Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2010 | 115 | 1466–1477 doi: 10.1111/j.1471-4159.2010.07051.x

Neurochemical profile of the developing mouse cortex determined by *in vivo* ^{1}H NMR spectroscopy at 14.1 T and the effect of recurrent anaesthesia

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Abstract

The neurochemical profile of the cortex develops in a region and time specific manner, which can be distorted by psychiatric and other neurological pathologies. Pre-clinical studies often involve experimental mouse models. In this study, we determined the neurochemical profile of C57BL/6 mice in a longitudinal study design to provide a reference frame for the normal developing mouse cortex. Using *in vivo* proton NMR spectroscopy at 14 T, we measured the concentrations of 18 metabolites in the anterior and posterior cortex on postnatal days (P) 10, 20, 30, 60 and 90. Cortical development was marked by alterations of highly concentrated metabolites, such as *N*-acetylaspartate, glutamate, taurine and creatine. Regional specificity was represented by early variations in the concentration of glutamine, aspartate and choline. In adult animals, regional concentration differences were found for *N*-acetylaspartate, creatine and *myo*-inositol. In this study, animals were exposed to recurrent isoflurane anaesthesia. Additional experiments showed that the latter was devoid of major effects on behaviour or cortical neurochemical profile. In conclusion, the high sensitivity and reproducibility of the measurements achieved at 14 T allowed us to identify developmental variations of cortical areas within the mouse cortex.

Keywords: anaesthesia, cerebral cortex, development, metabolism, NMR spectroscopy.

J. Neurochem. (2010) **115**, 1466–1477.

The mammalian brain matures gradually in a highly region specific manner. At particular developmental stages, brain cells differentiate and are integrated in complex functional networks. The degree of cellular differentiation (e.g. dendritic and axonal aborization and myelination) at any given developmental time point is reflected in regional alterations of the neurochemical profile as reported in the rat brain (Tkáč *et al.* 2003). Thus, metabolite composition and concentration changes across time allow us to make inferences about the degree of regional development. There is shared consensus that psychiatric disorders including schizophrenia (Tsuang 2000), autism (Keller and Persico 2003) and attention deficit/hyperactivity disorder (for review see Curatolo *et al.* 2009) result from interactions between genetic and environmental risk factors during neurodevelopment. For a better understanding of these disorders, it is crucial to characterize the aberrant developmental processes and to pinpoint sensitive periods during which they occur. One tool that allows *in vivo* monitoring of developmental changes in the neurochemical

Received June 15, 2010; revised manuscript received September 27, 2010; accepted October 5, 2010.

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Abbreviations used: Ala, alanine; Asc, ascorbate; Asp, aspartate; BBB, blood–brain barrier; Cr, creatine; CRLB, Cramér-Rao lower bound; Glc, glucose; Gln, glutamine; Glu, glutamate; Ins, *myo*-inositol; Lac, lactate; NAA, *N*-acetylaspartate; NAAG, *N*-acetylaspartatylglutamate; PCho, phosphocholine; PCr, phosphocreatine; PE, phosphoethanolamine; Tau, taurine; VOI, volume of interest.

profile is NMR spectroscopy. As such, it is particularly useful for longitudinal study designs to observe metabolite changes in the same subjects over time. Basic research aiming at understanding pathologies of development is predominantly conducted in animal models. This is mainly the result of great advances in genetic engineering which allow us to modify disease associated genes. The purpose of the present study was to provide a comprehensive account of the neurochemical profile in the developing mouse cortex using an *in vivo* non-invasive longitudinal study design. Although previous NMR studies have addressed metabolic modifications in the developing mouse brain (Larvaron *et al.* 2006; Weiss *et al.* 2009), a very limited number of metabolites was investigated, namely *N*-acetylaspartate (NAA), taurine (Tau), total creatine (Cr) and total glutamate plus glutamine. In addition, only the study by Weiss *et al.* (2009) employed a true longitudinal design scanning the same animals repeatedly. With ^{1}H NMR spectroscopy at 14.1 T, we evaluated concentration changes of 18 metabolites at five developmental stages – postnatal days (P) 10, 20, 30, 60 and 90 – for volumes in the anterior and posterior cortex.

This study design incorporated recurrent scans of the same animals, and thus required recurrent episodes of anaesthesia. To our knowledge, no study has yet investigated whether recurrent anaesthesia induces changes in mouse brain metabolism. Thus, to ensure an unbiased interpretation of our results, we conducted a control experiment in which we exposed animals to recurrent anaesthesia at the same developmental time points as mentioned above. Subsequently, we investigated whether recurrent anaesthesia affected the normal behaviour of the animals or the neurochemical profile.

