Neurotoxicology 74 (2019) 121–131

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

Maternal glyphosate-based herbicide exposure alters antioxidant-related genes in the brain and serum metabolites of male rat offspring

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ARTICLE INFO

Keywords:
Glyphosate-based herbicide
Antioxidant enzymes
Oxidative stress
Inflammation
Gene expression
Metabolomics

ABSTRACT

In response to the rapid development of genetically engineered glyphosate-tolerant crops, the use of glyphosate-based herbicides (GBHs), in agriculture, has increased substantially. Currently, it is estimated that 747 million kg of GBHs are applied per year. Although several epidemiological studies have demonstrated that there are health risks associated with GBH exposure, the effects these chemicals have on the oxidative and inflammatory response in the brain are still unclear. In fact, alterations in these processes could contribute to the development of neurological diseases, such as Alzheimer's disease and autism spectrum disorders. The present study exposed pregnant rats to GBH and evaluated changes in the expression of genes related to oxidnte defense and inflammation response and monitored the serum metabolome in the adult male offspring. Pregnant Wistar rats were administered distilled water or Roundup®, at either 5 and 50 mg/kg/day, (p.o.) from gestational day (GD) 18 to postnatal day (PND) 5. There was a significant increase in the gene expression levels of Neuroglobin (Ngb – oxygen storage and tissue protection) (105%, $p = 0.031$), Glutathione Peroxidase 1 (Gpx1 – oxidative stress) (95%, $p = 0.005$), Prostaglandin-Endoperoxidase Synthase 1 (Ptgs1 - inflammation) (109%, $p = 0.033$) and Hypoxia inducible factor 1 subunit alpha (Hif1α – oxygen sensor) (73%, $p = 0.017$), in the cerebellum of PND90 rats perinatally exposed to 50 mg GBH/kg/day. Moreover, both GBH-exposed groups displayed a significant decrease in the expression of Catalase (Cat – oxidative stress) (49%, $p = 0.003$; and 31% $p = 0.050$, respectively) expression, in the cortex. Serum metabolites analyses, from the same animals of each group, demonstrated that there were significant changes in the concentrations of lysophosphatidylcholine and phosphatidylcholine, which have been associated with neurodegenerative diseases. The results of the present study suggest GBH exposure during pregnancy alters the expression of genes associated with oxidant defense, inflammation and lipid metabolism. It is plausible that maternal GBH exposure could have lasting neuronal effects on the offspring later in life.

1. Introduction

Glyphosate-based herbicides (GBH), from which Roundup® is the principal representative, are the most commonly used agricultural products (Myers et al., 2016; Romano et al., 2012), and the most highly produced herbicides worldwide (Guyton et al., 2015). The role these compounds play as endocrine disruptors has been studied extensively. It is known that GBH affects aromatic amino acid metabolism through

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https://doi.org/10.1016/j.neuro.2019.06.004
Received 14 December 2018; Received in revised form 31 May 2019; Accepted 14 June 2019

the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase, which is lethal to the majority of plants (James, 2014). A variety of crops such as, soybeans, corn, cotton, canola, alfalfa and sugar beets (Green, 2016) have been genetically engineered to be glyphosate-tolerant, and these crops have been gradually increasing their the market share. In fact, current worldwide agricultural GBHs applications has risen to an estimated 747 million kg per year (Benbrook, 2016). Due to the fact that GBHs have the potential to contaminate the air, soil and water sources, the risk of human exposure to these chemicals is significantly increased.

Although previous studies have suggested that there are no harmful health effects associated with GBH exposure (Boocock and Coggins, 1983; Williams et al., 2012), there are several epidemiological studies demonstrating that GBH exposure is associated with human health risks (Aris and Leblanc, 2011; Benachour et al., 2007; Curwin et al., 2007; McQueen et al., 2012; Richard et al., 2005), including cancer (Malagoli et al., 2016; Myers et al., 2016), and the development of acute myeloid leukemia (Andreotti et al., 2018). Moreover, it has also been reported that a low dose GBH exposure, during the embryonic period, may have persistent effects even after the termination of the exposure, suggesting that these herbicides could be the basis for the manifestation of certain diseases in adulthood (Diamanti-Kandarakis et al., 2009; Schug et al., 2011). Previous work has also shown that GBHs can act as antioxidant defense system disruptors, resulting in lipid peroxidation and oxidative stress in the livers of both pregnant rats and their fetuses (Beuret et al., 2005). However, the cellular and metabolic consequences of GBH exposure, in the brain, have yet to be elucidated.

The glyphosate salt level reported as a limiting factor in long-term rat toxicity tests is 350 mg / kg bw / d (Germany Rapporteur Member State, 2015). Moreover, Niemann et al. (2015), proposed that the low-observed-adverse-effect level (LOAEL) that should be considered is 350 mg / kg bw / d, whereas the NOAEL should be set at 175 mg / kg bw / d. It was detected in human urine the approximately amount of 0.1–3.3 ug/kg bw/d (Niemann et al., 2015). It should be pointed out that animal doses do not accurately represent the environmental GBH-exposure levels in humans, which occur in quantities well below those of experimental animal models (Mesnage et al., 2015; Niemann et al., 2015). Additionally, humans are exposed to commercial formulations, which often contain other ingredients in the final formulation, that could potentially alter the toxicity (Owagboriaye et al., 2019).

Due to the overproduction of reactive oxygen and nitrogen species (ROS and RNS) that occurs during oxidative stress, interactions among cellular macromolecules are disrupted and signal transduction pathways are disturbed (Lenaz, 2012). In the brain, ROS are involved in a variety of cellular functions, including cell death and survival, however, due to the high metabolic rate and abundant supply of metals, the brain is also an ideal target for free radical attack (Ter-Minassian and Ter-Minassian, 2006). Biological and chemical oxidants have the potential of reacting with and damaging macromolecules, such as proteins, DNA and lipid membranes (Renis et al., 1996). To prevent oxidative damage, organisms have evolved enzymatic antioxidant defense systems, which are mainly represented by the enzymes superoxide dismutase 1 and 2 (SOD1 and SOD2), catalase (CAT), glutathione peroxidase (GPx1) and glutathione reductase. In a highly synchronized and cooperative manner these enzymes function to detoxify ROS and maintain a healthy redox state (Bayir, 2005; Choi, 1993).

Failure to maintain this redox balance can negatively impact the proper functioning of the nervous system, which is a well-vascularized organ that consumes 20% of the total oxygen (O_2). Importantly, O_2 consumption is essential for cellular metabolism and mitochondrial adenosine triphosphate (ATP) production. The antioxidant defense system ensures proper cell maintenance and activity, and prevents a breakdown of and damage to the central nervous system (Ziyin, 1998). Therefore, the structures that make up this system must be well established, and capable of receiving and sending information to other parts of the body (Zhang et al., 2002). Nevertheless, during ATP production, the formation of ROS and RNS inevitably occurs. If not controlled, the cell can enter a state of oxidative stress, which often associated with the oxidation and damage of lipids, proteins, nucleic acids, and other macromolecules (Hayashi et al., 2012). Oxidative stress also has an influence on the overall inflammatory response and can activate inflammatory reaction pathways that lead to a metabolic overproduction of ROS, further depleting the host antioxidant defenses. In fact, during the inflammatory response, there is a high consumption of oxygen and release of superoxide radicals (O_2^{-}).

