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Developmental and metabolic brain alterations in rats exposed to bisphenol A during gestation and lactation

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ABSTRACT

In recent years, considerable research has focused on the biological effect of endocrine-disrupting chemicals. Bisphenol A (BPA) has been implicated as an endocrine-disrupting chemical (EDC) due to its ability to mimic the action of endogenous estrogenic hormones.

The aim of this study was to assess the effect of perinatal exposure to BPA on cerebral structural development and metabolism after birth.

BPA (1 mg/l) was administered in the drinking water of pregnant dams from day 6 of gestation until pup weaning. At postnatal day 20, *in vivo* metabolite concentrations in the rat pup hippocampus were measured using high field proton magnetic resonance spectroscopy. Further, brain was assessed histologically for growth, gross morphology, glial and neuronal development and extent of myelination.

Localized proton magnetic resonance spectroscopy (^{1}H MRS) showed in the BPA-exposed rat a significant increase in glutamate concentration in the hippocampus as well as in the Glu/Asp ratio. Interestingly these two metabolites are metabolically linked together in the malate–aspartate metabolic shuttle.

Quantitative histological analysis revealed that the density of NeuN-positive neurons in the hippocampus was decreased in the BPA-treated offspring when compared to controls. Conversely, the density of GFAP-positive astrocytes in the cingulum was increased in BPA-treated offspring.

In conclusion, exposure to low-dose BPA during gestation and lactation leads to significant changes in the Glu/Asp ratio in the hippocampus, which may reflect impaired mitochondrial function and also result in neuronal and glial developmental alterations.

1. Introduction

In recent years, considerable research has focused on the association between exposure to endocrine-disrupting chemicals and the onset of disease and reproductive impairment. BPA (4,4′-isopropylidenediphenol), which is widely used in plastic food and drink containers, has been implicated as an endocrine-disrupting chemical due to its ability to mimic the action of endogenous estrogenic hormones (Paris et al., 2002; Ranjit et al., 2009; Vom Saal et al., 1998).

Several studies have confirmed the presence of BPA in urine in human populations (0.3–7.9 μg/day) (Calafat et al., 2005; Fujimaki et al., 2004; Takeuchi and Tsutsumi, 2002). BPA has been detected in the serum of pregnant women (1–2 ng/ml), fetus serum (0.2–9.2 ng/ml), amniotic fluid (8.3–8.7 ng/ml), placental tissue (1.0–104.9 ng/g), and breast milk (1.1 ng/l) (Ikezuki et al., 2002; Schonfelder et al., 2002; Ye et al., 2005), suggesting that the human fetus is readily exposed to this compound during pre- and postnatal development.

Furthermore, a number of studies have shown behavioural modifications after exposure to BPA during gestation and/or lactation at low and high doses (i.e. 40–400 μg/kg/day), such as masculinisation behaviour in female pups or hyperactivity (Dessi-Fulgheri et al., 2002; Farabollini et al., 2002; Ishido et al., 2004; Palanza et al., 2008). These behavioural changes suggest some effects of BPA on the developing central nervous system (CNS). The effect of BPA on the CNS may be of increased importance in the fetal and postnatal brain development and appears now as a public health concern (Vandenberg et al., 2009). To date, there have been few *in vivo* studies that have examined the impact of BPA exposure during

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Table 1
Primary associated role of the metabolites quantified by ^{1}H MRS and abbreviation in this study.

Primary associated role	Compound	Abbreviation
Antioxidants	Ascorbate	Asc
	Glutathione	GSH
Excitatory amino acid	Aspartate	Asp
Excitatory neurotransmitter	Glutamate	Glu
	N-Acetylaspartylglutamate	NAAG
Precursor of Glu located in astrocytes	Glutamine	Gln
Inhibitory neurotransmitter	γ-Aminobutyric acid	GABA
Membrane synthesis	Glycerophosphorylcholine	GPC
	Phosphorylcholine	PCho
	Phosphorylethanolamine	PE
Source of energy	D-Glucose	Glc
End product of anaerobic glycolysis	Lactate	Lac
Reservoir of generation of ATP	Creatine	Cr
	Phosphocreatine	PCr
Putative neuronal marker	N-Acetyl-aspartate	NAA
Osmoregulation	Taurine	Tau
	Myo-inositol	Ins

gestation and lactation on brain development (Facciolo et al., 2002). In addition, BPA (25–50 mg/kg/day, i.p. injection) has been shown to enhance oxidative stress and lipid peroxidation promoting the cellular death in several organs (e.g. brain, liver, and kidney) of exposed rodents (Aydogan et al., 2008; Kabuto et al., 2003).

