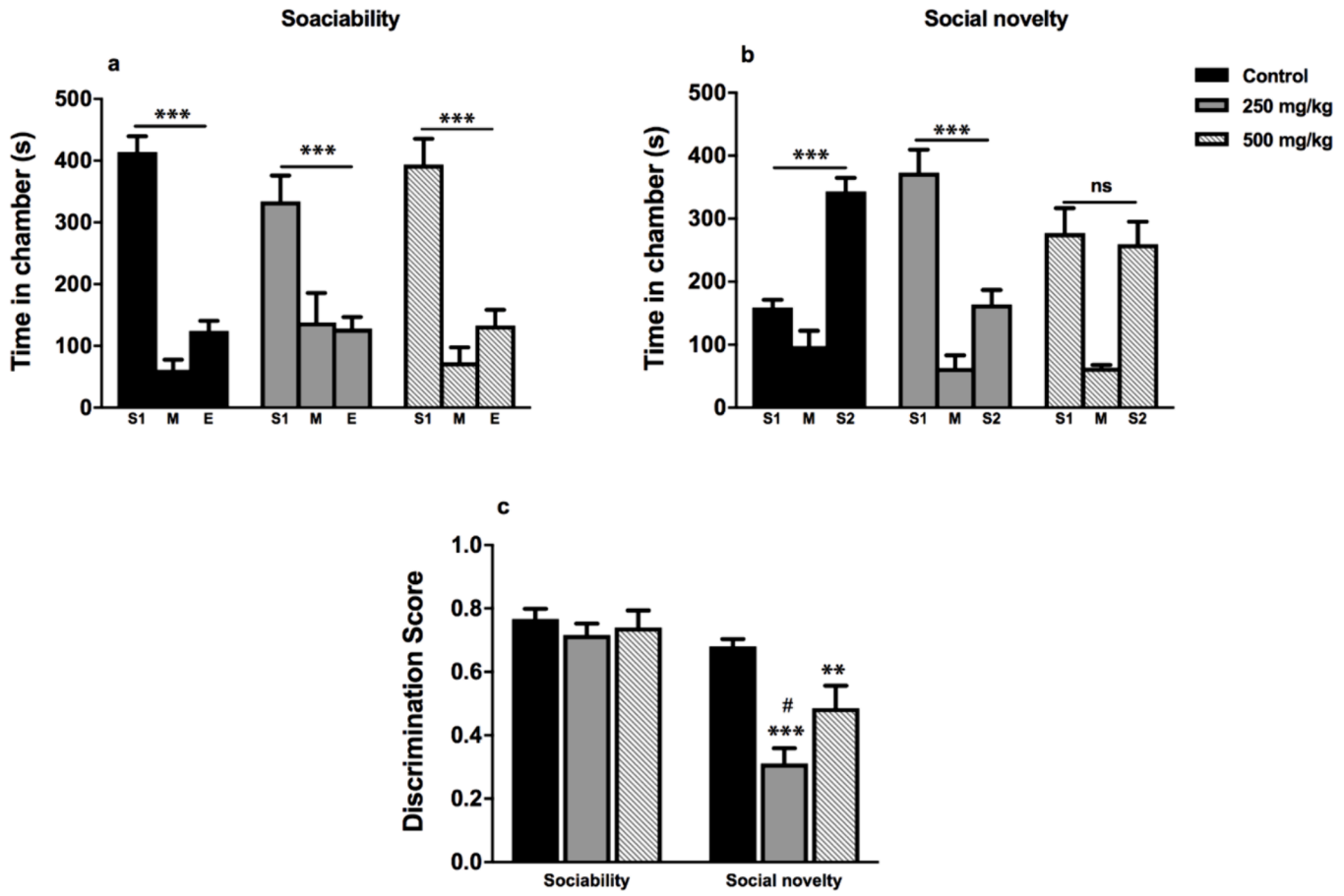

Fig. 1. GBH affected the social behavior of exposed mice. (a): Effect of GBH on the sociability. (b): Effect of GBH on the social novelty. (c): Discrimination score. Results are presented as mean ± SEM. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $^{\#}$ $p < 0.05$. The "*" refers to the 250 mg/kg or 500 mg/kg *vs* control groups comparison and the "#" refers to the 250 mg/kg *vs* 500 mg/kg groups comparison. *S1: Stranger 1; S2: Stranger 2; M: Middle; E: Empty.*

0.05) (Fig. 1c). Thus, these data substantiate the affected social novelty observed in GBH-treated mice.