In this context, globin proteins, which are present in different tissues of vertebrates, function as respiratory proteins. These small globular proteins have the ability to bind O_2 and deliver it to tissues (Wittenberg and Wittenberg, 2003). Currently, there are four heme proteins that have been shown to function as O_2 transporters. Hemoglobin (Hb), the primary component of red blood cells, transports O_2 from the respiratory surface to all tissues (Dickerson and Geis, 1983). Myoglobin, a protein found in skeletal and cardiac muscle tissues (Souza et al., 2016), stores and delivers O_2 to the mitochondria, and also plays an important role in nitric oxide (NO) detoxification (Wittenberg and Wittenberg, 2003). The two remaining globins, neuroglobin (Ngb) and cytoglobin (Cygb), were first identified and described in the early 2000s (Burmester and Hankeln, 2004; Burmester et al., 2002), and have been extensively studied since their discovery.

Both Ngb and Cygb can provide oxygen to the respiratory chain, supporting the production of ATP, and are involved in the detoxification of ROS and RNS, thus protecting neurons from irreversible oxidative injury (Brunori and Vallone, 2007; Fordel et al., 2007; Jin et al., 2010). These globins are expressed in the neurons of mice and rats (Fordel et al., 2004) as well as in humans (Xue et al., 2017), and carry oxygen in a manner similar to that of Hb and Mb (Fordel et al., 2004; Moens and Dewilde, 2000). Under hypoxic conditions, there is an increased expression of Cygb in the brain tissue of rats, which is induced by transcription factor hypoxia inducible factor 1 alpha (HIF1α) (Moeller et al., 2005).

Due to the high vascularization and neuronal activity, the cerebellum and cortex have been reposted to be highly susceptible to oxidative stress (Dellon et al., 2016, 2017; Kim and Byzova, 2014; Sugashi et al., 2014). Furthermore, it is known that genetic and environmental factors can interfere with the development and functioning of the cortex (Uzquiano et al., 2018). Environmental factors can also influence cerebellar functions, resulting in abnormal activity in this region and adjacent tissues (Hopfner and Helmich, 2018).

Based on these features, the present study evaluated the expression of genes related to oxidative stress and inflammation in the cortex and cerebellum of male rat offspring following perinatal GBH exposure. Serum metabolomics were also performed, with the intention of determining whether, changes in metabolite concentrations could be correlated with modifications in gene expression. Overall, the results suggest that maternal GBH exposure may play a role in irreversible neural damage later in the lives of their offspring.

2. Material and methods

2.1. Animals, experimental design and treatment

For this study, adult (90 days old) female Wistar rats (Rattus norvegicus) were bred with adult males, in breeding pairs consisting of one female and one male. The conception day or gestational day 1 (GD1) was determined by vaginal smear, with the rats having spermatozoa present in their smear being considered affirmative for gestation. The gestating animals were randomly divided into three groups (8 animals per group), which were administered water (control), 5 mg/kg per day (GBH 5) or 50 mg/kg per day (GBH 50) of Glyphosate Roundup® Transorb (Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, São Paulo, Brazil). The doses used in this study were the same as those employed in de Souza et al. (2017), in which Roundup® induced toxic thyroid axis effects. The GBH suspension was administered once a day, by oral gavage, to the pregnant rats from GD18 until the birth, and after giving birth the mothers continued receiving the treatments until the postnatal day 5 (PND5). The control group was only administered water, by gavage (de Souza et al., 2017; Romano et al., 2010, 2012).

The offspring were maintained at eight pups per female (4 males and 4 females), totaling 32 males per control or experimental group, until weaning, at PND21. Following weaning, the male offspring animals were subdivided into groups. The rats were fed standard rat chow and water ad libitum under dark/light cycle (12:12 h), in a temperature-controlled room (23 ± 1 °C).

At PND90 the male animals were euthanized and the cortex and cerebellum were collected for RNA extraction and Real – Time PCR (RT-PCR) analysis, and the blood was collected from the trunk (approximately 3 mL of total blood and 1,5 mL of serum), for serum separation and metabolomics analysis.

The experimental procedures were performed in accordance with the Brazilian College of Animal Experimentation and were approved by the Bioethical Commission at the Universidade Federal de São Paulo (protocol number 5097101316).

2.2. Network analysis

Genetic and physical interactions were investigated using the Esyn software, which is a free, open source tool for modeling biological networks and was configured to utilize the Biogrid Homo sapiens database. Additionally, it was also configured to perform genetic analyses and identify potential physical interactions from the list of tested genes. The genes were also subjected to protein-protein interaction network analysis using the STRING database (http://string-db.org). It provides direct (physical) and indirect (functional) associations by considering hierarchical and self-consistent ortholog annotations for all interacting proteins, and groups the proteins into families at various levels of phylogenetic resolution.

2.3. RNA extraction, and quantitative RT-PCR (qRT-PCR)

For total RNA extraction, the cortex and cerebellum were homogenized in TRIzol® solution (Invitrogen, Carlsbad, CA, USA), following the protocol of the manufacturer. After extraction of the total RNA, it was quantified, spectrophotometrically, using a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE, USA). Two μg of the total RNA were reverse transcribed using the M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Relative gene expression was analyzed by performing quantitative real-time PCR (qRT-PCR) using the SYBR Green master mix (Applied Biosystems, Foster City, CA, USA). Relative gene expression levels were determined by $2^{\Delta\Delta Ct}$ (Dussault and Pouliot, 2006), and all values were compared with the expression of the housekeeping gene cyclophilin A. For the Real Time qPCR, samples were run without cDNA, containing only water, primer and enzyme (negative control). All primers were designed to bind to sequences between two exons. The list of analyzed genes and their respective primers are presented in Supplementary Table 1.

2.4. Metabolomics extraction and identification

The metabolomics analysis profile was performed with the serum of five rats from each group: control, GBH 5 and GBH 50. The analytical tests were performed at the Innsbruck Medical University facility, in Austria, using a targeted metabolomics approach, which employed a combination of direct flow injection and liquid chromatography, as previously described by de Souza et al. (2017). Briefly, the AbsoluteIDQ p180 kit (Biocrates Life Sciences AG, Innsbruck, Austria) and MS/MS were used to quantify up to 188 endogenous metabolites from different compound classes. The concentration of each sample is stated in micro Molar (μM). Besides the comparative study among the studied groups we also compared animals exposed to GBH divided into three groups based on ΔΔCt levels of the studied genes.

