Maternal exposure of mice to glyphosate induces depression- and anxiety-like behavior in the offspring via alterations of the gut-brain axis

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Abstract

The past decade has been characterized by increased awareness and de-stigmatization of mental health issues, in particular the most common neuropsychiatric disorders depression and anxiety. Further, with growing understanding of neurodevelopmental disorders such as attention deficit and hyperactivity disorder and autism spectrum disorder, the number of diagnosed patients has increased. The pathogenesis of these behavioral disorders is multifactorial and early-life exposure to environmental chemicals has been proposed to be a relevant risk factor that might mediate these effects by disturbances on the gut-brain-axis. However, for glyphosate, the most widely used pesticide worldwide, there are only limited and inconsistent findings that link chronic low-dose exposure in particular during early life to neurobehavioral disorders. Here, we explored the impact of maternal oral glyphosate exposure (0.5 and 50 mg/kg body weight/day) during pregnancy and the lactational period on offspring's behavior, brain gene expression and gut microbiota using a cross-generational mouse model. Behavioural analyses revealed a depression- and anxiety-like behavior as well as social deficits most notably in adult female offspring of glyphosate-exposed dams. Furthermore, the expression of tryptophan hydroxylase 2, an enzyme discussed to be linked to behavioral problems, was reduced in the hippocampus of female offspring and correlated to a glyphosate-induced DNA hypermethylation of the gene. Moreover, maternal glyphosate exposure significantly altered the gut microbiota in the female offspring including a decreased abundance of *Akkermansia* and increased abundance of *Alistipes* and *Blautia*, bacteria involved in tryptophan metabolism and associated with depression- and anxiety-like disorders. Our results suggest that glyphosate might influence the gut-brain axis crosstalk following *in-utero* and lactational exposure. This study underlines the importance of understanding the impact of exposure to pesticides on the gut-brain axis and further

63 emphasizes the need for microbiome analyses to be compulsorily included in health
64 risk assessments of pesticides.
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1. Introduction

Around 300 million people are affected by depression and about 270 million by anxiety disorders globally (Dickerson et al., 2020). Additionally, diagnoses of neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention deficit and hyperactivity disorder (ADHD) have significantly increased over the past 25 years (Maleki et al., 2022; Moore et al., 2022). A common pathogenesis hallmark of these mental health disorders is multifactoriality with early-life exposure to environmental chemicals like pesticides proposed to be a relevant modifiable risk factor (Dickerson et al., 2020). The most commonly used pesticide worldwide both in terms of frequency of usage and overall quantity is glyphosate (Connolly et al., 2022). The general population is mostly exposed via food, although the quantities of glyphosate to which humans are exposed daily via food residues is still unclear (Connolly et al., 2020; Gillezeau et al., 2020). Recent data indicate that the average glyphosate intake in western~~ized~~ countries might be close to EFSA's acceptable daily intake (ADI) level (Connolly et al., 2020; Vandenberg et al., 2017), with exposures even higher in occupational settings or lower-income countries due to greater pesticide use and less ~~restrictive~~ stringent pesticide regulations (Buralli et al., 2020). Indeed, it was shown that the general population exposure has continuously increased over the past decades (Gillezeau et al., 2020).

To date, it is still under discussion whether low-dose glyphosate concentrations that are deemed acceptable for the common exposure by regulatory agencies, may have adverse effects in susceptible individuals like the unborn fetus exposed *in utero* or via breastmilk (Gillezeau et al., 2020; Mamane et al., 2015). While acute toxicity is well-established for glyphosate, little is known regarding early-life exposure scenarios (Requena-Mullor et al., 2021).

It has been shown that glyphosate is able to cross the placental and blood brain barrier (Martinez and Al-Ahmad, 2019; Poulsen et al., 2009) suspecting negative glyphosate effects on neurodevelopment or an association with the long-term pathogenesis of neuropsychiatric disorders (Bali et al., 2019; Winstone et al., 2022). Some epidemiological studies suggest that an offspring's risk of autism spectrum disorder increases following prenatal glyphosate exposure (He et al., 2022; von Ehrenstein et al., 2019). Another study reported an association between early-life exposure to glyphosate-based herbicide (GBH) and youth depression in a US birth cohort (Hyland et al. (2021). Overall, the number of epidemiological studies describing glyphosate exposure as a potential risk factor for neurodevelopmental disorders is still limited. Using experimental approaches with animal models, direct chronic and sub-chronic oral exposure to GHBs induced depression-like symptoms in adult rats (Ait Bali et al., 2017; Aitbali et al., 2018), while a developmental GBH exposure led to an increased immobility and decreased locomotor activity in young offspring (Cattani et al., 2017). However, it is noteworthy that GBHs contain many additional constituents like surfactants, which themselves have been shown to potentially induce adverse health outcomes. Therefore, this makes it imperative to dissect whether or not it is the actual active substance, glyphosate, causing undesired effects (Mesnage et al., 2019).

Environmental chemical exposures and their impact on neurodevelopmental disorders have been associated with disturbances of the gut microbiome on the gut-brain-axis (Giambò et al., 2022; O'Shaughnessy et al., 2021). Contaminant residues in food products like pesticides alter gut microbiome composition resulting in both inflammation and a disturbed gut permeability (Long et al., 2021). Gut dysbiosis also implies an altered amount and distribution of neurotransmitters and metabolites that ultimately can perturb the blood-brain-barrier, potentially in particular during the early postnatal phase leading to adverse neurodevelopmental outcomes (Balaguer-Trias et

al., 2022; Holland, 2017). For the common pesticide chlorpyrifos, it has been demonstrated that even a low-dose exposure during early developmental phases can lead to perturbations of the gut-brain-axis with adverse behavioral outcomes in the offspring using different mouse models (Guardia-Escote et al., 2020; Perez-Fernandez et al., 2020). Adverse behavioral outcomes have been linked to GBHs potentially affecting gut microbiome composition (Aitbali et al., 2018). So far, studies on the influence of *in-utero* and lactational exposure to the active substance glyphosate in the progeny related to behavioral outcomes are scarce with merely high-level GBH concentration proposed to alter relative abundance of various bacteria in the gut (Pu et al., 2020).

To provide insights into this topic, we conducted a well-established cross-generational mouse model (Jahreis et al., 2018; Junge et al., 2021; Junge et al., 2022) in which dams were chronically exposed to low doses of glyphosate during pregnancy and the lactational period. In this context, we studied the influence of maternal glyphosate exposure on behavior, brain gene expression and the gut microbiota in the F1 generation.

2. Methods

2.1. Mice

Balb/cByJ mice (6-8 weeks of age) were purchased from the Elevage Janvier Laboratory (Le Genest St Isle, France) with a 7-day adaption period before the start of experiments. Animals were maintained in groups of 3-6 mice per cage in the animal facility at the University of Leipzig (Germany) under conventional conditions with 21.5 - 23°C room temperature, an average of 55% humidity, and a 12-hour day/night rhythm. Exposed and control dams as well as the offspring of exposed and control mice were housed separately. All mice were kept in multiple sealed cages with HEPA filters by

144 Sealsafe® and bedded with LIGNOCEL® bedding material. Dams and pups received
145 a phytoestrogen-free diet (C1077 from Altromin, Lage, Germany) and water *ad libitum*.
146 All animal experiments were performed at least 3 times with at least 3 dams per group
147 resulting in ≥ 8 pups per group and sex (with a maximum of 4 male or 4 female pups
148 per dam). All animal experiments were conducted in accordance with institutional and
149 state guidelines. Animal protocols used in this study were approved by the Committee
150 on Animal Welfare of Saxony/Leipzig (animal authorization number: TVV14/18).
151
152 *2.2. Chronic exposure to glyphosate*
153 Female Balb/c mice were exposed to the active substance glyphosate (N-
154 (Phosphonomethyl)glycine; diluted in water) orally administered by gavage in 300 µl
155 distilled water three times per week. The intervention lasted from one week before
156 mating with BALB/c males until weaning of the pups at 3 weeks (Supplementary Figure
157 E1) . Female mice received a weekly glyphosate concentration of either 8.75 mg or
158 87.5 µg, respectively equating to the no observed adverse effect level (NOAEL; 50
159 mg/kg body weight/day, Gly_{NOAEL}) or human acceptable daily intake (ADI; 0.5 mg/kg
160 body weight/day, Gly_{ADI}) concentration of glyphosate (EFSA, 2015). Control dams
161 received distilled water also via gavage. Respective concentrations were chosen as
162 they, per definition, should not induce direct adverse effects on dams to allow an
163 unbiased analysis of the progeny. Directly after weaning serum and caecum samples
164 as well a brain tissue was collected for further analysis.
165
166 *2.3. Behavioral tests*
167 *2.3.1. Open Field Test*
168 After a two-day adaption period in the behavioral test room, the progeny was subjected
169 to the Open Field (OF) test in week 12 with males and females being kept separately

at all times. OF test was performed using an acrylic frame (45 x 45 x 38 cm) with evenly spaced infrared light beams that detect motions along the X and Y axis including another frame on top measuring rearing activities in the Z axis (ActiMot, TSE Systems, Berlin, Germany). The OF box is covered by an opaque quadratic box with two dimmed lights attached in the center to create a secure and quiet environment. For testing, the mouse was placed in the middle of the lower right corner and movements were automatically recorded for a test duration of ten minutes by the TSE system as soon as the mouse touched the floor. After each experiment, the box was extensively cleaned with a small amount of 70% EtOH for disinfection as well as to prevent distractions by olfactory cues, and kept open for ten minutes in order for the EtOH to be fully evaporated. A variety of parameters were determined to characterize the offspring's activity and anxiety levels. For activity, distance travelled and steps taken were measured. Active time was defined as subject's movements above 5 cm/s, movements below were defined as calm time. Overall speed was defined as average speed throughout the entire test period, whole locomotion speed was calculated as a measure of distance travelled during active times and total activity time. For anxiety measures, the center region was defined as the central square within the box yielding 25% of the total box area. Active times in the center, visits to the center, travelled distance in the center region and overall time spent in the center and rearing time were measured (Seibenhener and Wooten, 2015; Sestakova et al., 2013).