We found that recurrent anaesthesia during mouse development has no adverse effect on behaviour and does not cause major changes to the neurochemical profile in the adult animal. Our experiments show moreover that mouse cortical development is marked by strong alteration of highly concentrated metabolites such as NAA, Glu, Tau or Cr and by early regional variations in the concentration of Gln and aspartate (Asp) before P20, and choline between P20 and P60. In adult animals, anterior and posterior cortical areas were marked by differences in NAA, Cr and *myo*-inositol (Ins). These results expand our knowledge of normal mouse brain development and as such provide a valuable basis for the study of developmental defects in the cortex of a plethora of available mouse models of disorders affecting the CNS.

Materials and methods

Animals

All experiments were conducted under approval of the local ethics committee. For all experiments, we used C57BL/6 mice that were born in the local animal facility. The neurochemical profile of anterior and posterior cortical regions was determined in 18 mice (five males and 13 females) at postnatal days (P) 10, 20, 30, 60 and 90, under anaesthesia of 1–1.5% isoflurane in oxygen gas.

As *in vivo* NMR scanning of animals at different stages of development required recurrent anaesthesia, we determined whether multiple isoflurane anaesthesia sessions during development might affect the cortical neurochemical profile of the adult mice. For this purpose, a second cohort of mice (10 males and nine females) was anaesthetized at P10, P20, P30 and P60 with 2% isoflurane (in oxygen) for 90 min, the temperature being maintained at 37°C. Control mice (nine males and eight females) were not anaesthetized but were separated from the mother for the same period and maintained under identical temperature and housing conditions. The neurochemical profile was determined at P90. The mice were furthermore subjected to a comprehensive battery of behavioural tasks to investigate whether recurrent anaesthesia interferes with basic motor function, exploratory activity, anxiety-like behaviour, mood and learning capacity.

Behavioural testing

To test whether recurrent anaesthesia during postnatal development has adverse effects on the animals' general health status and functioning, we conducted a number of standardized behavioural tests between P70 and P85. The tests were conducted in the same order as they are described.

Grip strength test

With both forepaws each animal was placed onto a grip-strength meter (Columbus Instruments, Columbus, OH, USA). The experimenter gently pulled the mouse backwards by holding it at its tail. The device measured the peak force exerted by the action of the animal while gripping the sensor bar. For analysis, we used the maximum force the animal exerted at the moment of bar release. The grip strength for each animal was determined as the average of three successive trials.

Hanging wire test

With their forepaws, animals were individually placed onto a metal wire that was horizontally suspended between two wooden blocks at an elevation of 15 cm. The animal had then to move along the wire (a distance of 30 cm) to escape onto one of the wooden blocks. Latency to escape was recorded.

Rota-rod test

Four animals at a time were placed onto an automated rota-rod (Ugo Basile, Comerio, Italy). For 2 min, animals were allowed to habituate to walking on the rod at slow speed. Following the first 2 min, the rod accelerated linearly. Latency to fall was recorded.

Open field test

Each animal was placed into the centre of a white non-translucent rectangular Plexiglas open field arena of 60 cm × 40 cm (20 cm high) and exploration behaviour was monitored for 20 min. For the purpose of data analysis, the arena was virtually divided into centre and surround region. Locomotor velocity, distance and time spent within the centre were recorded with automated tracking software (Ethovision, Noldus, the Netherlands).

Elevated plus maze

The maze consisted of white Plexiglas runways (each 30 cm long and 5 cm broad) arranged in form of a plus at an elevation of 65 cm above the floor. Two opposing runways were protected by black Plexiglas side walls (10 cm high; closed arms), the other arms contained no walls (open arms). All four arms were joined by a center platform (5 cm × 5 cm). Animals were individually placed into the center of the maze facing an open arm and behaviour was monitored for 10 minutes with Ethovision tracking software. Latency to enter an open arm, entry frequency and time spent in the open and closed arms were recorded. An arm entry was recorded when the whole body (tail excluded) of the animal had entered a zone.

Y-maze spontaneous alternation

Based on rodents' natural preference for novelty, mice tend to explore the arms of a Y-maze successively resulting in an alternation pattern, which depends on intact spatial working memory. The maze consisted of three translucent equidistant Plexiglas tunnel arms (30 cm long, 5 cm wide and 7 cm high) with a sliding door at the distal end of each arm. Each animal was released at the distal end of one arm and behaviour was observed for 8 min. Start arms were randomized between animals and groups. All sessions were videotaped. The sequence of arm entries and total number of arm visits, were scored manually. An alternation was defined as three successive entries into the three separate arms (i.e. ABC, ACB, BAC, BCA, CAB, or CBA). The per cent alternation was calculated by multiplying the number of alternations with 100, divided by the total number of possible alternations (i.e. total number of arm entries minus 2).