Some investigators have reported the beneficial effects of BPA on brain development, mediated for the most part by estrogen receptors (ERs) (Kubo et al., 2004; Shikimi et al., 2004) (50–200 μM of BPA in cell culture or injection of 500 μg/day during 5 days in the rat cerebrospinal fluid, respectively). But most studies, however, have shown the potential risk of BPA exposure on brain development. *In vitro*, BPA exposure has been shown to inhibit oligodendrocyte precursor cell differentiation and myelin basic protein (MBP) expression (Seiwa et al., 2004), and to influence synaptogenesis and neuronal vulnerability in hippocampal cultures (Sato et al., 2002). *In vivo*, BPA inhibits estrogen-activated hippocampal spine synapse formation (Maclusky et al., 2005; Zhou et al., 2009) (subcutaneous injection of 40–400 μg/kg of BPA in the rat and from gestational day (GD) 8 to PND21 20 μg/kg/day of BPA, respectively) and disrupts normal neocortical development by accelerating neuronal differentiation/migration (Nakamura et al., 2006) (subcutaneous injection of 20 μg/kg/day from GD0). It is therefore of substantial interest to investigate the effects of pre and post-natal exposures of BPA on brain development. In particular, in view of the potential translation to human studies, the approach through non-invasive techniques is attractive.^{1}H MRS is an *in vivo* investigational approach that has been used to study the metabolic changes during rat brain development (Tkac et al., 1999). This technique allows the assessment of the concentration of 21 metabolites, termed "neurochemical profile" (Pfeuffer et al., 1999) (cf. Table 1). Previous studies have shown that impaired nutrient supply, such as iron deficiency (Rao et al., 2003) or chronic hypoxia (Raman et al., 2005) leads to specific changes in the neurochemical profile that implicate delayed myelination and impaired development of the neuropil.

The aim of the current study was to assess the effects of BPA exposure during gestation and lactation on cerebral metabolism and development by using *in vivo* localized ^{1}H MRS in combination with quantitative histopathology.

2. Materials and methods

2.1. Animals

All animal procedures were reviewed and approved by the university and state animal ethics boards. Virgin female (275–300 g) and male (300–325 g) Sprague–Dawley (Taconic Europe, The Netherlands) rats were used for breeding. Pregnant dams were housed individually under standard conditions (12 h:12 h light–dark cycle). To avoid BPA induced potential malformation during the first days of gestation (Nakamura et al., 2006; Xing et al., 2010), the administration of BPA started from day 6 of gestation through postnatal day 20 (PND20) of lactation, where dams were given 1 mg/l BPA (dissolved in 1% ethanol) in their drinking water (Rubin et al., 2001; Somm et al., 2009). The mean level dosage of intake of the dams was estimated to approximately 70 μg/kg/day by weighting the drinking bottle every day. This dose is in the range that does induce neural and behavioural changes. The dams of control group received drinking water containing the same amount of ethanol (1%) without BPA. The end of the treatment coincided with the weaning age (PND20).

To control for dietary phytoestrogen intake, control and BPA-treated dams were given a chow diet low in phytoestrogens, with genistein content below the detection limit (Kliba Nafag 3250, Provimi Kliba, Kaiseraugst, Switzerland) 10 days prior to mating and throughout the gestation and lactation. BPA-free cages and drinking bottles were used to avoid contamination. Male and female data were combined for all analyses since no gender differences between BPA-treated and control-offspring's neurochemical profiles were seen. Furthermore, the number of offspring per litter and the ratio of male to female offspring at birth were not statistically different between control and BPA-treated rats.

2.2. Nuclear magnetic resonance (NMR)

Localized ^{1}H MRS was focused on the investigation of metabolic changes in the hippocampus region since several studies have reported neuronal alterations in hippocampal cultures after BPA exposure (Choi et al., 2007; Lee et al., 2008; Maclusky et al., 2005; Ogiue-Ikeda et al., 2008; Sato et al., 2002; Zhou et al., 2009). Pups from the two animal groups (n = 8 BPA-treated group, n = 6 control), were studied at PND20. Pups were anesthetized using a mix of isoflurane (3%) and oxygen, and then maintained under anesthesia using a nose cone with 1–1.5% of isoflurane during the MRS acquisition (on average 90 min). During the MRS experiment pups were maintained in a specific home-built holder to restrain head motion. The body temperature was maintained at 37.5 ± 1 °C by circulating heated water and breath rate was monitored during the whole experiment. To control for basic physiological status of the animal, whole blood lactate measurements were done before and after the MRS acquisition on a GM7 Micro-Stat (Analox Instruments Ltd., UK) by tail-bleeding; no difference in blood lactate was observed.