2.3.2. Light Dark Test

The Light Dark (LD) test is based on rodents' aversion towards brightly lit areas versus their innate exploratory drive and is therefore not only suitable to explore activity levels but particularly for the analysis of anxiety-like behavior (Takao and Miyakawa, 2006). The LD test was conducted one day after the OF test using the same experimental

set-up and equipment. In addition to the OF box, a black partition box with an open door and a lit was place inside the OF separating the box into a dark and lit area. At the start of the experiment, mice were placed in the middle of the dark compartment, then the lit was closed with the mouse being free to transit between the dark and lit compartment via the open door. The ActiMot system subsequently tracked the mice's movement for 10 minutes. As measures of anxiety, the number of transits into the lit compartment was determined as well as latency to enter the lit compartment, denoting the time until the mouse first transited to the lit compartment. Additionally, distance travelled in the lit compartment was measured.

2.3.3. Three-chamber sociability test

To study general sociability and interest in social novelty of mice the three-chamber sociability (SOC) test was used. It is based on the fact that mice prefer to explore a novel mouse rather than objects or an already known mouse (Kaidanovich-Beilin et al., 2011). The SOC test was conducted between week 9 and 10 in the offspring using a video-tracking system (VideoMot 2, TSE Systems) surrounded by dimmed lighting. For social partners, BALB/cByJ mice aged 8 - 20 weeks were regularly handled for familiarization. One day before the experiment, social partners of the same sex were habituated in the SOC test box for 10 minutes each. The SOC test box consisted of three opaque equally-sized chambers à 20 x 40 x 25 cm with two seven-centimeter-diameter cylindric cages including removable lids for social partners in the right and left chambers. The social partners' cylindric cages were comprised of metal-wired walls to prevent direct physical contact between social partners and test mice but still allow for social interaction. Chambers were divided by opaque inserted walls with mouse-sized doors. SOC testing was performed starting with a 30-minute habituation of test mice and social partners within a separated section of the behavioral test room. The

SOC test consists of three phases: 1) Habituation: The test mouse was carefully placed into the middle chamber with doors to the other two compartments closed. The test mouse was habituated for 5 minutes. 2) Sociability: One social partner was quietly inserted into the right-hand social partner cage, chamber doors were carefully removed and the video tracking was started immediately. The test mouse was then free to either explore the social partner or the so-called novel object in the form of the left-hand empty cage for 10 minutes. 3) Social novelty: The test mouse was subsequently carefully nudged in the direction of the middle chamber, doors were closed again and the second social partner was placed into the second cage. Again, doors were quietly removed, video tracking started immediately for 10 minutes with the test mouse now free to either explore the known or the new social partner. After each test round, the SOC box and social partner cages were cleaned with 70% EtOH.The parameters determined were time spent in each compartment. To calculate the sociability index, time spent exploring the novel mouse area divided by the overall time spent exploring (novel mouse and novel object) x 100. For the social novelty index, time spent exploring the second social partner was divided by overall exploration time of both social partners x 100 (Lo et al., 2016).

2.4. Gene expression analysis

For gene expression analysis whole hippocampi were carefully dissected, RLT buffer was added (350 µl for hippocampus samples) and frozen at -80°C for later analysis. Briefly, total RNA was extracted from hippocampus samples using TRIzol reagent according to manufacturer's instructions. 1 µg of RNA was used for cDNA synthesis applying the ImProm-II™ Reverse Transcription System. Primers (Supplemetary Table E1) were designed using appropriate UPL probes as describes earlier (Bauer et al., 2022). Comprehensive gene expression was determined using 48.48 Dynamic Array

248 on the BioMark™ HD System (Standard BioTools, South San Francisco, USA).
249 Expression was determined via $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *GAPDH,*
250 *RPLP0, UBS* and *ACTB* were used as reference genes and data were normalized to
251 the lowest measured value (Bauer et al., 2022).
252
253 *2.5. Pyrosequencing*
254 Whole hippocampi of the progeny were fully dissected and immediately frozen for later
255 analysis. PyroMark Assay Design Version software was applied for primer design for
256 CpG-sites-containing regions of interest for the differentially expressed gene *tph2*
257 (Supplementary Table E2). Pyrosequencing was conducted as described by Leppert
258 et al. (2020). Briefly, genomic DNA (gDNA) of hippocampus samples was isolated
259 using the DNeasy Blood and Tissue Kit and subsequently bisulfite-converted using the
260 EZ DNA Methylation™ Kit. Bisulfite-treated gDNA was then amplified via the HotStar
261 Taq DNA Polymerase Kit. DNA methylation was quantified by pyrosequencing utilizing
262 a PyroMark Q48 according to manufacturer's instructions (Leppert et al., 2020).
263
264 *2.6. Serotonin assay and cytokine production*
265 Serotonin (5-hydroxytryptamine, 5-HT) levels were quantified in hippocampus tissue
266 using Abcam's Serotonin ELISA Kit according to accompanying instructions (Abcam,
267 2023). For tissue homogenization, 100 µl of PBS was added to the right hippocampus
268 with samples subsequently being mechanically ground. Another 200 µl of PBS was
269 added, followed by a centrifuging step at 4°C, 5 minutes and 5 g. 50 µl of sample
270 homogenate were taken for protein quantification using Bradford Reagent for the
271 calculation of serotonin levels. Samples were added to the serotonin assay at a dilution
272 of 1:10. In addition, Interleukin (IL)-6, IL-1β, and Tumor necrosis factor (TNF)-α were
273 measured using DuoSet ELISA kits (R&D Systems, Wiesbaden-Nordenstadt, DE)

according to the manufacturer's instructions (Jahreis et al., 2017) in relation to the protein content.

2.7. Microbiota assessment

To assess microbiota community structure we used 16 S rRNA gene profiling of caecum samples of dams as well as 3-week-old offspring upon sacrificing. DNA was extracted with QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) as previously described (Haange et al., 2020). V3-V4 variable regions of the 16 S rRNA genes were amplified by PCR and a library was constructed, followed by paired-end 2x250bp Illumina sequencing (StarSEQ GmbH, Mainz, Germany). Raw data were processed by Starseq using the QIIME 2 workflow (Bolyen et al., 2019). Here, data was de-multiplexed and quality checked, primers removed, paired-end reads were joined, and low-quality reads removed (de Sena Brandine and Smith, 2019). Operational Taxonomic Units (OTUs) were obtained, after read correction and chimera removal using the deblur workflow (Quast et al., 2013). The read counts per OTU with taxonomic annotation were normalized and relative abundances of each OTU and taxa were calculated using the R scripts Rhea (Lagkouvardos et al., 2017).

2.8. Metabolomics

The metabolite response in the female offspring of glyphosate-exposed dams was determined by a targeted metabolic approach. Metabolome analyses were performed in 10 µl of serum using the AbsoluteIDQ® p180 kit by Biocrates Life Science enabling the quantification of biogenic amines, amino acids, acylcarnitines, glycerophospholipids and sphingolipids. Biogenic amines and amino acids were analyzed via LC-MS, while acylcarnitines, glycerophospholipids and sphingolipids were analyzed using flow injection MS-MS analysis (FIA-MS/MS) as described in detail

elsewhere (Fries et al., 2022; Huber et al., 2016). Biocrates' MetIDQ software provided quality measures and quantification. Endotoxin (lipopolysaccharide, LPS) was determined using a Limulus amebocyte lysate-based (LAL) assay (Pierce Chromogenic Endotoxin Quant Kit, ThermoScientific) according to themanufacturer's instructions.

2.9. Statistical analysis

Experimental data were processed and analyzed in GraphPad PRISM 9.1.2 for macOS (Dotmatics, Boston, USA). Data were illustrated as mean ± SEM and p values of < 0.05 were considered significant. For statistical inference, either Welch's t-test or Wilcoxon-Mann-Whitney test were used according to individual data sets as indicated in result figures (Rasch et al., 2011). For microbiome analysis, alpha-diversity (Richness and Shannon-Effective) and beta-diversity (Principal Component Analysis) of taxa were determined. For genus level, data with a relative abundance/sample at > 0.2 were included and analyzed using ANOVA followed by Dunnett's correction for multiple comparison (Buchenauer et al., 2022). Heatmaps for microbiome and metabolomic analysis were generated in R (version 3.6.1) using the *pheatmap* and *ggplotify* packages after data were z-scored.

3. Results

3.1. Effects of maternal glyphosate exposure on weight development in the offspring

Chronic exposure to Gly_{NOAEL} led to a significantly lower weight within the first three weeks in the female offspring and about the entire observation period in the male offspring (Supplementary Figure E2A). Female offspring of Gly_{NOAEL}-exposed dams showed significantly increased weight change (%) starting at week 7 compared with

offspring of control mothers. At 4 weeks of age, however, these mice catched up and had a similar weight compared to control mice. Male offspring had, similar to female offspring, a diminished weight compared to the controls but no catch up was observed and the male weights remained lower throughout the whole experiment (Supplementary Figure E2B). Maternal exposure to Gly_{ADI} did not affect the weight development in the F1 generation. Moreover, none of glyphosate concentrations used changed significantly fat or lean mass in the offspring of exposed dams (Supplementary Figure E2C).