2.5. Metabolomics statistical data analysis

The MetaboAnalyst v3.5 software (www.metaboanalyst.ca) was used to analyze the metabolomics analysis profile data (Xia et al., 2015). The data type was defined as “concentrations”. Data were normalized using the values obtained from the pooled control group and generalized by log transformation. To determine the statistical significance, the normalized data were subjected to a parametric *t*-test (group-variance equal) with an adjusted p-value (FDR) cutoff of 0.05. The volcano plots were constructed by combining the fold change alterations (threshold = 2.0), followed by statistical *t*-tests. Samples were defined as unpaired, the X-axis was defined as log scale (FC), the Y-axis was defined as -log10 (p-value), and the analysis was based on the FDR adjusted p-values (threshold = 0.1). The controls were then compared to the GBH-exposed samples (GBH 5 and GBH 50), and indicated that the group variance was equal. For correlating gene expression data with metabolite data the PatternHunter software was employed. The pattern is specified as a series of numbers separated by "-". Each number corresponds to the expected gene expression pattern in the corresponding group. For example, a 1–2-3-4 pattern is used to search for features that increase linearly with time in a time-series data with four-time points (or four groups). The order of the groups is given as the first item of the predefined patterns. The distance measure was Pearson r. A predefined profile was used at the order Control – Treated (GBH 5 and GBH 50). The results shown are only for the compounds that displayed a 1–2 correlation pattern, and had statistical significance, when compared to controls or between treated samples.

2.6. Metabolite sample clustering analysis

Sample clustering analysis was performed using the partial least squares discriminant analysis (PLS-DA) and sparse partial least squares discriminant analysis (sPLS-DA) methods. All sample metabolites from controls and GBH-exposed animals were configured to display 95% of the confident regions, which were selected based on 2D score plots. Heat maps were used to plot the significantly altered metabolites that were detected by the *t*-test analysis. Distance measurements were based on Euclidian distribution, Ward was used for the clustering algorithm, and both group average data and per-sample data were selected.

2.7. Data analysis

The data are reported as means ± SEM (standard error of the mean) values. The experiments were subjected to a normality test (Shapiro-Wilk), followed by an analysis of variance (one-way ANOVA). When the results passed the normality test, the Student-Newman-Keuls post hoc test was employed, and when the results did not pass the normality test, the Dunn's Multiple Comparison post hoc test was used. Pearson’s correlation analysis was used to evaluate the strength of a relationship among variables obtained from the same subject. The range of r values is from $+1$ to -1, in which a value of $+1$ is an exact correlation, a value of -1 is an exact inverse correlation, and a value equal to zero is a completely random association. The Software Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of perinatal GBH exposure on the expression of Neuroglobin and Cytoglobin genes in the cortex and cerebellum of male rats

As shown in Fig. 1, the relative gene expression levels of Ngb and Cygb were examined in the cortex and cerebellum of male rats perinatally exposed to GBH. In the cortex of the GBH-exposed animals, the

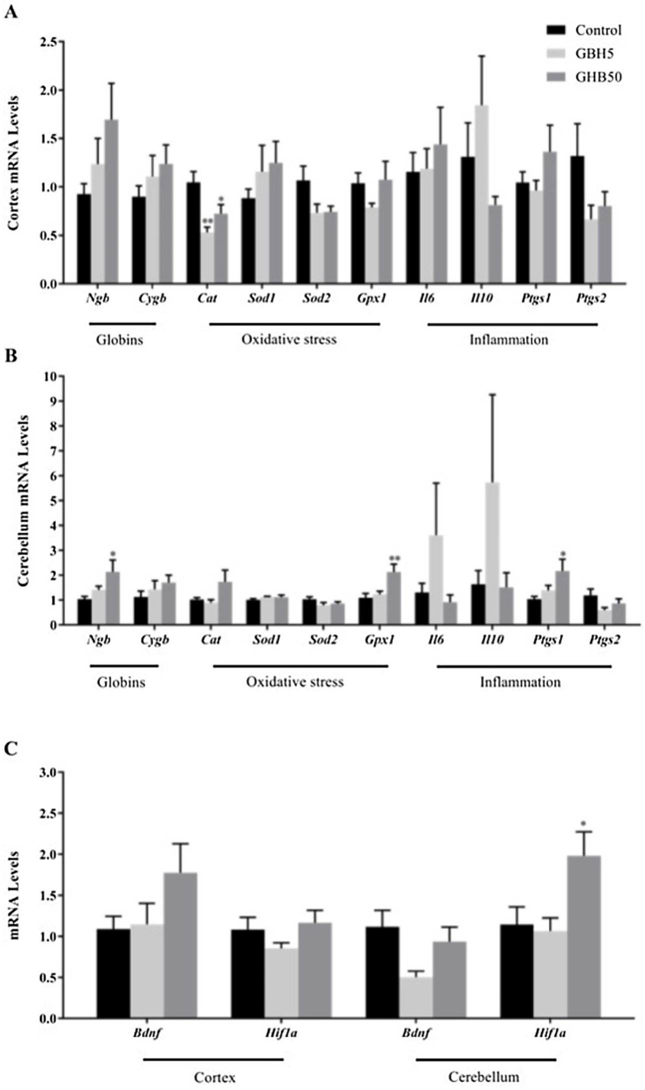

Fig. 1. Gene profile in the cortex and cerebellum of GBH-treated animals. (A) Cortex. The mRNA levels of globins, oxidative stress and inflammation related genes. (B) Cerebellum. The mRNA levels of globins, oxidative stress and inflammation related genes. (C) Bdnf and Hif1α gene expression in the cortex and cerebellum. One-way ANOVA and Newman-Keuls Multiple Comparison Test.Values are the mean ± SEM (n = 8). $^*P < 0.05$ and $^{**}P < 0.01$ compared to control rats.

gene expression of Ngb and Cygb was unaffected and did not change in either GBH-treated group, when compared to controls animals (Fig. 1A). However, in the cerebellum of animals from the GBH 50 group, the gene expression of Ngb was significantly increased by approximately 105% (2.129 ± 0.482), when compared to control rats (1.037 ± 0.108) (Fig. 1B).

3.2. Effect of perinatal GBH exposure on the expression of genes related to oxidative stress in the cortex and cerebellum of male rats

The gene expression of four important genes related to oxidative stress: Sod1, Sod2, Gpx1 and Cat were also analyzed. Expression of the Sod2 gene decreased by 31% (0.662 ± 0.065) and 28% (0.693 ± 0.040), in the GBH 5 and GBH 50 groups, respectively. The expression of Catalase (Cat) was reduced in the cortex of GBH 5 animals by 49% in the GBH 5 group (0.530 ± 0.055) and by 31% in the GBH 50 group (0.723 ± 0.093) when compared to relative controls (1.047 ± 0.109) (Fig. 1A).

In the cerebellum of both groups of GBH-exposed animals there was a significant increase in glutathione peroxidase 1 (Gpx1) expression (Fig. 1B). This augmented expression was found to be 95% greater in the GBH 50 group (2.127 ± 0.315), when compared to control group (1.091 ± 0.172) and 73% greater when compared to GBH 5 group (1.227 ± 0.127).