2.2.1. Proton spectroscopy

All ^{1}H MRS examinations were performed on a Varian INOVA console (Varian, Palo Alto, CA) connected to an actively shielded 9.4 T/31 cm magnet (Magnex Scientific, Abingdon, UK) with actively shielded gradients (400 mT/m in 120 μs, 12 cm inner diameter). A home-built 17 mm-diameter ^{1}H quadrature surface coil was used for radio frequency transmission and signal reception. A fast spin echo magnetic resonance imaging (MRI) sequence (echo train length = 16, echo spacing = 10 ms, effective echo time = 80 ms) was used to obtain anatomical images with an in plane resolution of ~80 μm and a slice thickness of 0.8 mm. Based on these images, the volume of interest (VOI) (2 mm × 3 mm × 2 mm) for spectroscopy was positioned on the left hippocampus. An ultra-short echo time (TE = 2 ms) localized STEAM-based sequence was used for *in vivo* ^{1}H MRS. The STEAM sequence is based on three 90° radio frequency pulses affording a robust spatial localization and allows short echo time measurements (Tkac et al., 1999). The water signal was suppressed by a VAPOR

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scheme integrating three outer volume suppression modules (Tkac et al., 1999). First and second order shim current were adjusted with an EPI-based FASTMAP sequence (Gruetter, 1993) on a volume of 3 mm × 4 mm × 3 mm centered on the VOI. The typical line width of water resonance (FWHM) was 8–10 Hz. All spectra were acquired with a spectral width of 5 kHz, 4096 complex data points, mixing time of 20 ms and repetition time (TR) of 4 s. Water spectrum was acquired as reference for quantification (4 averages) and then the metabolite spectrum was acquired using water suppression based on VAPOR, applied before the localized spectroscopy sequence. Spectra were acquired in 30 blocks of 12 averages stored separately on disk for a total acquisition time of 24 min.

2.2.2. Data analysis

The 30 blocks were corrected for B_0 shift correction before summation and then an eddy-current correction was applied. Metabolite concentrations were quantified using LCModel as in previous studies (Tkac et al., 2003). This software is based on a frequency domain analysis, fitting the *in vivo* spectra with a linear combination of spectra of the individual metabolites (Provencher, 1993). The water signal was used as reference for quantification as in previous studies (Tkac et al., 2003) assuming a water concentration of 82.3% in the rat brain at PND20 (Tkac et al., 2003). The results provided the quantification of the following 17 metabolite concentrations: ascorbate (Asc), aspartate (Asp), creatine (Cr), γ-aminobutyric acid (GABA), glucose (Glc), glutamate (Glu), glutamine (Gln), glutathione (GSH), glycerophosphorylcholine (GPC), phosphorylcholine (PCho), myo-inositol (Ins), lactate (Lac), N-acetyl-aspartate (NAA), N-acetylaspartylglutamate (NAAG), phosphocreatine (PCr), phosphorylethanolamine (PE), and taurine (Tau). The uncertainty on the quantification was estimated from the Cramér–Rao lower bounds (CRLBs) provided by the LCModel program. Only metabolites with a CRLB lower than 20% were considered in the statistical analysis.

Results are expressed as mean of means ± SEM (histological parameters). Significant differences between controls and BPA-treated animals were assessed using a Mann–Whitney *U* test for independent samples. A probability of $p < 0.05$ was considered to be significant. Two animals were rejected from the MRS study (one in each group) based on poor spectral quality and health criteria.

2.3. Histological analysis

At PND20, pups from each group ($n = 7$ BPA-treated animals, $n = 4$ controls), taken from 2 to 4 different litters, were deeply anesthetized using ketalar (50 mg/ml; 0.2–0.5 ml, i.p.). Animals were perfused intracardially with 0.9% NaCl, then 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde overnight, then 20% in sucrose for 24 h, and stored at −80 °C until sectioned. Coronal sections (10 μm) at the level of the dorsal hippocampus were cut on a cryostat (Microm Cryo-Star HM 560M, Microm International, Germany). Three sections at 200 μm intervals were collected from each animal and were alternately processed for haematoxylin and eosin (H&E) staining and specific immunochemistry.

2.3.1. Histology

Sections were stained with H&E and assessed qualitatively for the presence of haemorrhages, lesions or infarcts.

2.3.2. Immunohistochemistry

The areal densities of astrocytes and neurons were assessed using glial fibrillary acidic protein (GFAP) and neuronal nuclei (NeuN), respectively. Non-specific binding was blocked by incubating sections in PBS containing 4% bovine serum albumin (BSA) for 30 min at room temperature. Sections were then incubated in rabbit anti-GFAP (1:400, Z334, Dako Cytomation, Denmark), and mouse anti-NeuN (1:400, MAB377, Chemicon, USA) in 0.25% BSA in PBS overnight at 4 °C. This was followed with a 2 h incubation at room temperature with Alexa 555 anti-mouse (1:200) or Alexa 488 anti-rabbit (1:200) secondary antibodies, respectively (Molecular Probes, Invitrogen, Paisley, UK). Sections were rinsed in PBS and mounted using PBS–glycine media.

The extent of myelination was assessed using myelin basic protein (MBP). Activity of endogenous peroxidases was blocked with 0.3% H_2O_2 in methanol for 20 min. Nonspecific binding was blocked by incubating the slides in 4% BSA for 30 min at room temperature. Sections were then incubated in primary antibody for MBP (1:400, MAB386, Chemicon, USA) overnight at 4 °C. This was followed by 60 min incubation at room temperature in secondary antibody (1:200 anti-mouse immunoglobulin G; Vectastain kit, Vector Laboratories, Burlingame, CA) and the avidin–biotin complex (1:200, Vector Laboratories). Sections were then developed with the chromagen, 3,3 diaminobenzidine (DAB) in 0.01% hydrogen peroxide.