3.2. Effects of maternal glyphosate exposure on behavior disorders of the offspring

To assess whether maternal exposure to glyphosate has an impact on neurobehavior in the offspring we performed three behavioral tests including OF, DL and SOC. Exposure of dams to Gly_{NOAEL} significantly reduced the following activity paramaters in the female offspring: distance, locomotion speed, locomotion time and the number of steps compared to control animals measured in the OF (Figure 1A-E). Furthermore, also anxiety-related parameters like center activity and center visits were significantly reduced in the female offspring from Gly_{NOAEL}-exposed dams within the OF test, and the number of transitions to the light compartment in trend as measured by the DL test (Figure 2A-E). In the male offspring of Gly_{NOAEL}-exposed dams, only the locomotion speed during the OF test was reduced, the other activity as well anxiety-related parameters were not affected (Fig. 1 F-J, Fig. 2F-J). Maternal exposure to the lower Gly_{ADI} concentration significanty reduced the number of center visits, but had no further effects on the activity nor on anxiety-related parameters in female offspring. For sociability, female offspring from Gly_{NOAEL}-exposed dams showed a reduced interest for a social partner in the SOC test compared to the control mice (Figure 3A, B),

something that was not observed for the male offspring (Figure 3E, F). In addition, no difference in preferring the old SP1 or new SP2 was observed comparing the female or male offspring from glyphosate-exposed dams to their respective un-exposed controls (Figure 3C, D, G, H). Similarly, maternal exposure to Gly_{ADI} was without any effects on offspring's behavior.

3.3. Effects of maternal glyphosate exposure on expression of behavior-relevant genes and serotonin and cytokine levels in the offspring's brain

To investigate whether the exposure to Gly_{NOAEL} during pregnancy and the lactational period induced changes in the expression of behavior-relevant genes in the brain of the F1 generation we isolated RNA from hippocampus of 3-week-old mice directly after weaning. Gene expression analysis was performed for 20 genes that have been described to be related to neurobehavior (Supplementary Table E3). In female offspring of Gly_{NOAEL}-exposed dams, we observed a significant reduced expression of trypthophan hydroxylase 2 mRNA (*tph2*- the rate-limiting enzyme in cerebral serotonin synthesis) while in the male offspring no significant changes could be detected (Figure 4A,B). Furthermore, two regulatory CpG regions for *tph2 c*ould be identified and the percentage methylation levels were evaluated in hippocampus samples of 3-week-old female offspring. Here, a significant hypermethylation in *tph2* was identified after maternal glyphosate exposure (Figure 4C). An additional analysis of serotonin protein levels in the hippocampus of 12-week-old female offspring revealed reduced serotoinin without reaching significance level (Figure 4D). Interestingly, inflammatory cytokines IL-6 and TNF-α were increased in the hippocampus of 12-week-old and IL-6 already in the 3-week-old female offspring from Gly_{NOAEL}-exposed dams compared to control mice (Figure 4E).

3.4. Effects of glyphosate exposure on gut microbiota

Since recent advances demonstrated the importance of the the gut microbiota for the development of neurobehavior disorders (Sharon et al., 2016) and glyphosate is supposed to induce alterations in microbiota (Rueda-Ruzafa et al., 2019), we have compared the bacteria community structure in the 3-week-old female offspring from glyphosate-exposed dams directly after weaning with the female offspring from control mice using 16S rRNA gene profiling of caecum samples. While alpha-diversity of the gut microbiome (Figure 5A) as well as the relative abundance of bacterial families (Figure 5B) remained unaffected in the female offspring by maternal glyphosate exposure, beta-diversity analyses indicated that glyphosate exposure during pregnancy and lactation led to a significantly differentiated clustering of the samples in the offspring (Figure 5C). To characterize mechanisms which may explain the Gly_{NOAEL} effects on neurobehavior the relative abundance of the gut microbiota on genus level in female F1 offspring was investigated (Supplementary Table E2). Here, we observed a significantly increased relative abundance of *Alistipes, Blautia,* and *Lactobacillus* (CON vs. Gly_{NOAEL}) while *Akkermansia and Parabacteroides* showed a decrease in relative abundance at the genus level in the juvenile offspring after maternal Gly_{NOAEL} exposure compared to the offspring from control dams (Figure 5D).

To determine if the changes oberserved in the F1 generation constitue a mere transmission of the maternal microbiota, we also analyzed the dams' microbiota composition (Supplementary Table E3). Interestingly, the relative abundance of *Blautia* was similarly altered in the Gly_{NOAEL}-exposed dams and their offspring. Apart from that, Gly_{NOAEL} expsosure of dams only led to a reduced relative abundance of *Enterococcus* and was without any other effects on genus level at this concentration.

3.5. Effects of glyphosate exposure on serum metabolites in the offspring

To investigate whether maternal glyphosate exposure has an impact on the offspring´s metabolic response, metabolites were measured in serum samples of 3-week-old female offspring in a targeted metabolomics approach. The metabolite profile of acylcarnitines, glycerophospholipids and sphingolipids showed a significant downregulation of glutarylcarnitine, two sphingolipids (C16:1, C18:1), and various phosphatidylcholines (C36:4-6, C38:5) within the Gly_{NOAEL} group (Figure 6A,B). In contrast, analysis of biogenic amines and amino acids yielded no significant alterations except for an increase in creatinine for Gly_{NOAEL}-exposed female offspring compared to control (Figure 6C,D). Interestingly, serum endotoxin levels were significantly increased in the 3-week-old female offspring from Gly_{NOAEL}-exposed dams (Figure 6E).

4. Discussion

The impact of glyphosate on human health has been a topic of great interest and discussed controversely in recent years. While studies investigating adverse health effects of glyphosate have long been focused on glyphosate's potential carcinogenicity, current epidemiological data also suggest an association between an exposure to GBH and neurobehavioral problems (He et al., 2022; Hyland et al., 2021; von Ehrenstein et al., 2019). However, most of the epidemiological and experimental studies focused on an exposure scenario in an occupational context with high glyphosate concentrations. Nevertheless, similar to studies using GBH (450 mg/kg bw/day) (Barbosa et al., 2022; Ren et al., 2019) we also observed a significantly lower weight within the first three weeks in the female and male offspring of Gly_{NOAEL} (50 mg/kg bw/day)-exposed dams. The later significantly increased percentage weight gain may be explained by glyphosate's potential effects on adiposity (Amato et al., 2021; Barbosa et al., 2022). The absolute weight not reaching the same level as controls may be explained by glyphosate simultaneously reducing muscle weight and bone density as shown before

(Barbosa et al., 2022; Diaz-Martin et al., 2021; Hamdaoui et al., 2020). As the main outcome in focus of the present study, we showed that low-dose glyphosate exposure during pregnancy and the lactational period induced depression- and anxiety-like behavior and social deficits in the female offspring. During the OF test, particularly female offspring releaved decreased activity levels in terms of a reduction of speed and distance, indicating a reduced exploratory behavior, which can be interpreted as depression-like symptoms (Becker et al., 2021). Parameters contrasting whereabouts in the center *vs* the periphery, like percentage time spent in the center and visits to the center or time spent in the periphery, are generally considered indications for explorative or anxious behavior (Belovicova et al., 2017). Since we saw a decrease in these measures for maternally exposed offspring, an anxiety-like phenotype can be concluded. Reduced activity levels and stays in a lit compartment are commonly related to anxiety disorders during the DL test, which further compounds the interpretation of an anxiety phenotype within the glyphosate offspring (Takao and Miyakawa, 2006). Generally, reduced social approach is regarded as a central symptom of autism-spectrum disorder, which is a parameter we observed in the female offspring maternally exposed to the glyphosate NOAEL concentration, allowing for the cautious interpretation of an autism-like phenotype (Lo et al., 2016).

In our study, the behavioral alterations were primarily observed in the female offspring of Gly_{NOAEL}-exposed dams, although also the male offspring showed a trend to an anxiety-like behavior in some parameters which could potentially reach significance by an increased sample size. However, several *in-vitro* and animal studies might be of interest that indicate endocrine disrupting properties of glyphosate like interfering with estrogen receptor-α activity (de Araújo-Ramos et al., 2021; Mesnage et al., 2017) that might explain sex-specific effects. Otherwise, there is a lack of consensus within the

scientific community whether or not glyphosate is to be considered an endocrine disrupting chemical (EDC). To this day, no international institution has officially classified glyphosate as an endocrine disruptor (de Araújo-Ramos et al., 2021). In our studies, we did not investigate the binding of glyphosate to hormone receptors. Further, while we used glyphosate to expose pregnant mice, in epidemiological studies an effect for the unknown additional components of commercially available glyphosate preparations can not be excluded.

It is important to state that the glyphosate effects on offspring's behavior addressed here were observed using the NOAEL concentration, a dosis that actually should have no adverse impact. However, there are also other studies describing that maternal exposure to low-dose glyphosate concentrations can lead to adverse health outcomes like a disturbed reproduction (Guerrero Schimpf et al., 2021; Kubsad et al., 2019; Rossetti et al., 2021) or an altered immune response in the next generations (Buchenauer et al., 2022). There are two aspects that are rarely addressed by regulator when classifying substances according to their harmful potential. First, studies conducted for regulation of chemicals mostly investigate short term toxicity but not the long-term effects of low-dose exposure in complex organisms. Second, studies often do not consider the impact on vulnerable populations, during critical life periods or transgenerational effects.