3.3. Effect of perinatal GBH exposure on the expression of genes related to inflammation in the cortex and cerebellum of male rats

With regards to inflammation, the expression levels of interleukin 6 (Il6), interleukin 10 (Il10) and prostaglandin-endoperoxidase synthase 1 and 2 (Ptgs 1 and 2) were analyzed. There were no significant alterations in the expression of these genes, when comparing either GBH 5 or GBH 50 groups to the control animals (Fig. 1A) in the cortex. In contrast, when compared to control animals (1.037 ± 0.105), the GBH 50 rats exhibited a 109% increase in the expression of the Ptgs1 gene in the cerebellum (2.168 ± 0.471).

3.4. Effect of perinatal GBH exposure on the expression of the hypoxia-inducible factor 1 alpha (Hif1α) gene in the cortex and cerebellum of male rats

In the cortex, the relative gene expression levels of Hif1α and Bdnf did not differ between controls and GBH-treated groups (Fig. 1C). However, in the cerebellum, the relative gene expression of Hif1α gene was found to be increased by 73% in the GBH 50 group (1.980 ± 0.292), when compared to control (1.144 ± 0.213) and by 86% when compared to the GBH 5 group (1.064 ± 0.158) (Fig. 1C).

3.5. Genes related to oxidative stress and inflammation form a protein interaction network

The analyzed genes associated with oxidative stress and inflammation, were then subjected to protein interaction network analysis, using the String webserver (Fig. 2A). Interactions were evaluated by three different methods (experimentally-based, text-mining-based or database-based). Proteins such as Ptgs1 and Ptgs2, as well as Sod1 and Sod2 were shown to interact by all three methods, while Il10 and Il6 and Bdnf and Cat were identified by the text-mining-based method, thus supporting their interactions. Alternatively, using the same list of genes, another network-based analysis was performed, using the Biogrid database, which focused on genetic and physical interactions (Fig. 2B).

3.6. Correlated gene expression in the cortex and cerebellum using Pearson's correlation coefficients

In the cortex of the analyzed animals, there were some genes that had a correlated variation in expression following GBH exposure (Table 1). For example, there was a correlation between Ngb and Cygb (r = 0.860), which is interesting, since the String protein interaction network analysis identified these two proteins as having a physical interaction (Fig. 2A). A positive expression correlation was also observed for Sod1 and Ngb (r = 0.810), and Sod1 and Cygb, (r = 0.92), however, direct interactions between these protein pairs were not identified by the network analyses. Furthermore, significant results were also obtained for Hif1α and Il6 (r = 0.720), and Il6 and Il10 (r = 0.490) (Fig. 2A), Hif1α and Ptgs1 presented a significant correlation of r = 0.750, and the correlation between Ptgs1 and Il6 was r = 0.860. Hif1α still has a significant correlation with Gpx1 (r = 0.850). Gpx1 has significant correlation with Il6 (r = 0.700) and Ptgs1 (r = 0.880). Ptgs2 presents significant correlation with Sod2 (r = 0.580), with Ptgs2 and Sod2 having a predicted interaction in the presented network.

In the cerebellum, another correlation pattern among the studied genes was identified (Table 2). The Ngb and Cat genes were

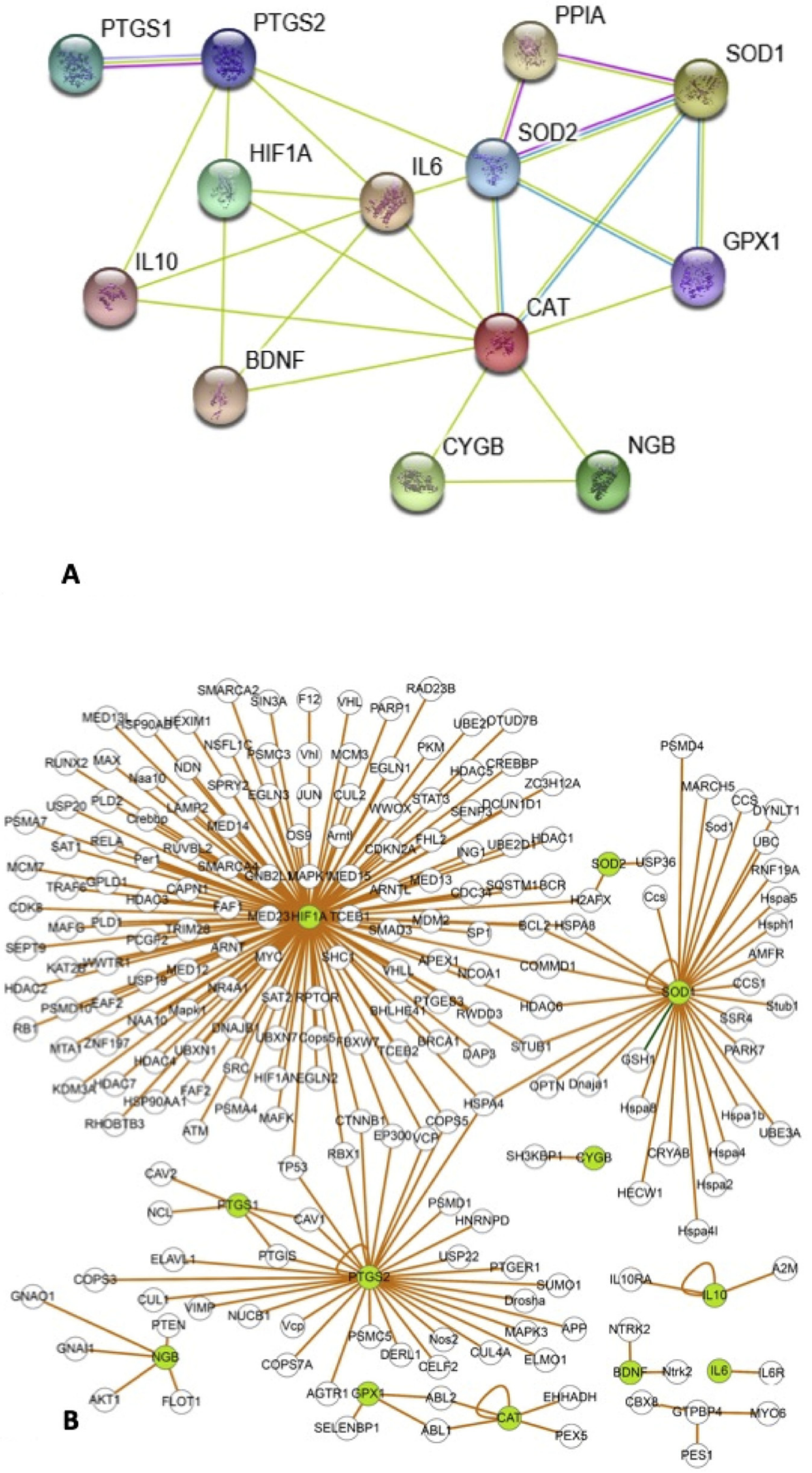

Fig. 2. Oxidative stress and inflammation gene network interactions.
A) Protein-protein interaction network of gene products associated with oxidative stress and inflammation. Lines connecting proteins correspond to a validation method that was used to show that an interaction exists B) Genetic and physical interaction between genes. Genes in green circles are related to oxidative stress and inflammation.