3.2. GBH-exposed mice showed impaired spatial memory

The two-way repeated measures ANOVA found a significant effect of treatment ($F_{(2,17)} = 20.64$, $p < 0.01$) and time ($F_{(2,17)} = 465.47$, $p < 0.001$) on the latency to the escape box. However the interaction was not significant ($F_{(2,17)} = 1.95$, $p = 0.07$). *Post hoc* analysis showed that animals treated with 500 mg/kg manifest higher latency to find the escape box compared to the controls ($t = 2.79$, $p < 0.05$) (Fig. 2a, b). Subsequently, we compared the time spent by the animals in the target quadrant. The analysis revealed a significant effect of the treatment ($F_{(2,17)} = 9.38$; $p < 0.01$) as well as a significant interaction between the two factors ($F_{(2,17)} = 9.38$; $p < 0.05$), while no significant effect of time was observed ($F_{(2,17)} = 9.38$; $p = 0.054$). *Post hoc* analysis showed that the 500 mg/kg group spent less time in the target quadrant compared to the control and 250 mg/kg groups ($t = 3.91$; $p < 0.01$; $t = 3.50$; $p < 0.01$, respectively), but no difference was observed between the control and the 250 mg/kg groups ($t = 0.40$; $p = 0.68$) (Fig. 2b). Finally, we analyzed the number of errors made by the animals before finding the escape hole. The two-way repeated measures ANOVA analysis revealed a significant effect of the treatment ($F_{(2,17)} = 5.61$, $p < 0.05$), the time ($F_{(2,17)} = 51.47$, $p < 0.001$) as well as a significant interaction between the two factors ($F_{(2,17)} = 4.51$, $p < 0.05$). Multiple comparisons did not show any differences between groups although a trend toward increased errors made by the 500 mg/kg group was observed (Fig. 2c). Thus, the data obtained during the probe trial suggest that GBH exposure adversely affected the spatial memory of mice exposed to 500 mg/kg.

3.3. GBH-exposed mice showed affected cognitive flexibility

We found that the latency to identify the escape box was affected by the treatment ($F_{(2,17)} = 20.64$, $p < 0.01$) and time ($F_{(2,17)} = 465.47$, $p < 0.001$). However the interaction between the two factors was not significant ($F_{(2,17)} = 1.95$, $p = 0.07$). *Post hoc* comparisons showed that animals in the 250 mg/kg group ($t = 4.95$; $p < 0.001$) as well as those in the 500 mg/kg group ($t = 3.81$; $p < 0.01$) showed higher latency compared to the controls (Fig. 3a, b). Next, we analyzed the time spent by the animals in the target quadrant and we revealed a significant effect of the treatment ($F_{(2,17)} = 9.38$; $p < 0.01$) as well as a significant interaction between the two factors ($F_{(2,17)} = 9.38$; $p < 0.05$). However, no significant effect of time was observed ($F_{(2,17)} = 9.38$; $p = 0.054$). *Post hoc* comparisons showed that 250 mg/kg and 500 mg/kg groups spent less time in the target quadrant compared to the control group ($t = 3.33$; $p < 0.01$; $t = 2.26$; $p < 0.01$, respectively) (Fig. 3b). Finally, we analyzed the number of errors made by the animals before reaching the target box. The two-way repeated measures ANOVA analysis revealed a significant effect of treatment ($F_{(2,17)} = 5.61$, $p < 0.05$), time ($F_{(2,17)} = 51.47$, $p < 0.001$), and interaction between the two factors ($F_{(2,17)} = 4.51$, $p < 0.05$). Interestingly, multiple comparisons analysis showed that animals treated with 500 mg/kg had a higher number of errors compared to the controls ($t = 4.28$; $p < 0.01$), while no significant difference was observed between the control and the 250 mg/kg groups ($t = 1.96$; $p = 0.06$) (Fig. 3c). Thus, these data obtained through the reversal probe trial indicated that GBH exposed mice exhibited cognitive