The *in-utero* environment is characterized by rapid cell division and organ growth in the developing fetus and is therefore particularly susceptible to certain low-dose chemical exposures that would otherwise not exert adverse effects in children or adults (Maddalon et al., 2021; Peillex and Pelletier, 2020). In this context, it has been shown that glyphosate can pass the placental barrier, is present in amniotic fluid and placenta

and is also found in breastmilk (Muñoz et al., 2021; Serra et al., 2021). Glyphosate has long been believed to be harmless in humans, as it targets the 5-enolpyruvylshikimate-3-phosphate synthase (ESPS) - an enzyme which exists in plants and bacteria only and not in human cells. However, the human gut consists of a wealth of bacteria that are dependent on ESPS (Marques et al., 2007). Therefore, it has been proposed that, mechanistically, glyphosate may exert its adverse effects by causing dysbiosis in the gut microbiome and thus inducing further changes, likewise in the gut-brain crosstalk (Aitbali et al., 2018; Maddalon et al., 2021). Here, we observed a decreased abundance of *Akkermansia* in the gut of female F1 offspring of Gly_{NOAEL}-exposed dams, that showed anxiety- and depression-like sypmtoms. Several mouse studies have demonstrated that a reduced relative abundance of *Akkermansia* in the gut correlates with anxiety- and depressive-like behavior (Ding et al., 2021; McGaughey et al., 2019). Moreover, the abundance of *Akkermansia* was also significantly decreased in ulcerative colitis patients with depression (Chen et al., 2021). Furthermore, maternal Gly_{NOAEL} exposure led to an increased abundance of *Alistipes* and *Blautia* in the offspring's gut microbiota. While the relative abundance of *Blautia* was similarly affected in directly exposed dams, all other changes on the gut microbiota observed in the female offspring were not detectable in the dams. Therefore, a simple transmission of the dams' microbiota might not be the sole reason for the Gly_{NOAEL}-induced alteration in the offspring's microbiota. There might be e.g. a direct glyphosate effect on the neonates via breastmilk that can shape the microbiota (Qi et al., 2022). *Alistipes* and *Blautia* both have been shown to be elevated in patients with anxiety and depression (Chen et al., 2018; Jiang et al., 2018; Zhu et al., 2021). Interestingly, *Akkermansia*, *Alistipes* and *Blautia* are all associated with an impaired tryptophan metabolism (Dhaliwal, 2019; Ding et al., 2021; Golubeva et al., 2017) as a dysregulation of tryptophan metabolites, in particular 5-hydroxytryptamin (5-HT, serotonin), plays a

central role in the pathogenesis of many neurologic and psychiatric disorders including anxiety and depression (Roth et al., 2021). An enzyme crucial for the serotonin synthesis is the tryptophan hydroxylase (Tph) with its isoform Tph2 exlusively expressed in neuronal cell types (Roth et al., 2021). Tryptophan is catalysed by Tph to form 5-hydroxytryptophan and then decarboxylased via tryptophan decarboxylase to form 5-HT (Bian and Wang, 2023). In the current study we found a significantly reduced Tph2 mRNA expression in the cerebellum and the hippocampus of the female offspring from Gly_{NOAEL}-exposed dams. Since it is known that DNA methylation can alter Tph2 expression and thereby affecting Tph2 enzyme activity and preventing Trp from being further catalysed into 5-hydroxytryptophan (Bian and Wang, 2023), we assessed DNA methylation of two CpG regions within Tph2 gene. The observed DNA hypermethylation might be responsible for the decreased Tph2 gene expression in the female offspring from Gly_{NOAEL}-exposed dams. Analysing the serotonin levels in the offspring's brain we detected a diminished concentration in the hippocampus of females from Gly_{NOAEL}-exposed dams, but this did not reach significance. Next to that, the levels of 2 SM (C:1, 18:1) and 4 PC (C36:4-6, C38:5) were significantly reduced in the serum of females from Gly_{NOAEL}-exposed dams compared to the control mice. This is in line with a Dutch family-based study showing an inverse correlation between SM and PC and depression- and anxiety-like symptoms (Demirkan et al., 2013). Increased serum creatinine levels as found in our study in 3-week-old offspring from Gly_{NOAEL}-exposed dams were only reported in context of an impaired kidney function that has been shown to be associated with depressive symptoms (Zhang et al., 2021). Although this correlation can be bidirectional leading to higher creatinine levels as response to behaviroal problems (Liu et al., 2022) we cannot fully exclude that glyphosate might have affected the kidney in the offspring since an association

between glyphosate exposure and changes in renal function or kidney injury has been shown before (Jayasumana et al., 2015).

Another possibility as to how an increased abundance of *Alistipes* might affect behavioral outcomes is the known production of lipopolysaccharide (LPS) (d'Hennezel et al., 2017) that can reach the bloodstream via an enhanced intestinal permeability as a result of an altered gut microbiota (Mangiola et al., 2016). LPS is known to cause systemic and psychiatric changes like depression-like behavior (Haba et al., 2012). LPS also stimulates the production of inflammatory cytokines in the brain which can alter the activity and the synthesis of neuropeptides, both of which are associated with psychiatric disorders like depression (Mangiola et al., 2016). In line with that, we found increased endotoxin concentrations in the serum of the juvenile female offspring from Gly_{NOAEL}-exposed dams and also higher levels of the proinflammatory cytokines IL-6 and TNF-α in the hippocampus in 12-week-old and for IL-6 also already in the 3-week-old female offspring. However, it should also be noted here that glyphosate might exert effects directly in the brain since this pesticide was shown to infiltrate there and increase pro-inflammatory cytokines like TNFα (Winstone et al., 2022).

Bacteria that promote the intestinal barrier integrity are *Parabacteroides* leading to beneficial effects in mouse models for inflammatory arthritis or colonic tumorigenesis (Koh et al., 2020; Sun et al., 2023). In parallel, in an experimental model for stress-induced anxiety and depression the relative abundance of *Parabacteroides* was significantly decreased (Jiang et al., 2023), something that we also observed in the female offspring from glyphosate-exposed dams.

In contradiction to the Gly_{NOAEL}-induced changes in the relative abundance of *Akkermansia, Alistipes, Blautia* and *Parabacteroides* that offer mechanistic explanations for the observed anxiety- and depression-like behavior the increased abundance of *Lactobaccillus* is difficult to interpret. Several experimental studies in

559 animals with anxiety-like bahavior could demonstrate anxiolytic effects after treatment
560 with *Lactobacillus* as a probiotic (Bharwani et al., 2017; Bravo et al., 2011). However,
561 a meta-analysis of clinical studies revealed that probiotics did not significantly reduce
562 symptoms of anxiety in humans (Reis et al., 2018). In this context, also possible
563 differences between an exposure to glyphosate as active substance or to glyphosate-
564 based herbicide products containing additional ingredients have to be evaluated to
565 clearly identify the risk driver for behavioral diseases being influenced by the gut-brain
566 axis.
567

568 **5. Conclusion**

569 Low-dose glyphosate exposure in a cross-generational mouse model resulted in dose-
570 dependent and sex-specific effects in the offspring. In particular, Gly_{NOAEL} exposure of
571 dams induced depression- and anxiety-like behavior and social deficits in the female
572 offspring, while maternal Gly_{ADI} exposure provoked no significant effects.The observed
573 behavioral issues might be caused by influencing the gut-brain-axis leading to an
574 impaired trpytophan metabolism and proinflammatory conditions in the brain. Further,
575 our data emphasize the urgent need to include studies on microbiome effects in health
576 risk assessments of pesticides.
577

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Zhu J, Li M, Shao D, Ma S, Wei W. Altered Fecal Microbiota Signatures in Patients With Anxiety and Depression in the Gastrointestinal Cancer Screening: A Case-Control Study. Front Psychiatry 2021; 12: 757139.

Figure legends

Figure 1: Effects of maternal glyphosate exposure on activity behavior in the offspring.

Shown are distance travelled (A, F), the locomotive speed (B, G), the percentage of global test period during which mice were active (C, H), the amount of steps taken (D, I) and the time mice spent rearing (E, J), all in female and male mice. Data are presented in violin plots - dashed line: median, dotted lines: quartiles, Welch's t-test or Wilcoxon-Mann-Whitney test, *p < 0.5, female: CON n = 10, Gly_{ADI} n = 14, Gly_{NOAEL} n = 10; male: CON n = 14, Gly_{ADI} n = 14, Gly_{NOAEL} n = 8

Figure 2: Effects of maternal glyphosate exposure on anxiety-related parameters in the offspring.

Shown are center activity (A, F), and center visits (B, G) derived from the OF test, as well as transitions to lit compartment (C, H), the distance travelled in the lit compartment (D, I) and the latency to enter the lit compartment (E, J) as measured in the DL test all in female and male mice. Data are presented in violin plots - dashed line: median, dotted lines: quartiles, Welch's t-test or Wilcoxon-Mann-Whitney test, *p < 0.5, female: CON n = 10, Gly_{ADI} n = 14, Gly_{NOAEL} n = 10; male: CON n = 14, Gly_{ADI} n = 14, Gly_{NOAEL} n = 8

Figure 3: Effects of maternal glyphosate exposure on social behavior in the offspring.

Presented are percentage time spent in each area during the first test phase (A, E), sociability index calculated as time spent with social partner 1 (SP1) divided by overall exploration time of either SP1 or novel object (NO, B, F), percentage time spent in

each area during test phase 2 (C, G), and social novelty index calculated as time spent with the second social partner (SP2) divided by overall exploration time of SP1 and SP2 (D, H). Data are shown as means ± SEM, Welch's t-test or Wilcoxon-Mann-Whitney test with *p < 0.5, female: CON n = 12, Gly_{ADI} n = 16, Gly_{NOAEL} n = 14; male: CON n = 9, Gly_{ADI} n = 13, Gly_{NOAEL} n = 11

Figure 4: Effects of maternal glyphosate exposure on behavior-relevant genes and serotonin and cytokine levels in the offspring's brain.

Depicted are the gene expression in the hippocampus of 3-week-old female (A) or male (B) offspring and the percentage of *Tph2* DNA methylation in 3-week-old female offspring (C), serotonin (D) and cytokine levels (E) in the hippocampus of females from Gly_{NOAEL}-exposed dams *vs.* control mice. Data are shown as volcano plots (plotted are fold changes *vs.* significance on the x and y axes respectively for all measured genes) or as box plots, Wilcoxon-Mann-Whitney, *p < 0.05, (A, B): n = 4, (C, D, E): n = 5

Figure 5: Effects of maternal glyphosate exposure on the gut microbiota in female offspring.