2.4. Quantitative analysis

Quantitative analyses were performed using MetaMorph® Imaging System (Meta Imaging Software, Molecular Devices Corporation, PA, USA). Measurements were made on coded slides blinded to the observer with the codes not being disclosed until the conclusion of analyses.

2.4.1. Areal density of neurons

The density of NeuN-positive neurons was assessed in 6 fields within the motor and somatosensory cortices, respectively. The density of neurons in the hippocampus was also assessed (9 fields per animal). Values for each animal in each region were pooled and a mean value was calculated. A mean of means ± SEM was calculated for each group.

2.4.2. Areal density of astrocytes

The density of GFAP-positive astrocytes was assessed in the cingulum. Six fields per animal were examined; values for each animal were pooled and a mean value was calculated. A mean of means ± SEM was calculated for each group.

2.4.3. Myelination

The optical density (OD) of MBP-stained fibres was measured in the corpus callosum and cortex using ImageJ (Rasband, 1997–2009). Eight fields within the corpus callosum, and six fields within the cortex, were examined. Optical density was measured at grey levels. Non-specific background ODs were measured at each brain level in a region devoid of MBP-immunostaining and were subtracted from the corpus callosum and cortex values. The area of MBP-positive fibres

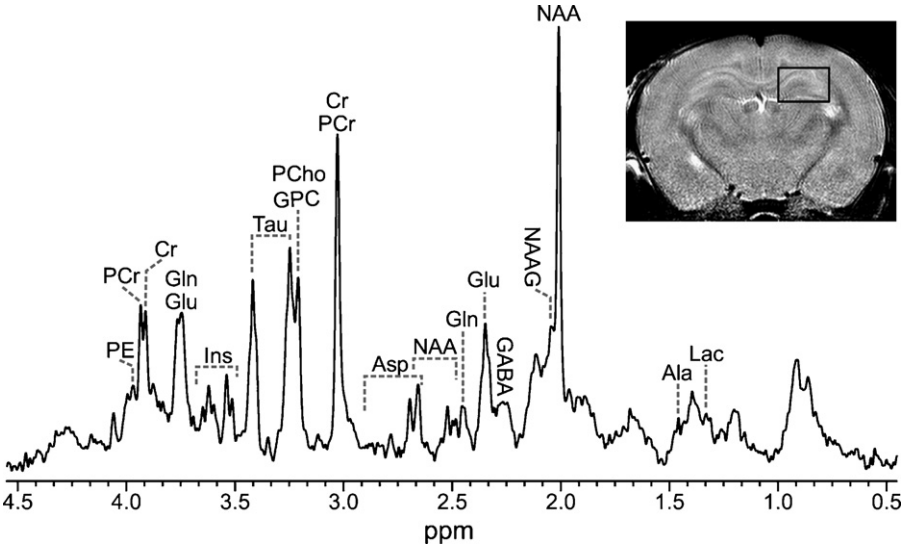

Fig. 1. Proton nuclear magnetic spectroscopy (^{1}H NMR). Typical ^{1}H NMR spectrum acquired in a volume of 12 μl on the control group showing the metabolite resonances. Note the excellent spectral resolution, evidenced for example by the separation of PCr, Cr at 3.9 ppm and NAA, NAAG at 2 ppm. Processing for display: Gaussian filtering with Gaussian width = 0.12 and shift = 0.05. Acquisition parameters: echo time = 2 ms, repetition time = 4 s, mixing time = 20 ms, number of transient = 360. No baseline correction was applied. The inset shows *in vivo* T_2-weighted fast-spin echo image (16 echo train with 10 ms echo spacing, effective echo time = 80 ms, repetition time = 6 s, acquisition matrix = 256 × 256, FOV = 20 mm × 20 mm, slice thickness = 1 mm). The black box indicates the voxel position placed on the hippocampus (3 mm × 4 mm × 3 mm).

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in the cortex, relative to cortical size, was assessed using MetaMorph® Imaging System.

3. Results

3.1. Nuclear magnetic resonance

During the *in vivo* MRS measurements, the whole blood lactate measurement done before and after the experiment did not show any significant difference. Moreover, excellent spectral quality was achieved, as judged from water line width, ranging from 8 Hz to 10 Hz (9 ± 0.7 Hz) in the hippocampus (Fig. 1). Water suppression typically resulted in a residual water signal below that of N-acetyl-aspartate (NAA) and a highly stable baseline. Signal to noise ratio was on average 20 ± 3. In addition, the separation of NAAG from NAA and PCr from Cr was consistently observed (Fig. 1). Such high spectral quality allowed the quantification of the concentration of 21 metabolites (neurochemical profile), of which 14 had a precision better than 20% and especially Cr, Tau, Cr + PCr, Glu, NAA, NAA + NAAG had a precision better than 5% (Table 2). Only these 14 metabolites were analysed for the study.