significantly correlated (r = 0.760), and also presented a physical interaction (Fig. 2). Sod1 had a significant inverse correlation with Il6 (r = -0.530) and Il10 (r = -0.550). Interestingly, the Il6 and Il10 also presented a correlation (r = 0.850), and, as mentioned previously, Il6 and Il10 also interact within the network in Fig. 2. Sod2 had a significant correlation with Ptgs2 (r = 0.570) and Bdnf (r = 0.750), with Sod2 and Ptgs2 also present interaction in the network. Gpx1 had a significant correlation with Hif1α (r = 0.770), Ptgs1 (r = 0.740) and

Table 1

Pearson correlation matrix of cortical gene expression.

r	Ngb	Cygb	SOD1	CAT	SOD2	HIF1α	IL6	IL10	PTG S1	PTG S2	BDNF	GPx1
Ngb	1											
Cygb	0.86***	1										
SOD1	0.81***	0.92***	1									
CAT	0.02	−0.02	0.1	1								
SOD2	−0.23	−0.10	−0.21	−0.1	1							
HIF1α	0.07	0.09	−0.04	−0.04	0.42	1						
IL6	−0.04	−0.06	−0.27	−0.28	0.14	0.72***	1					
IL10	−0.19	−0.18	−0.23	−0.09	−0.23	0.059	0.49*	1				
PTG S1	0.06	0.03	−0.18	−0.28	0.26	0.75***	0.86***	0.23	1			
PTG S2	−0.39	−0.25	−0.12	0.29	0.58**	0.25	−0.12	−0.28	−0.11	1		
BDNF	−0.19	−0.04	0.1	0.19	0.03	0.29	−0.06	−0.28	−0.01	0.58***	1	
GPx1	0.19	0.10	−0.05	−0.06	0.37	0.85***	0.7***	−0.01	0.88***	0.04	0.06	1

r, Pearson coefficient, and correlations (two-tailed) that are significant at the 0.05(*), 0.01(**) or 0.001(***).

Ptgs2 (r = 0.780). Bdnf not only had a significant correlation with Hif1α (r = 0.620), but was also predicted to physically interact with this protein (Fig. 2).

3.7. *Glyphosate exposure alters the levels of oxidative stress related metabolites*

After n metabolite data normalization (Figs. 4 and 5), using the approach described in the Methods section, statistical analyses were employed for identifying significant alterations in metabolite concentrations. When comparing the GBH 5 group to controls, it was found that the levels of 17 metabolites were significantly altered (Table 3, Supplementary Fig. S1). Seven of these metabolites were significantly increased in both GBH-exposed groups, and 10 were reduced in the GBH 5 group. In all exposed samples we found the same concentration standard for GBH 5 (Fig. 3A and B). Additionally, 11 out of the 17 metabolites with altered expression in the GBH 5 group correspond to different types of phosphatidylcholine (PC). The GBH 5 group also displayed altered acylcarnitine (C102) concentrations.

On the other hand, when comparing the GBH 50 group to control rats, we found that there were 13 altered metabolites significantly changed (Table 4, Supplementary Fig. S1). Five of these metabolites were significantly increased in the GBH-exposed animals, and eight were reduced in the GBH 50 group. In all exposed samples we found the same concentration standard for GBH 50 (Fig. 3C and D). Furthermore, 6 out of the 13 metabolites were associated with PC. Similar to the GBH 5 group, the GBH 50 group also displayed altered acylcarnitine (C102) concentrations.

3.8. *Differential gene expression influences metabolite levels in male rats perinatally exposed to glyphosate*

The altered metabolite concentrations found in both the GBH 5 (Table 3) and GBH 50 (Table 4) animals demonstrate that cellular metabolism has been compromised. In fact, most of the observed alterations were associated with cellular oxidative stress and inflammation. Interestingly, the GBH-induced alterations of serum metabolite concentrations were found to be directly correlated with genes that had displayed altered expression profiles (i.e. Ptgs1, Ngb, Cat and Gpx1) in the cortex and cerebellum of the investigated rats.

4. Discussion

It is known that environmental factors experienced by pregnant mammals are capable of reprograming the metabolic responses of their progeny (Stone et al., 2016). In this context, the primary goal of our work was to determine if maternal GBH exposure could induce long-term changes in the expression of genes related to the antioxidant defense system and inflammation, in the cortex and cerebellum of adult male rat offspring. It is important to mention that the chow consumption and the body growth of dams and their offspring were not affected by GBH treatment (data not shown), which could potentially compromise the conclusions of the presented data. Furthermore, these results corroborate our previous studies (Romano et al., 2010, 2012) and are consistent with other reports in the literature (Caglar and Kolankaya, 2008; Stout and Ruecker, 1990).

In the present study, it was demonstrated that perinatal GBH exposure altered the gene expression of antioxidant enzymes in both the cortex and cerebellum of adult offspring, and identified important gene expression correlation patterns in both brain regions. For example, catalase gene expression, which encodes for an important hydrogen

Table 2

Pearson correlation matrix of cerebellar gene expression.

R	Ngb	Cygb	SOD1	CAT	SOD2	HIF1α	IL6	IL10	PTG S1	PTG S2	BDNF	GPx1
Ngb	1											
Cygb	0.44	1										
SOD1	0.31	0.13	1									
CAT	0.76***	0.21	0	1								
SOD2	−0.21	0.09	0.06	0.01	1							
HIF1α	0.2	0.42	0.16	0.26	0.57*	1						
IL6	−0.21	−0.08	−0.53*	0.09	0.08	−0.02	1					
IL10	−0.23	−0.24	−0.55*	0.04	0.06	−0.05	0.85***	1				
PTG S1	0.53	0.14	0.45	0.35	0.04	0.34	−0.22	−0.39	1			
PTG S2	−0.31	0.34	0.26	−0.14	0.57*	0.35	−0.13	−0.12	−0.05	1		
BDNF	−0.33	0.28	−0.14	−0.08	0.75***	0.62**	0.08	0.14	−0.15	0.72	1	
GPx1	0.41	0.38	0.26	0.26	0.31	0.77***	−0.27	−0.39	0.74***	0.078**	0.3	1

r, Pearson coefficient, and correlations (two-tailed) that are significant at the 0.05(*), 0.01(**) or 0.001(***).

Table 3
Metabolites with a significant alteration in expression in the GBH 5 samples. Negative t.stat indicates higher concentration (fold-change) in GBH 5 treated samples, when compared to controls.