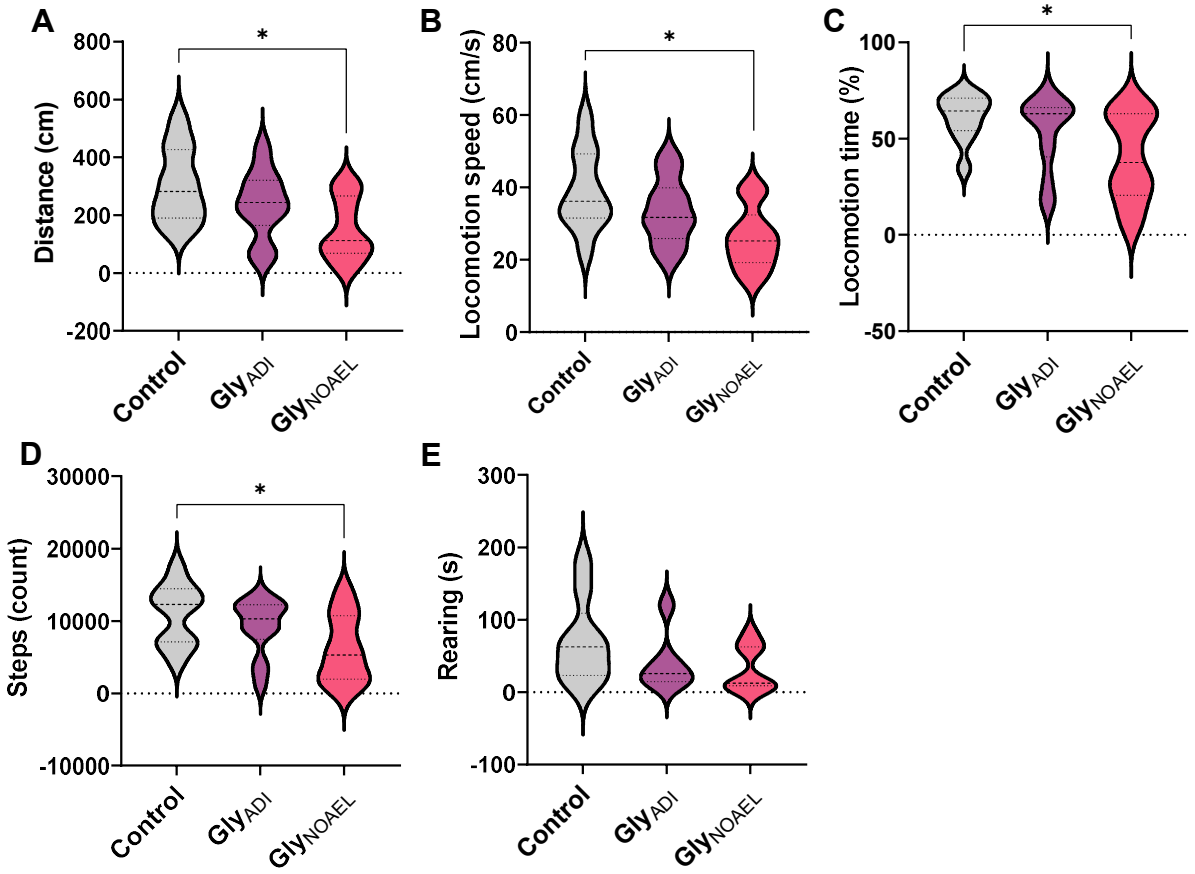
Alpha diversity (A), distribution of bacterial families (mean relative abundance) (B), Beta diversity (PERMANOVA) (C), and relative abundance of significantly affected bacteria on genus level (D) in caecum samples of female offspring from glyphosate-exposed dams are shown. Data are shown as violin blots, Wilcoxon-Mann-Whitney, *p < 0.05, n = 4 (CON), n = 5 (Gly_{ADI}, Gly_{NOAEL})

Figure 6: Effects of maternal glyphosate exposure on serum metabolites in female offspring.

Shown are the heatmap of relative abundances (z-scores) of Acylcarnitines, Glycerophospholipids & Sphingolipids (A) with significantly altered metabolites within the Gly_{NOAEL} group (B), the heatmap of relative abundances (z-scores) of Amino acids & Biogenic amines (C) with significantly increased creatinine levels in the Gly_{NOAEL} group (D) and serum Endotoxin concentration (E) all measured in 3-week-old female offspring from Gly_{NOAEL}-exposed dams compared to control animals . Shown are violin and box plots, Wilcoxon-Mann-Whitney, *$p < 0.05$, (A, B): CON n = 4, Gly_{ADI} n = 5, Gly_{NOAEL} n = 5; (C, D) CON n = 3, Gly_{ADI} n = 4, Gly_{NOAEL} n = 5; (E) n = 8