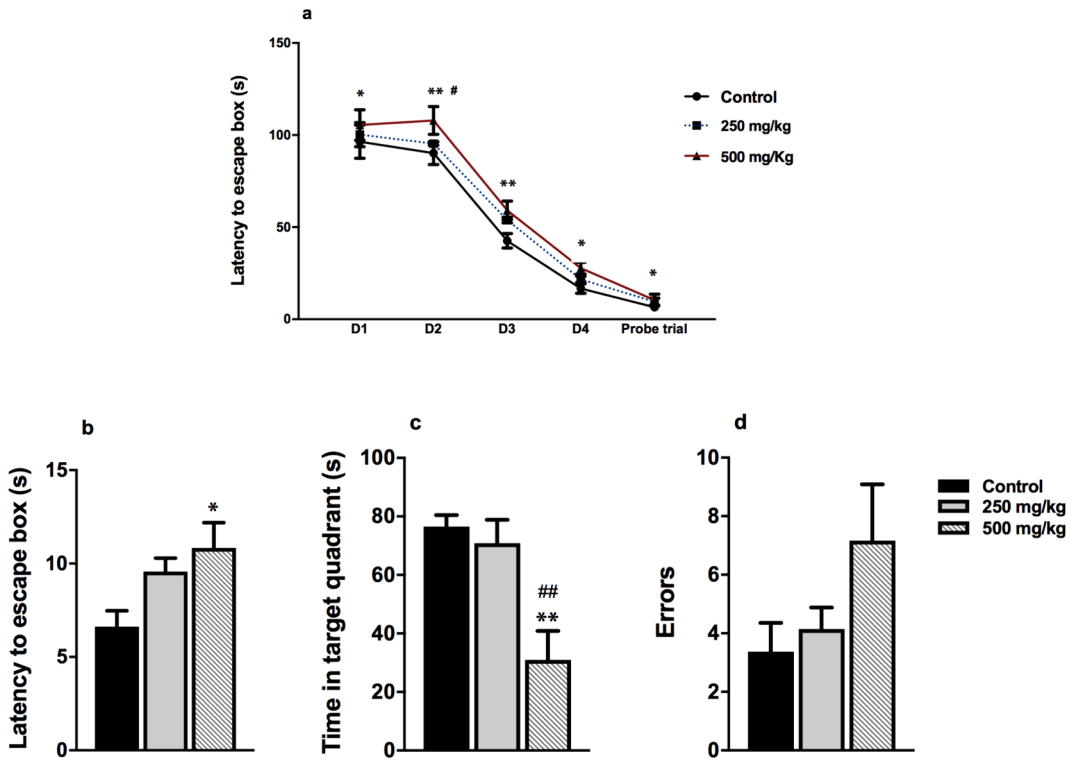

Fig. 2. GBH affected the spatial memory of exposed mice in the Barnes maze probe trial. (a): Latencies to identify the escape box during the forward acquisition. b): Latencies to identify the escape box during the probe trial. (c): Time in the target quadrant. (d): Number of errors. Results are presented as mean ± SEM. *$p < 0.05$; *** $p < 0.001$; $^{\#\#}$ $p < 0.01$. The "*" refers to the 250 mg/kg or 500 mg/kg *vs* control groups comparison and the "#" refers to the 250 mg/kg *vs* 500 mg/kg groups comparison.

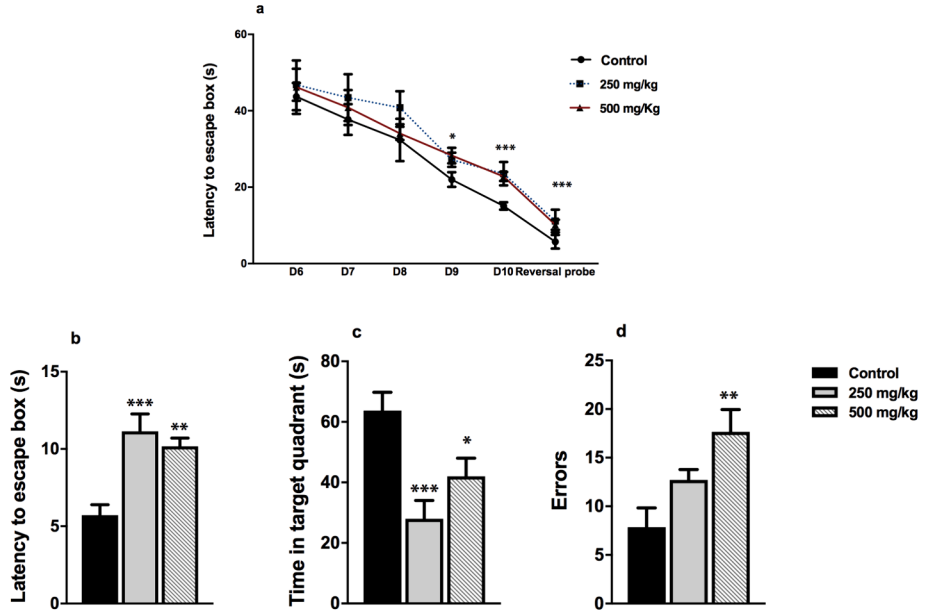

Fig. 3. GBH affected the cognitive flexibility of exposed mice in the Barnes maze reversal probe trial. (a): Latencies to identify the escape box during the reversal learning. (b): Latencies to identify the escape box during the reversal probe trial (c): Time in the target quadrant. (d): Number of errors. Results are presented as mean ± SEM. ** $p < 0.01$; *** $p < 0.001$. The "*" refers to 250 mg/kg or 500 mg/kg *vs* control groups comparison.

flexibility deficits.