Description	Abbreviation	t.stat	p.value	-LOG10(p)	FDR
free carnitine	C0	−4.6101	0.001732	2.7614	0.045257
phosphatidylcholines diacyl	PC aa C384	−4.3655	0.002395	2.6207	0.04611
phosphatidylcholines acyl-alkyl	PC ae C404	−4.2576	0.00277	2.5575	0.04611
phosphatidylcholines diacyl	PC aa C366	−4.2218	0.002909	2.5363	0.04611
phosphatidylcholines diacyl	PC aa C344	−4.1779	0.003089	2.5102	0.04611
phosphatidylcholines diacyl	PC aa C385	−4.0406	0.003733	2.428	0.049972
phosphatidylcholines diacyl	PC aa C364	−3.9979	0.003962	2.4021	0.049972
phosphatidylcholines acyl-alkyl	PC ae C340	3.9387	0.004304	2.3661	0.049972
Acylcarnitines	C102	3.9435	0.004275	2.3691	0.049972
phosphatidylcholines acyl-alkyl	PC ae C362	4.2415	0.002832	2.548	0.04611
phosphatidylcholines acyl-alkyl	PC ae C343	4.2491	0.002803	2.5524	0.04611
lysophosphatidylcholines	lysoPC a C182	4.6384	0.00167	2.7773	0.045257
phosphatidylcholines acyl-alkyl	PC ae C363	4.929	0.001151	2.939	0.040087
Phospholipase A2	PLA2 Act	4.995	0.001059	2.9749	0.040087
Tetrahydrocannabinolic acid	D9 Arac PC ae	5.6254	0.000495	3.3051	0.034511
Threonine	Thr	7.2645	8.68E-05	4.0614	0.009073
phosphatidylcholines acyl-alkyl	PC ae C342	9.4491	1.29E-05	4.8882	0.002704

peroxide (H_2O_2) reducing enzyme, was decreased in both GBH-exposed groups. Such a decrease could potentially inhibit the elimination of H_2O_2, which could accumulate and damage the brain (Partadiredja et al., 2005, 2009; Stone et al., 2016). The decrease in Cat expression may reduce the antioxidant potential of the tissue, leading to neuronal oxidative stress in the brain region. The significant correlation with Ngb, Cygb and Sod1 expression indicates that these genes are involved in protecting the tissue against oxidative stress.

In the cerebellum, Gpx1 was significantly upregulated in the GBH 50 group. Similar to Cat, Gpx1 detoxifies H_2O_2 and is an important human antioxidant enzyme (Lubos et al., 2011). Recently, Gallegos et al. (2018) showed that the Gpx1 activity is increased in the brains of adult offspring, of mothers administered GBH in the drinking water (1.30 g/l) during pregnancy. GPx1 probably affords protection against GBH exposure (Ishibashi et al., 2002), since these chemicals can act as oxidants. Together, these results suggest that a long-term increase in GPx1 expression could afford protection against perturbations in neuronal metabolic homeostasis at least in the cerebellum. With regards to the metabolite data, oxidative stress-related metabolites, including lysophosphatidylcholine (LPC) and PC, were found to be misregulated, which could be due to the increased Gpx1 expression, or a compensatory mechanism in response to downregulation of the Cat gene.

In the cerebellum, there were alterations in the expression of Ptgs1, Ngb and Hif1α, genes related to inflammation an hypoxia. Previous studies have shown that increased oxidative stress and inflammation in the brain might be related to the manifestation of some neurodegenerative diseases, since neurons are highly sensitive to oxidative stress (Andersen, 2004; Floyd and Hensley, 2002). Therefore, in order to

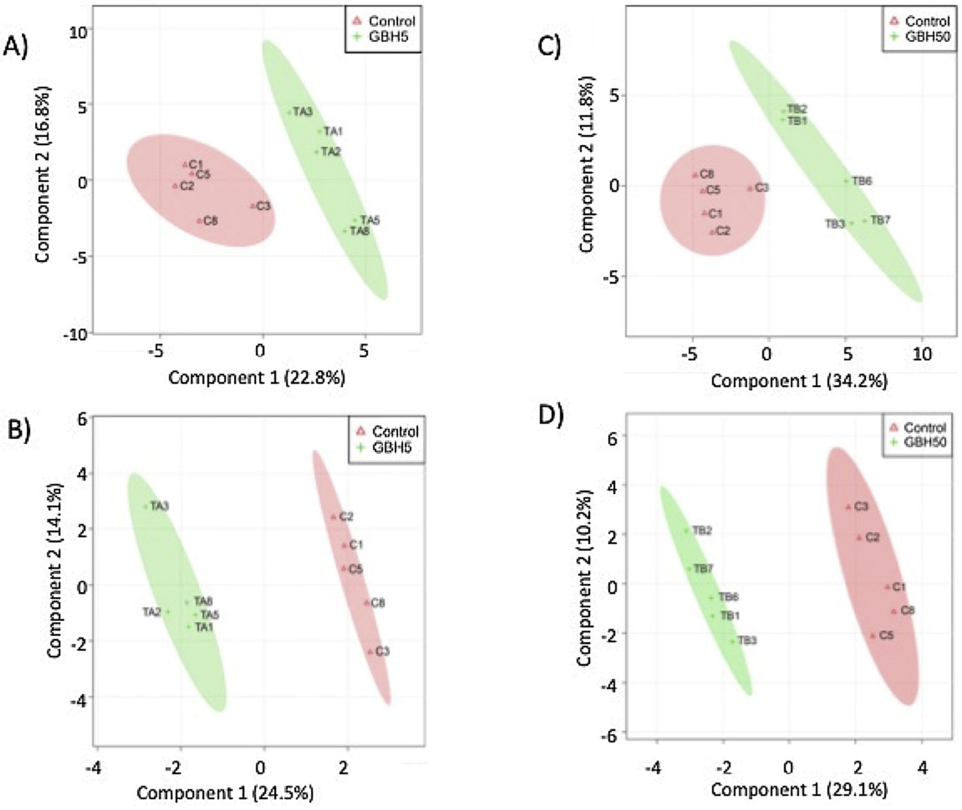

Fig. 3. Metabolite profile of control and GBH-exposed animals.
A) Control and GBH 5 animals using the PLSDA clustering method B) Control and GBH 5 animals using the sPLSDA clustering method C) Control and GBH 50 animals using the PLSDA clustering method D) Control and GBH 50 animals using the sPLSDA clustering method.