Figure 1

Activity female offspring



Activity male offspring

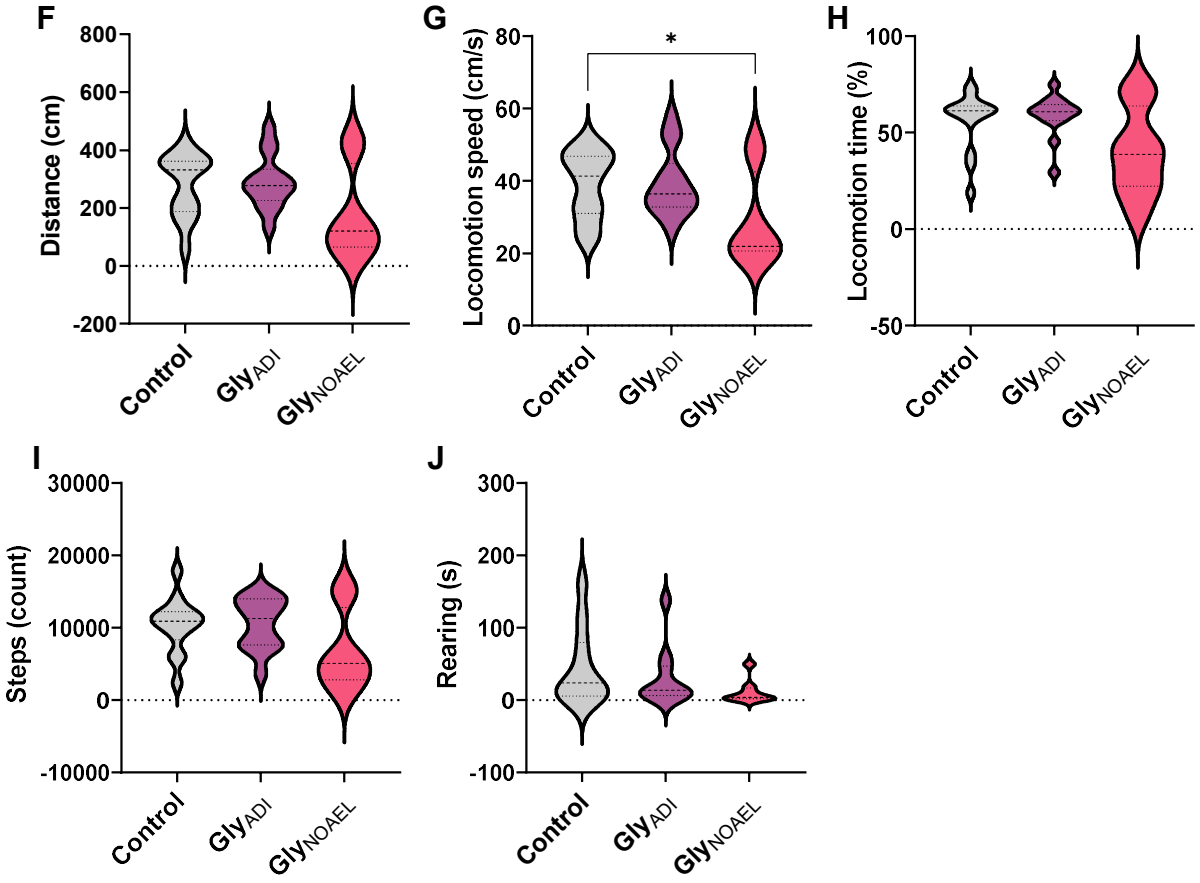


Figure 2

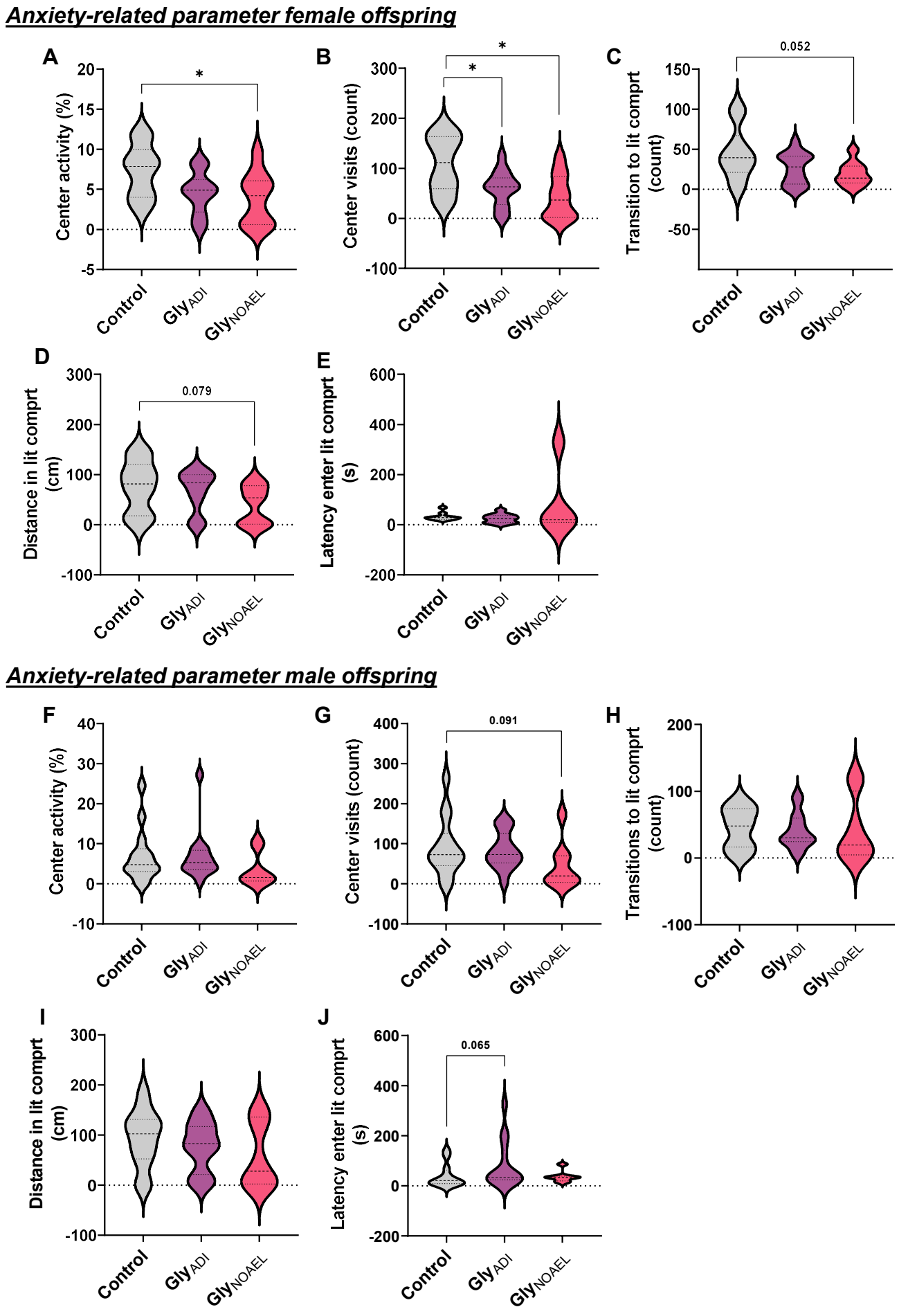
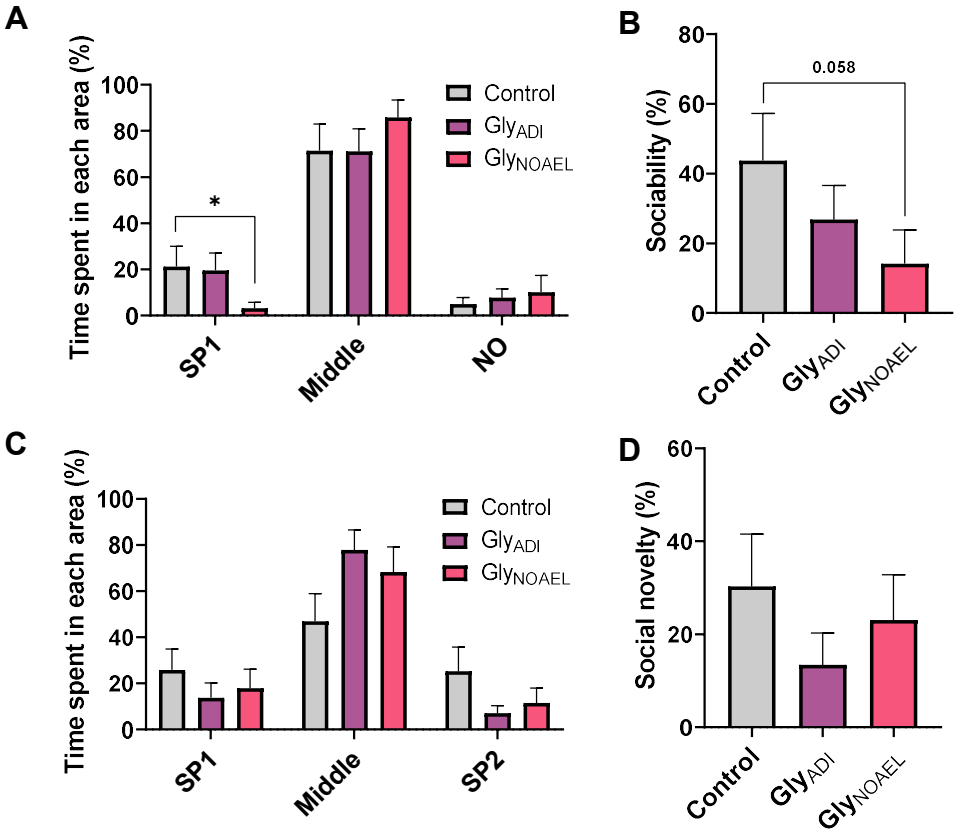

Anxiety-related parameter female offspring
A
Center activity (%)
20
15
10
5
0
-5
*
Control
GlyADI
GlyNOAEL
B
Center visits (count)
300
200
100
0
-100
*
*
Control
GlyADI
GlyNOAEL
C
Transition to lit comprt (count)
150
100
50
0
-50
0.052
Control
GlyADI
GlyNOAEL
D
Distance in lit comprt (cm)
300
200
100
0
-100
0.079
Control
GlyADI
GlyNOAEL
E
Latency enter lit comprt (s)
600
400
200
0
-200
Control
GlyADI
GlyNOAEL
Anxiety-related parameter male offspring
F
Center activity (%)
40
30
20
10
0
-10
Control
GlyADI
GlyNOAEL
G
Center visits (count)
400
300
200
100
0
-100
0.091
Control
GlyADI
GlyNOAEL
H
Transitions to lit comprt (count)
200
100
0
-100
Control
GlyADI
GlyNOAEL
I
Distance in lit comprt (cm)
300
200
100
0
-100
Control
GlyADI
GlyNOAEL
J
Latency enter lit comprt (s)
600
400
200
0
-200
0.065
Control
GlyADI
GlyNOAEL