Overall, the neurochemical profile of the BPA-treated animals was very similar to that of the control group (Table 3). However, a statistically significant increase of glutamate (Glu) from $[Glu]_{control} = 9.0 \pm 0.3$ μmol/g, to $[Glu]_{BPA} = 9.7 \pm 0.2$ μmol/g ($p = 0.035$, unpaired *t*-test) was noted. Interestingly, aspartate (Asp), which is metabolically linked to Glu by the transaminase reaction showed a trend towards a decrease. Consequently, the Glu/Asp ratio significantly increased from $[Glu/Asp]_{control} = 4 \pm 0.3$ μmol/g to $[Glu/Asp]_{BPA} = 5.5 \pm 0.4$ μmol/g ($p = 0.02$) (Fig. 2), whereas the sum of Asp + Glu concentrations did not change significantly ($p > 0.5$). Glu and Asp resonances were well separated in the ^{1}H spectra, therefore the correlation between these two metabolites during the quantification was very low (average correlation coefficient of 0.04). However, it is important to notice that Asp concentration was quantified with a CRLB of 35% in the BPA-treated animals. Due to the biological implication of Asp in this model, its quantification was kept as a significant result. Furthermore, the Glu/Asp ratio was still significant despite the higher variability on the Asp concentration.

Table 2
Cramér–Rao lower bound (CRLB) of the metabolite concentration quantified with LCModel.

Metabolite	CRLB [%]	
	Control	BPA
Asc	10	11
Asp	19	35
Cr	7	7
PCr	5	5
GABA	12	12
Glc	13	15
Gln	13	13
Glu	4	3
GSH	18	19
Ins	6	6
Lac	25	24
NAA	2	2
NAAG	19	21
PE	8	9
Tau	3	3
NAA + NAAG	2	2
Glu + Gln	4	3
GPC + PCho	9	8
Cr + PCr	2	2

All selected metabolites have a CRLB lower than 20% assuring a robust quantification of metabolite concentrations.

Table 3
Metabolite concentration.

Metabolite	Concentration [μmol/g] ± SE	
	Control	BPA
Asc	3.3 ± 0.1	3.2 ± 0.1
Asp	2.3 ± 0.2	1.9 ± 0.2
Cr	3.8 ± 0.1	3.8 ± 0.1
PCr	5.2 ± 0.1	5.3 ± 0.1
GABA	1.9 ± 0.1	1.9 ± 0.1
Glc	3.4 ± 0.2	3.2 ± 0.1
Gln	2.0 ± 0.2	1.9 ± 0.1
Glu	9.0 ± 0.2	9.7 ± 0.2*
GSH	1.0 ± 0.1	0.9 ± 0.1
Ins	4.0 ± 0.1	4.1 ± 0.2
Lac	0.7 ± 0.1	0.9 ± 0.2
NAA	10.0 ± 0.2	9.6 ± 0.2
NAAG	0.9 ± 0.1	0.8 ± 0.1
PE	3.1 ± 0.1	3.3 ± 0.1
Tau	12.0 ± 0.3	12.00 ± 0.1
NAA + NAAG	10.8 ± 0.1	10.44 ± 0.2
Glu + Gln	11.0 ± 0.4	11.59 ± 0.2
GPC + PCho	1.0 ± 0.1	1.1 ± 0.1
Cr + PCr	8.9 ± 0.2	9.2 ± 0.2
Glu + Asp	11.2 ± 0.9	11.57 ± 0.5
PCr/Cr	1.4 ± 0.1	1.4 ± 0.1
Glu/Gln	4.6 ± 0.3	5.3 ± 0.2
Glu/Asp	4.0 ± 0.3	5.5 ± 0.4*

Concentrations of brain metabolites present in the hippocampus at PND20 in the control and BPA groups (mean ± SEM).
* $p < 0.05$, versus control offspring.

3.2. Histological analysis

There were no gross morphologic changes (haemorrhages, lesions or infarcts) in the brains of either control or BPA-treated offspring.

The morphology and density of NeuN-positive neurons were assessed in the motor and somatosensory cortices, and hippocampus. In both the motor and somatosensory cortices, no striking changes in neuronal morphology could be seen and there was no significant difference between control and BPA-treated offspring in the density of neurons ($p > 0.05$, Table 4). In the hippocampus, neuronal morphology appeared to be similar in the two groups but the NeuN-positive neuron density was decreased in the BPA-treated offspring when compared to controls ($p < 0.05$).

The density of GFAP-positive cells was assessed in the cingulum. Astrocytes appeared with increased processes in the BPA treated pups compared to controls. GFAP density in astrocytes was increased in BPA-treated offspring compared to controls ($p < 0.05$; Table 4).