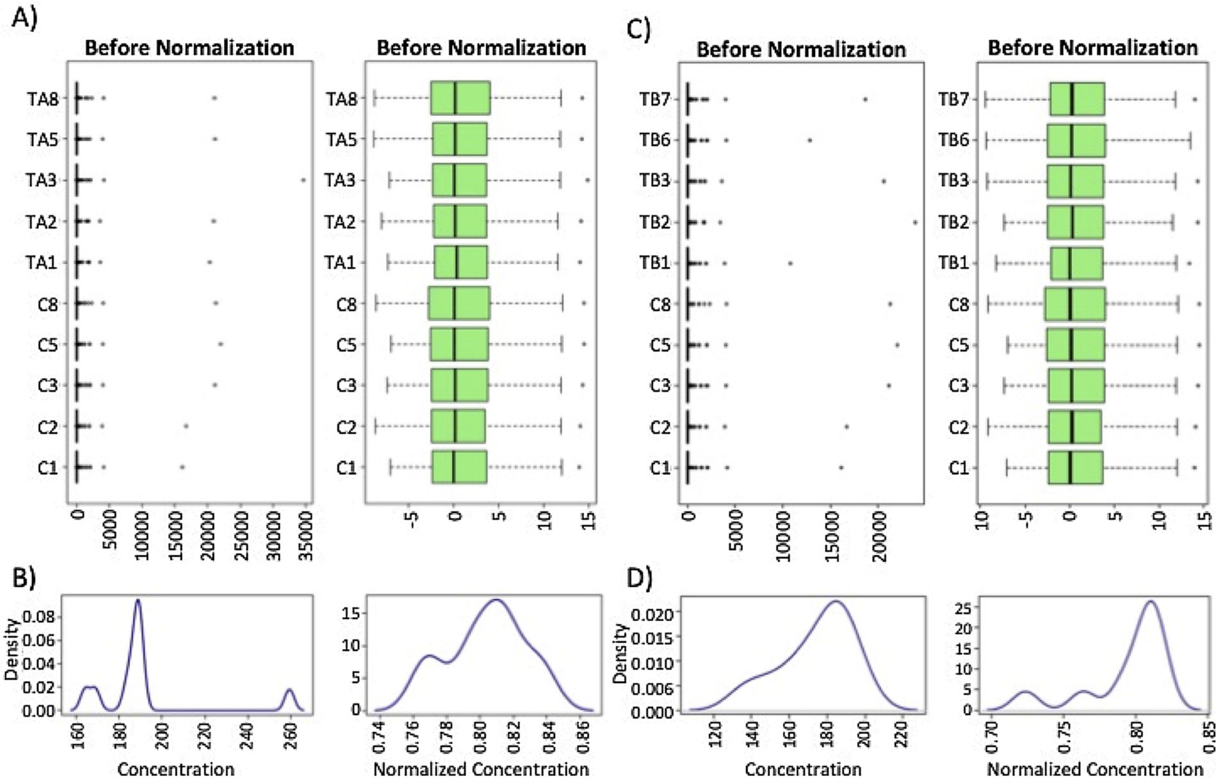

Fig. 4. Metabolite normalization for GBH 5 and GBH 50 animals.
A) Boxplot normalization with control and GBH 5 samples B) Normalized metabolite concentration for control and GBH 5 samples C) Boxplot normalization for control and GBH 50 samples D) Normalized metabolite concentration for control and GBH 50 samples.

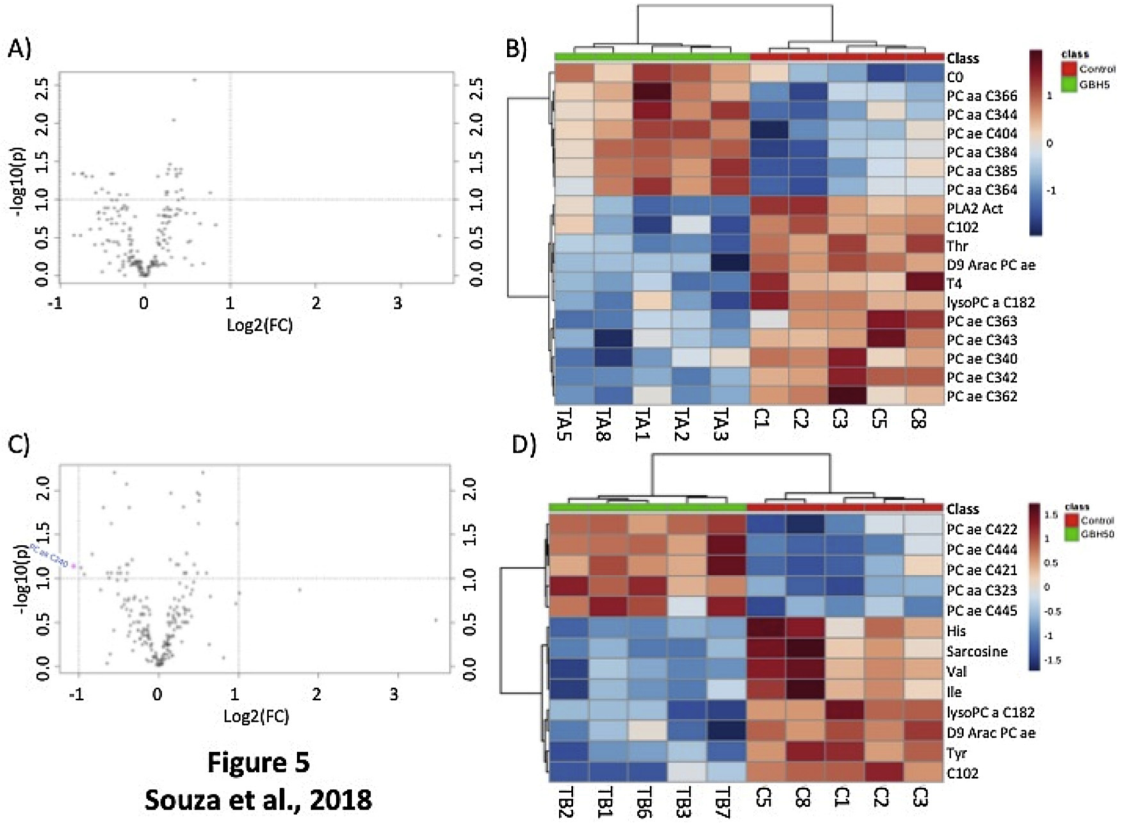

Fig. 5. Sample clustering for GBH 5 and GBH 50 animals.
A) Volcano plot of control and GBH 5 samples B) Heatmap and dendrogram of metabolites with significant alterations in GBH 5 samples, as compared to controls C) Volcano plot of control and GBH 50 samples D) Heatmap and dendrogram of metabolites with significant alterations in GBH 50 samples, as compared to controls.

Table 4
Metabolites with a significant alteration in expression in the GBH 50 samples. Negative t.stat indicates higher concentration (fold-change) in GBH 50 treated samples, when compared to controls.

Description	Abbreviation	t.stat	p.value	-LOG10(p)	FDR
phosphatidylcholines diacyl	PC aa C323	−7.664	5.94E-05	4.2263	0.0062062
phosphatidylcholines acyl-alkyl	PC ae C444	−6.9345	0.00012027	3.9198	0.0083789
phosphatidylcholines acyl-alkyl	PC ae C445	−5.3472	0.00068812	3.1623	0.015471
phosphatidylcholines acyl-alkyl	PC ae C422	−5.2866	0.00074025	3.1306	0.015471
phosphatidylcholines acyl-alkyl	PC ae C421	−4.7383	0.0014671	2.8335	0.023586
L-Isoleucine	Ile	4.7446	0.0014553	2.837	0.023586
Sarcosine	Sarcosine	4.8277	0.0013084	2.8832	0.023586
phosphatidylcholines acyl-alkyl	D9 Arac PC ae	5.4861	0.00058324	3.2341	0.015237
L-Histidine	His	5.7358	0.00043606	3.3604	0.01302
Valine	Val	6.015	0.00031803	3.4975	0.011078
Acylcarnitines	C102	6.2184	0.00025427	3.5947	0.010629
lysophophatidylcholine	lysoPC a C182	6.4365	0.00020117	3.6964	0.010511
L-Tyrosine	Tyr	8.149	3.82E-05	4.4176	0.0062062

avoid factors that can induce irreversible physiological changes, it is important to control the excessive GBH use.