Figure 3

Social behaviour female offspring



Social behaviour male offspring

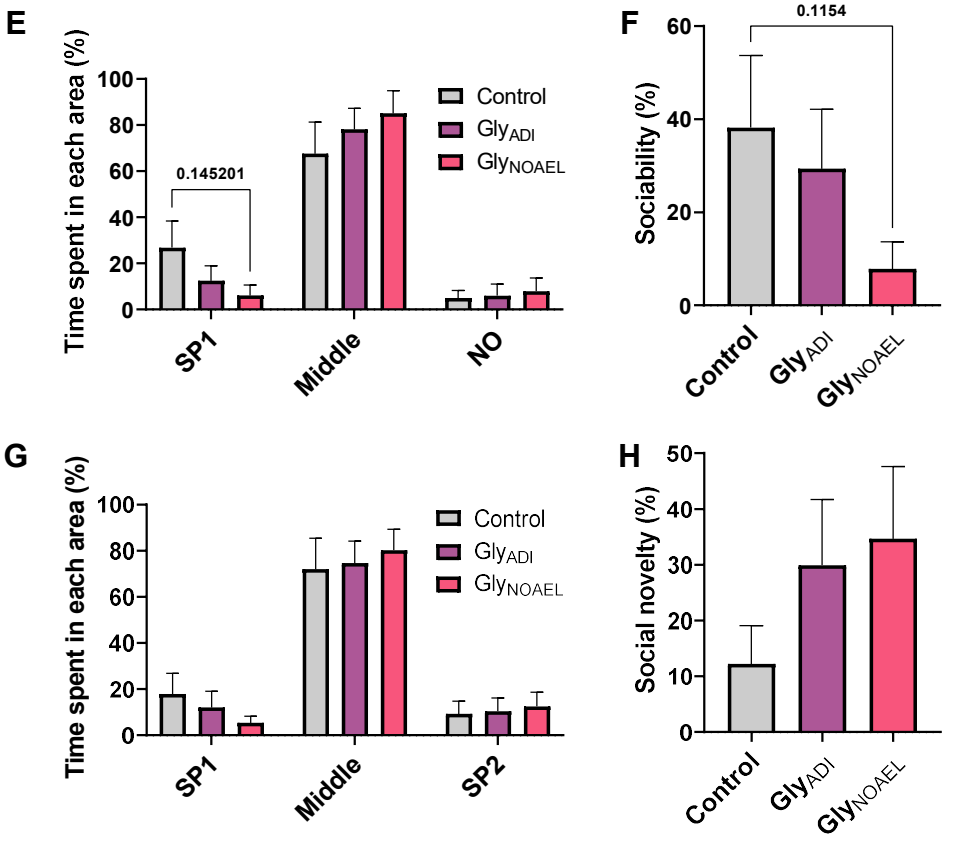


Figure 4

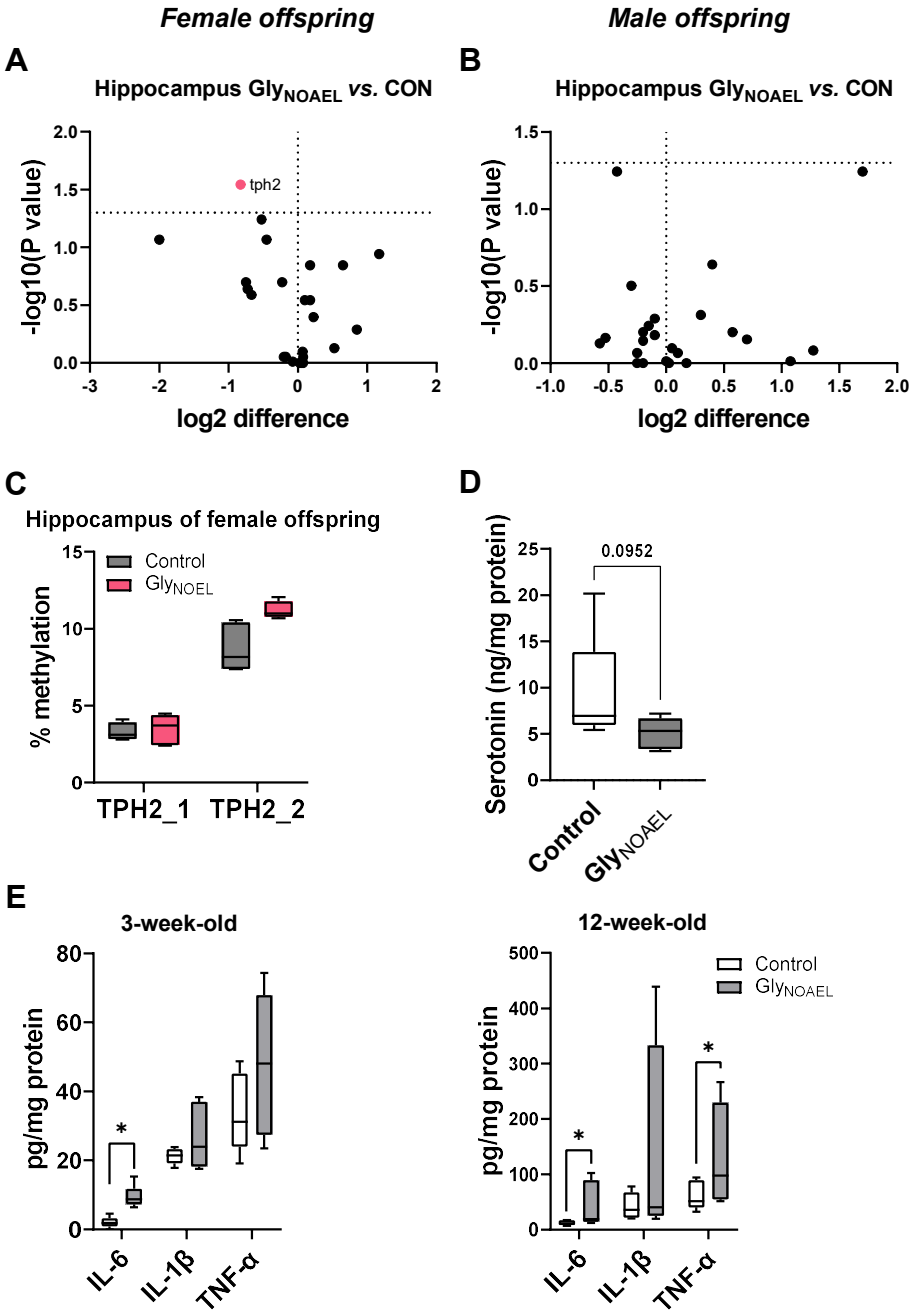

Female offspring
Male offspring
A
Hippocampus Gly$_{NOAEL}$ vs. CON
-log10(P value)
log2 difference
tph2
B
Hippocampus Gly$_{NOAEL}$ vs. CON
-log10(P value)
log2 difference
C
Hippocampus of female offspring
% methylation
Control
Gly$_{NOEL}$
TPH2_1
TPH2_2
D
Serotonin (ng/mg protein)
0.0952
Control
Gly$_{NOAEL}$
E
3-week-old
pg/mg protein
IL-6
IL-1β
TNF-α
12-week-old
pg/mg protein
Control
Gly$_{NOAEL}$
IL-6
IL-1β
TNF-α

Figure 5

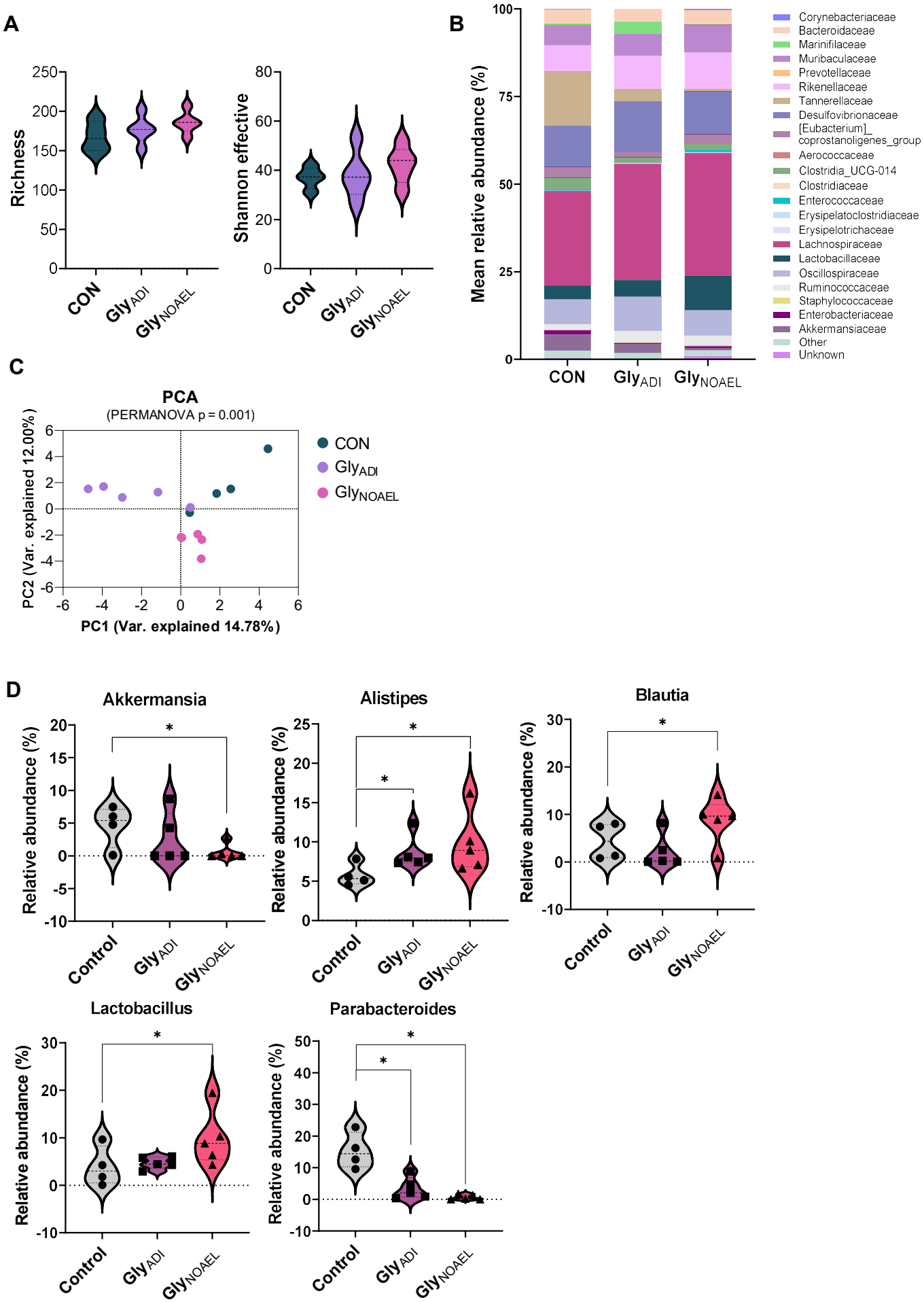
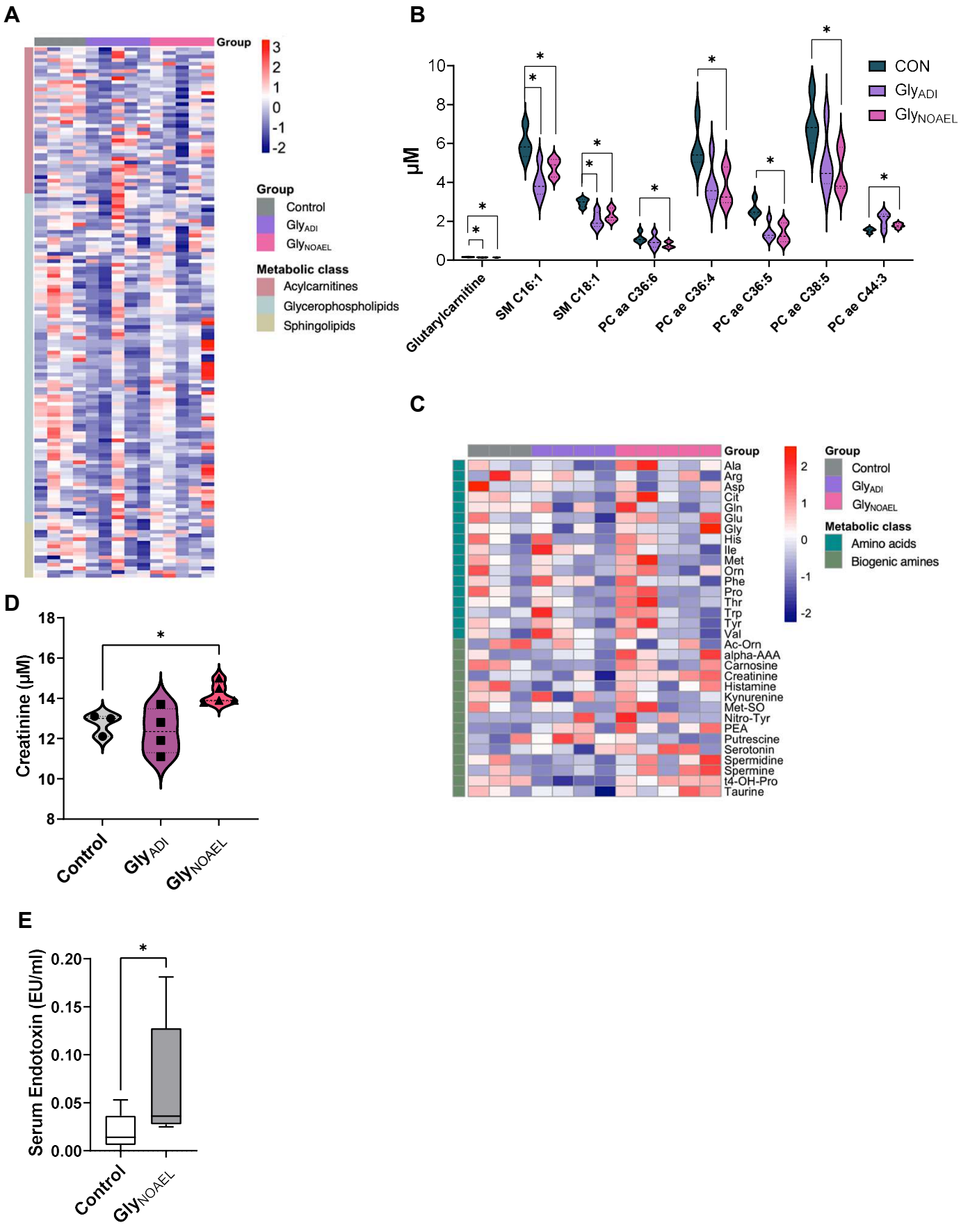
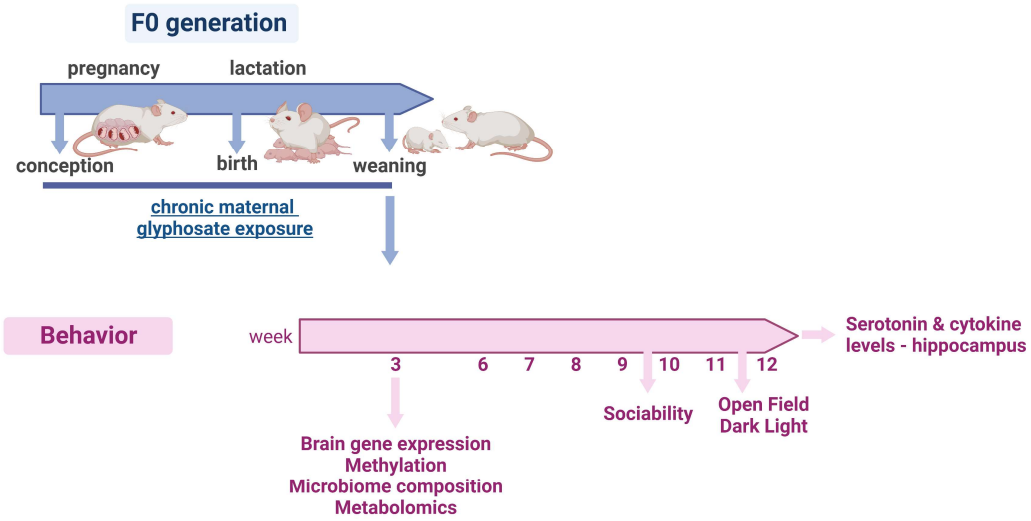