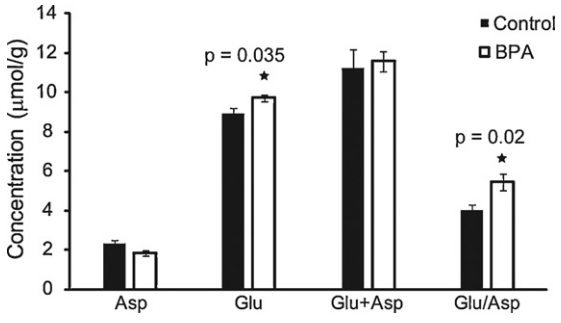

Fig. 2. ^{1}H NMR metabolite concentrations. Concentration of glutamate (Glu) and aspartate (Asp) in hippocampus at PND20 in the control group (black) and in the BPA group (white). Glu was significantly increased in the BPA group ($p = 0.035$). The Glu/Asp ratio shows a significant decrease ($p = 0.02$). Error bars are the error of the mean. * $p < 0.05$, versus control offspring.

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Table 4
Histology results.

	Control	BPA
Motor cortex (neurons/mm^2)	1281.0 ± 72.0	1162.9 ± 44.1
Somatosensory cortex (neurons/mm^2)	888.7 ± 44.1	882.3 ± 45.1
Hippocampus (neurons/mm^2)	4437.6 ± 136.0	3753.0 ± 83.0*
Astrocyte density (GFAP/mm^2)	664.6 ± 102.7	1080.6 ± 104.3*
Myelin: cortex (OD)	0.135 ± 0.014	0.125 ± 0.004
Myelin: corpus callosum (OD)	0.291 ± 0.026	0.317 ± 0.017
Percent of myelination fibres in cortex (%)	53.1 ± 1.4	56.2 ± 0.7

Density of neurons and glia in the cortex and hippocampus of control and BPA-treated offspring.
* $p < 0.05$, versus control offspring.

In order to determine the extent of myelination, MBP staining in the corpus callosum and cortex was assessed. There was no difference in MBP staining in the corpus callosum or cortex ($p > 0.05$; Table 4) between the groups. In addition, the area of MBP-positive fibres in the cortex, relative to cortical area, was not significantly different between the groups ($p > 0.05$).

4. Discussion

In the present study, a combination of ^{1}H MRS and histopathology was used in order to characterize different mechanisms likely to contribute to adverse neurodevelopmental outcomes following BPA exposure. This study has shown that offspring from dams exposed to BPA during pregnancy and lactation, exhibit alterations in localized cerebral metabolism, in neuronal and glial development. The NMR *in vivo* results show that a consistent neurochemical profile can be obtained from small volumes in the developing rat hippocampus, with a precision of better than 20% allowing the investigation of the neurochemical profile sequelae of environmental disruptors on brain development. Treatment of rat pups with BPA altered the Glu/Asp ratio reinforcing the determination of the neurochemical profile as a robust investigational tool in environmental science.

The stable concentration of whole blood lactate during the ^{1}H MRS confirms the non-invasive capability of the MRS techniques.

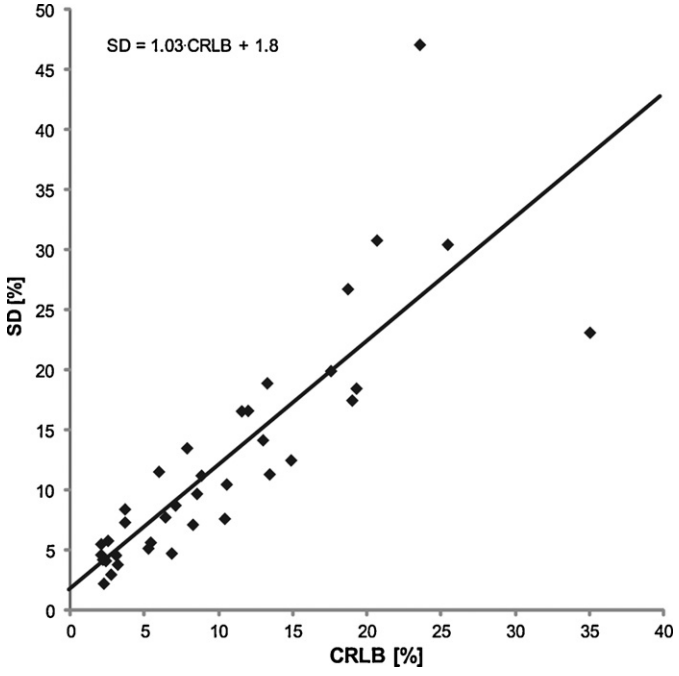

Fig. 3. Correlation graph between SD and CRLB. The CRLB follows very closely to the standard deviation within the two groups with a correlation factor close to 1.

The neurochemical profile and average precision of 12% of the control group was in excellent agreement with a previous study on developing rat brain (Tkac et al., 2003). The precision of the measurement was determined from the CRLB, a standard output of the LCModel program. The CRLB follows very closely the SD inside the group with a correlation factor close to one (Fig. 3). Consequently this implies negligible inter-animal variability.