4. Discussion

The results obtained in this study indicate that the social behavior abilities of mice treated with GBH did not undergo substantial changes. Indeed, during the sociability session, animals from both control group and those from GBH groups spent more time in the chamber containing the stimulus mouse compared to the empty chamber. These results contradict our previous data [14], where we showed that prenatal exposure to GBH caused a decrease in sociability in adult mice. However, this difference can be parsimoniously explained by the different

age periods of exposure to GBH. The developing brain is particularly vulnerable to toxic substances, and this sensitivity is likely greatest *in utero* and throughout early childhood. Indeed, the central nervous system of young individuals undergoes rapid growth and development, and these processes are accompanied by a high degree of both plasticity and vulnerability in brain cells. Additionally, immature metabolic pathways make young individuals less capable than adults of breaking down and excreting toxic compounds [18].

Our data on the effect of GBH on social memory demonstrate that animals exposed to this compound exhibit an inability to distinguish the new social partner from the familiar one. Indeed, animals from the control group spent more time near Stranger 2 and had a higher discrimination index compared to animals exposed to GBH. These results are consistent with those of Pu et al. [19], who showed that prenatal exposure to Gly at a dose of 50 mg/kg increased the risk of developing the typical signs of ASD, including the severe impairment of social memory. These findings are further supported by those of Biosca-Brull et al. [20], who established a connection between pesticide exposure and the emergence of ASD-like behaviors, including the perturbation of social cognitive abilities.

Our results obtained by Barnes maze test clearly showed that although the error number was unchanged, the animals treated with 500 mg/kg of GBH exhibited increased latency to reach the escape box and a decreased time in the target quadrant, the more sensitive measure of performance in the probe trial [21], suggesting that spatial memory is affected by GBH. This finding supports the negative role of GBH exposure on cognitive functions. Indeed, GBH can affect both recognition and working memory in rodents [22,23]. Furthermore, our results obtained during the reversal probe session revealed that treated animals were unable to relearn the new location of the target hole compared to the controls indicating an impairment of cognitive flexibility. These findings are in agreement with previous studies pointing to a causal association between pesticide exposure and the impairment of executive functions. Indeed, individuals exposed to OP pesticides showed an alteration in executive function performance [24].

The mPFC has emerged as a crucial neural substrate of social cognition [25] and cognitive flexibility [26]. Patients with lesions of the mPFC exhibit severe social behaviour impairment [27] and previous work demonstrated that an increase in excitatory/inhibitory (E/I) balance in the rodent mPFC leads to social behavior deficits, which can be partially rescued by stimulation of parvalbumin (PV) interneurons [28]. Moreover, Cao et al. [29] demonstrated that in mice mPFC exhibiting autistic behaviors, including impairment in social novelty gamma oscillations, closely linked to the activity of PV-positive interneurons, are dysfunctional and are associated with decreased excitability of PV cells. Moreover, the integrity of E/I balance, dependent on PV interneuron activity, is required for typical behavioral responses underlying cognitive flexibility [26]. Accordingly, previous data from our laboratory showed that GBH can induce hyperactivation of mPFC neurons [30], while a previous report highlighted the sensitivity of GABAergic neurons to Gly exposure in the C. *elegans* animal model [31]. Based on the aforementioned data, we suggest that PV cells, the most abundant GABAergic interneuron population of the mPFC, could be severely affected by GBH exposure.

In conclusion, the results obtained in this work suggest that exposure to GBH impacts the integrity of both social cognition and cognitive flexibility functions. Nevertheless, further research is needed to shed light on the cellular and molecular mechanisms underlying the observed alterations.

Significance statement

We used mice to investigated the impact of glyphosate-based herbicide (GBH) on social cognition and cognitive flexibility to contribute addressing GBH safety on higher brain function integrity.

CRediT authorship contribution statement

Yassine Ait bali: Writing – original draft, Software, Methodology, Investigation, Data curation, Conceptualization. **Fatiya Alfari Madougou:** Methodology, Investigation. **Saadia Ba-M'hamed:** Writing – review & editing, Supervision, Investigation, Data curation, Conceptualization. **Maurizio Giustetto:** Conceptualization, Formal analysis, Writing – review & editing, Supervision. **Mohamed Bennis:** Writing – review & editing, Supervision, Project administration, Investigation, Data curation, Conceptualization.

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.

Data availability

No data was used for the research described in the article.

Acknowledgments

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