A
Richness
Shannon effective
CON
GlyADI
GlyNOAEL
B
Mean relative abundance (%)
Corynebacteriaceae
Bacteroidaceae
Marinifilaceae
Muribaculaceae
Prevotellaceae
Rikenellaceae
Tannerellaceae
Desulfovibrionaceae
[Eubacterium]_coprostanoligenes_group
Aerococcaceae
Clostridia_UCG-014
Clostridiaceae
Enterococcaceae
Erysipelatoclostridiaceae
Erysipelotrichaceae
Lachnospiraceae
Lactobacillaceae
Oscillospiraceae
Ruminococcaceae
Staphylococcaceae
Enterobacteriaceae
Akkermansiaceae
Other
Unknown
C
PCA
(PERMANOVA p = 0.001)
PC2 (Var. explained 12.00%)
PC1 (Var. explained 14.78%)
CON
GlyADI
GlyNOAEL
D
Akkermansia
Alistipes
Blautia
Lactobacillus
Parabacteroides
Relative abundance (%)
Control
GlyADI
GlyNOAEL

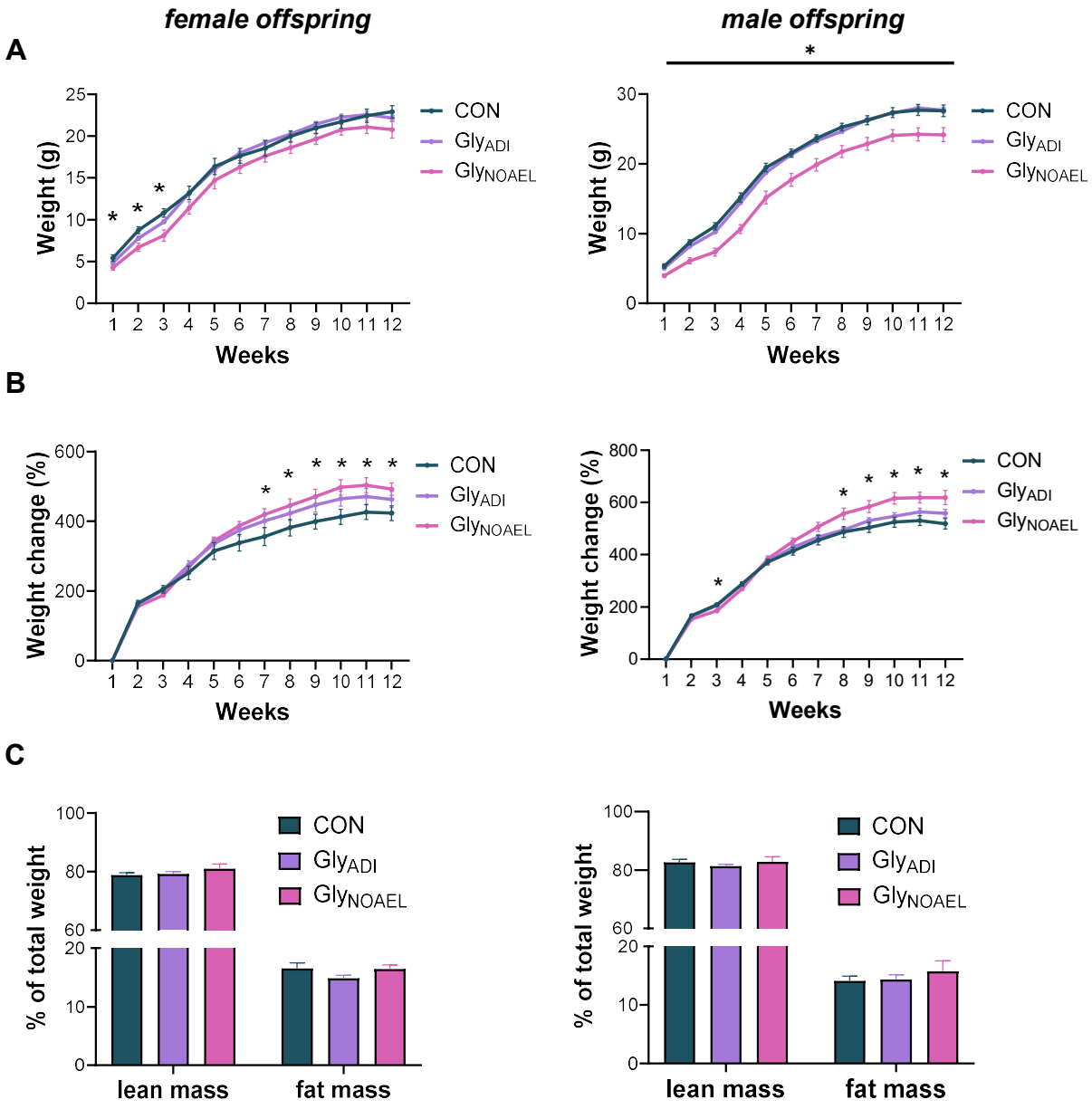
Figure 6


A
Group
3
2
1
0
-1
-2
Group
Control
Gly$_{ADI}$
Gly$_{NOAEL}$
Metabolic class
Acylcarnitines
Glycerophospholipids
Sphingolipids
B
μM
10
8
6
4
2
0
CON
Gly$_{ADI}$
Gly$_{NOAEL}$
Glutarylcarnitine
SM C16:1
SM C18:1
PC aa C36:6
PC ae C36:4
PC ae C36:5
PC ae C38:5
PC ae C44:3
C
Group
Ala
Arg
Asp
Cit
Gln
Glu
Gly
His
Ile
Met
Orn
Phe
Pro
Thr
Trp
Tyr
Val
Ac-Orn
alpha-AAA
Carnosine
Creatinine
Histamine
Kynurenine
Met-SO
Nitro-Tyr
PEA
Putrescine
Serotonin
Spermidine
Spermine
t4-OH-Pro
Taurine
2
1
0
-1
-2
Group
Control
Gly$_{ADI}$
Gly$_{NOAEL}$
Metabolic class
Amino acids
Biogenic amines
D
Creatinine (μM)
18
16
14
12
10
8
Control
Gly$_{ADI}$
Gly$_{NOAEL}$
E
Serum Endotoxin (EU/ml)
0.20
0.15
0.10
0.05
0.00
Control
Gly$_{NOAEL}$

Supplementary Figure E1



Supplementary Figure E2



Supplementary Figure E3