BPA pre and post-natal treatments resulted in small changes in the neurochemical profile that reached statistical significance for glutamate, where an increase was noted. This Glu increase could be reflecting an alteration of Glu uptake by the astrocytes through ERs (Sato et al., 2003). We further observed a trend for Asp to decrease and thus evaluated the Glu/Asp ratio, which was significantly increased. However, it is important to notice that Asp concentration was quantified with a CRLB of only 35% in the BPA-treated animals, which may explain why Asp decrease was not significant. Glu and Asp fulfil many roles in mammalian cells of the nervous system and are metabolically linked by the transaminase reaction. The altered Glu/Asp ratio may thus indicate an impaired aspartate amino-transferase reaction, a key step in the malate–aspartate shuttle, a scheme of which is shown in Fig. 4. The malate–aspartate shuttle serves to transport reducing equivalents across the inner mitochondrial membrane and is thus a crucial component in glucose respiration, which powers almost all energy demands in the brain. The malate–aspartate shuttle has a rate-controlling step at the Glu/Asp antiporter, which transports a proton and glutamate in exchange for Asp across the inner mitochondrial membrane. The observation of increased Glu is consistent with excess glutamate that is not adequately transported into the mitochondrion. Likewise, the reduced Asp is consistent with insufficient transport of Asp out of the mitochondrion, compared to the activity of the cytosolic dehydrogenases. It is thus likely that the altered Asp/Glu ratio indicates impaired malate–aspartate shuttle activity and thus implies impaired mitochondrial function.

In this context, it is of interest to note that BPA has been reported to interfere with mitochondrial integrity. Nakagawa and Tayama (2000) demonstrated in isolated rat's liver mitochondria that the intracellular levels of ATP are decreased due to an inhibition of NAD^+- and FAD-linked respiration. The malate–aspartate shuttle is central to NAD^+ regeneration. Bindhumol et al. (2003) have also reported a decrease in the activity of antioxidant enzymes, which further support impaired mitochondrial function. Furthermore, it has been shown that BPA induced ROS production and oxidative stress, which compromised mitochondrial function (Ooe et al., 2005).

In vivo, BPA exposure during pregnancy and lactation has been shown to decrease neuronal density in the hippocampus (Choi et al., 2007; Ogiue-Ikeda et al., 2008; Sato et al., 2002). Several studies have reported alterations in hippocampal morphology with altered estradiol and testosterone induced spine synapse formation not only in rats but also in non human primates following BPA exposure (Lee et al., 2008; Leranth et al., 2008; Maclusky et al., 2005; Zhou et al., 2009). Synapse plasticity in the striatum is also perturbed after pre- and postnatal exposures with alteration of the function of dopaminergic receptors and disturbance in the developmental pattern of synaptic plasticity, which causes controlling deficits in motor behaviour (Zhou et al., 2009). Earlier studies (Ikemoto et al., 2004) reported that the exposure to BPA during pre- and postnatal developments has long-lasting effects on central dopaminergic systems linked with behavioural rewarding effects. *In vitro* exposure to BPA results in a marked influence on synaptogenesis and potentially neuronal vulnerability in hippocampal cultures (Sato et al., 2002; Zhou et al., 2009). The effects of BPA on synaptogenesis could be mediated through several mechanisms, including a number of receptor systems, such as estrogen (ER-α and ER-β) and thyroid receptors (TRs). As synapse remodelling in the

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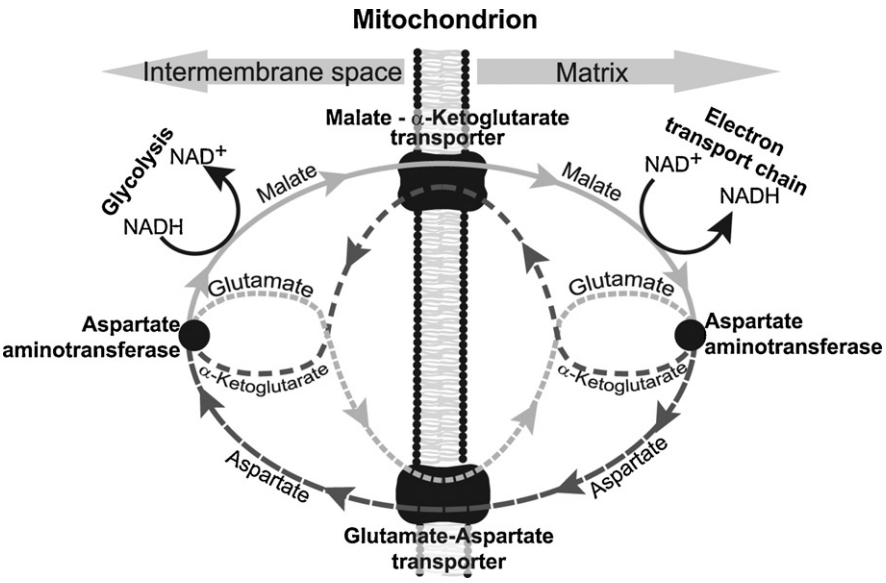

Fig. 4. The malate–aspartate shuttle. It recycles the reducing equivalents, links together Glu and Asp during the aspartate aminotransferase. An impaired Glu/Asp ratio alters the malate–aspartate shuttle function.

hippocampus is implicated in memory acquisition, retention and learning (Silva, 2003), it is likely that BPA exposure during critical periods of development could lead to altered cognitive function.