A previous study showed that HIF1α induces inflammatory responses in the brain and plays an important neuroprotective role following brain injury (Fan et al., 2009); however, it is specific tissue and can also be neurotoxic (Fan et al., 2009; Helton et al., 2005). Herein, HIF1α was found to be upregulated in the cerebellum of the GBH 50 animals, which may represent adaptive and protective mechanisms against altered energy metabolism and/or increased oxidative stress, as evidenced by the increased expression in Ngb, Ptgs1 and Gpx1 in the tissue.

The increased Ngb expression in the cerebellum is interesting because it has been shown to be potentially critical in the maintenance of neural integrity (Brunori and Vallone, 2007). In fact, this protein plays an important role in the detoxification of ROS that have been implicated in the development of neurodegenerative diseases (Ahmed et al., 2008). Ngb has been demonstrated to be essential for the recovery of the central nervous system (CNS), after traumatic hypoxia-induced lesions in the brain of rodents (Chuang et al., 2010). Additionally, Oliveira et al. (2015) and Khot and Tirschwell (2006) both showed that both Ngb and Cygb play roles in CNS protection against oxidative stress and hypoxia, as well as other conditions caused by hypothyroidism and hyperthyroidism.

Since it is known that some cytokines can inflict tissue damage through the accumulation of ROS, the expression of molecules involved in the production of pro-inflammatory cytokines was also investigated. The activation of anti-inflammatory cytokines was also monitored, since they may represent a counterbalance effect (de Bilbao et al., 2009). IL6 is a key inflammatory facilitator, which functions as an anti- and pro-inflammatory cytokine, and is one of the first cytokines synthesized in the brain following tissue damage (Ley et al., 2011). IL10 is an anti-inflammatory cytokine that protects the brain (de Bilbao et al., 2009). In the present study, neither cytokine presented a significant alteration in gene expression. However, after analyzing the gene expression data from the cortex and cerebellum samples, we did find that IL6 and IL10 have a positive correlation in gene expression. This result suggests that uprefulation of these cytokines may have neuroprotective effects (de Bilbao et al., 2009).

Finally, we analyzed the serum metabolomics, since oxidative stress can be activated by phosphatidylcholine (PC) (Frey et al., 2000; Mateos et al., 2008). Another study found low levels of PC in patients with ulcerative colitis, a chronic inflammatory disorder of the colon (Treede et al., 2007). The authors went on to ahow that there was a positive correlation between of phosphatidylcholine and the human anti-inflammatory response, and that PC therapy had beneficial effects in these patients (Treede et al., 2007). Additionally, lysophosphatidylcholine (LPC) induces endothelial cell injury by altering nitric oxide (NO) production, thereby increasing ROS (Kim et al., 2009). Together, these studies suggest that alterations in PC levels, and possibly other derived compounds, can modulate cellular function. Herein, LPC concentrations were significantly reduced in both GBH-exposed groups when compared to controls. Interestingly, several independent studies have demonstrated that PC can directly interfere with the central nervous system normal function. For example, in mice with dementia, PC treatment increased acetylcholine concentrations in the brain and improved memory (Rao et al., 2015). PC supplementation has also been shown to alleviate symptoms associated with hypomania and insomnia symptoms (Rao et al., 2015). More recently, Ross et al. (2016), reported that maternal PC treatment during pregnancy could increase α7-nicotinic acetylcholine receptor (CHRNA7) activation in the offspring, which could be link to the onset and development of schizophrenia, autism, and attention deficit hyperactivity disorder (Ross et al., 2016).

Free carnitine (C0) concentrations increased in the GBH 5 group, and could have substantial implications on fatty acid oxidation (Karlic and Lohninger, 2004). In fact, a retrospective study involving 100 autistic children showed that reduced carnitine levels were associated with mitochondrial dysfunctions (Filipek et al., 2004). Later, a similar study presented evidence for carnitine levels being reduced in autistic individuals (Mostafa and Al-Ayadhi, 2015). Furthermore, carnitine and acylcarnitine supplementation had beneficial effects in the treatment of neurological disorders, increasing antioxidant activity and enhancing cholinergic neurotransmission (Jones et al., 2010). Although reduced levels of free carnitine have been associated with autism, increased levels of this metabolite have been associated with metabolic disorders (Makrecka-Kuka et al., 2017), obesity and insulin resistance (Schooneman et al., 2016), in humans and rodents. Taken together, these results demonstrate the importance of carnitine homeostasis, and suggest that the increased carnitine levels detected in the GHB 5 group could potentially impact the health of the animals.

Other misregulated metabolites included valine and isoleucine, which are members of the branched-chain amino acids (BCAAs). It has been previously shown that reduced BCAA levels may affect the glutamate-glutamine pool in the body, resulting in the tissue being more vulnerable to oxidative stress (Tamanna and Mahmood, 2014). Additionally, experimental evidence suggests that sepsis, cancer, trauma, and burns enhance BCAA oxidation and reduce BCAA concentrations (Tamanna and Mahmood, 2014).

PTGS1 was found to be highly expressed in the GBH 50 samples. It catalyzes the conversion of arachidonic acid to prostaglandin H2, which is subsequently metabolized to various biologically active prostaglandins (Lee et al., 2007). The misregulation of several phosphatidylcholines has been observed in animal models of Parkinson's disease (Farmer et al., 2015), and another study also found alterations in the LPC/PC ratio in the cerebrospinal fluid from Alzheimer disease patients (Mulder et al., 2003). Thus, it is plausible that these molecules are involved in the onset and/or pregression of these neurological diseases. According to the previous reports, we also hypothesized that Ptgs1 could increase the free arachidonic acid conversion rate. Consequently, reducing the amount of deposited phospholipids, and increasing the production of PCs, a situation that was observed in the in the metabolite analyses for both the GBH 5 and GBH 50 groups (Tables 3 and 4).

In summary, the findings from the present study demonstrate that perinatal GBH exposure can alter the expression of several important genes related to oxidative stress and inflammation, and metabolite concentrations in adult male offspring. Additionally, the differential gene expression results were shown to be correlated with the altered metabolite concentrations. Together, our results indicate that maternal GBH exposure can impact subsequent generations, and increase the risk for developing neurodegenerative diseases later in adulthood.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Funding

This project was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, process number 18952-12-7) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, process number 2017/07053-3) to JSS, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, process number 2013/26851-7) to MIC, CAPES – AUXPE Pró-integração (process number 3160/2013-98) to MRDS and Fundação Araucária (FA grant number FA09/2016) to RHH.

Acknowledgments

The authors are grateful for the financial support of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; 18952-12-7) and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP process #2017/07053-3) to JSS and R_2G English Editing, for the English content of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neuro.2019.06.004.

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