Further, direct neuronal toxicity of BPA induced apoptotic cell death have been suggested to be through calcium, ROS, ERK, and JNK *in vitro*. In contrast, NF-kappaB cascade was activated for survival signalling after BPA treatment (Lee et al., 2008). Therefore the mechanisms leading to decrease of hippocampal volume and neuronal density seen in our study are likely multi-factorial not only with a direct toxicity of BPA on the developing neurons, but also with BPA's ability to alter synaptogenesis that would lead to increased developmental pruning of neurons unable to establish sufficient synaptic activity for survival.

The decrease in hippocampal neuronal density and volume may reflect inadequate accelerated neuronal differentiation and maturation with an increase in the normal developmental process of programmed cell death. On the other hand, BPA exposure has also been shown to increase cell growth of immature neurons and neurospheres *in vitro* (Kubo et al., 2004), and *in vivo*, to promote Purkinje dendritic growth (Shikimi et al., 2004) and accelerate neocortical neuronal differentiation/migration (Nakamura et al., 2006). This neuronal differentiation/migration could be transitory and counter-balanced by the altered synaptogenesis and increased neuronal pruning. The current study cannot differentiate between these mechanisms of altered neuronal development but certainly confirms that gestational and postnatal exposition to BPA provides altered hippocampal development.

The areal density of GFAP-positive astrocytes was increased in BPA-treated offspring compared to controls (Cai et al., 2006). Several mechanisms of astrocyte activation have been linked to BPA exposure. Hydroxy radical formation has been shown to be increased in adult mouse brain following 5 days of BPA exposure (Kabuto et al., 2003) and BPA exposure during pregnancy and lactation increases lipid peroxidation in the postnatal mice brain (Kabuto et al., 2004). Thus, the activation of astrocytes in the white matter observed in the present study may be due to BPA-induced tissue oxidative stress and peroxidation. *In vitro*, low-level BPA has been shown to increase the production of GFAP protein in differentiating astrocyte progenitor cells, an effect mediated through the activation of signal transducer and activator of transcription 3 (STAT3), and mothers against decapentaplegic homolog 1 (Smad1) (Yamaguchi et al., 2006). Several recent reports show that BPA binds to thyroid hormone receptors (THRs), and has selective effects on thyroid function (Bindhumol et al., 2003; Zoeller, 2007). Thyroid hormone (TH) is a pivotal factor in astrocyte development (Adachi et al., 2002; Seiwa et al., 2004), thus, the increase in GFAP expression in the present study may reflect BPA's ability to influence TH signalling in the developing brain. The effects of BPA on brain development are also mediated for the most part by ERs and astrocytes are important target cells for estrogens and express all types of ERs during development and in the adult brain (Chaban et al., 2004; Hosli et al., 2001; Sato et al., 2003). Treatment of mouse purified astrocytes with BPA *in vitro* causes astrocyte activation, as detected by an increase in the levels of GFAP-immunoreactivity (Miyatake et al., 2006). Thus, GFAP upregulation and increase in astrocyte observed in the present study may also be ER-mediated. Further, estrogens and BPA down-regulate L-Glu uptake activity of astrocytes through ERs, which regulates the L-Glu concentration in the synaptic clefts, thereby altering synaptic transmissions and glutamate excitotoxicity. BPA might then alter not only directly synapse formation and activity but also through ERs on astrocytes (Sato et al., 2003). In summary, BPA has the capability of modulating astrocyte development and function through several different mechanisms leading to changes in the glial role and function within the brain.

Taking into account the significant change in the Glu/Asp ratio in the hippocampus observed by MRS, it is possible that the decrease in neuronal density in the hippocampus may be secondary to impaired mitochondrial function and reduced ability of the brain to oxidize glucose, thus leading to cell death. A critical experiment for future studies will be to examine the long-term effects of BPA-exposure on the brain, as alterations in brain structure may appear secondary to further impairments in cerebral metabolism, which may exacerbate during normal aging.

In conclusion, exposure to low dose BPA during gestation and lactation results in subtle and regional neuronal and glial alterations in brain development in offspring. Furthermore, BPA exposure leads to significant changes in the Glu/Asp ratio in the hippocampus, which is postulated to reflect impaired mitochondrial function and probably implicates a reduced ability of the brain to oxidize glucose especially in conditions of elevated energetic demand. The changes we observed in brain structure and metabolism after prenatal BPA exposure will likely imply long-lasting effects on cognitive development and function.

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