Forced swim test

Animals were individually placed into a transparent Plexiglas cylinder (inner diameter: 18 cm; height: 25 cm) filled with water (25°C) to a height of 15 cm. Swimming behaviour was monitored with a camera and Ethovision tracking software for 5 min. Swim velocity and distance as well as latency to float were recorded. Floating was defined as a complete absence of movements of all four paws.

Spatial and object recognition memory

We used an adapted version of the object exploration paradigm originally described by Buhot and Naïli (1995). The test apparatus was a grey non-translucent square Plexiglas arena (in cm: 50 × 50 × 30). The walls contained a pattern of white stripes that was interrupted by an area (10 cm wide, 30 cm high) of white circles on only one wall, which served as internal spatial cue. The test consisted of one habituation and six test sessions. Each session lasted 8 min. Between sessions, animals were placed into a holding cage with access to food and water. Intersession intervals lasted 5 min before familiarization sessions and 15 min before memory test sessions. Following the habituation period, three dissimilar objects were secured at pre-determined positions within the arena. Test sessions 1, 2 and 3 served as familiarization sessions, during which all objects remained at the same positions, and animals could establish a stable memory of the objects and their particular spatial arrangement. After the third familiarization session, one object was displaced to a new position. Test session 4 evaluated spatial memory (i.e. whether the animal recognized the position change and displayed an exploratory preference for the displaced object). Subsequently, animals were familiarized to the novel spatial arrangement in one session (test session 5). Following that, one object that had previously remained in its original position was replaced by an unfamiliar yet un-encountered object to test object recognition memory in session 6. Animals that formed a stable representation of previously present objects, should now preferentially explore the novel object. Test sessions 1, 3, 4 and 6 were video recorded and the number of object contacts was scored.

NMR spectroscopy

Spontaneously breathing mice under isoflurane anaesthesia (1.0–1.5% in oxygen gas) were fixed in a custom-made holder with foam cushion for head immobilization (at P10, P20 and P30) or in a mouse holder with a bite bar and two ear inserts (RAPID Biomedical, Rimpar, Germany; at P60 and P90). Body temperature was maintained at 37°C by warm water circulation. The duration of each NMR scanning session was restricted to 100 min maximum.

All experiments were carried out in a horizontal 14.1 T/26 cm magnet (Varian/Magnex Scientific, Abingdon, UK), with a 12-cm inner-diameter gradient (400 mT/m in 200 ms, minimized eddy currents), interfaced with a DirectDrive console (Varian Inc., Palo Alto, CA, USA). Radio frequency transmission and reception was achieved with a home-built quadrature surface coil composed of two geometrically decoupled single-turn loops of 8 mm inner diameter resonating at 600 MHz. The mouse brain was positioned in the isocentre of the magnet and located with fast-spin-echo images with repetition time of 4 s, echo time of 52 ms and echo train length of 8. Field homogeneity in the region of interest was achieved with FAST(EST)MAP (Gruetter 1993; Gruetter and Tkáč 2000). Volumes of interest (VOI) were placed in anterior and posterior regions of the cortex of the C57BL/6 mice (respectively, at ∼0.62 and ∼−2.0 mm relative to Bregma, according to the mouse brain atlas by Franklin and Paxinos 2008). As shown in Fig. 1, the anterior VOI was placed above the frontal part of the ventricles whyle the posterior VOI was above the hippocampal formation. The size and position of the VOIs were adjusted proportionally to the brain size of the mice at different ages to include similar tissue composition (see illustration in Fig. 1). Thus, the total volume of the VOIs ranged from 2.5 to 3.8 μL (Table 1). ^{1}H NMR spectra were acquired using SPECIAL (Mlynárik *et al.* 2006) with echo time of 2.8 ms, repetition time of 4 s and typically with 480 scans.

Metabolite concentrations were determined using the linear combination analysis method LCModel (Provencher 1993), including a macromolecule spectrum in the database, as in previous studies (e.g. Mlynárik *et al.* 2006). The unsuppressed water signal measured from the same VOI was used as an internal reference for the quantification of metabolites, using corrections for water content variations during development. Namely, water content was assumed 86.5% at P10 and 80.0% from P20 onwards (e.g. Tkáč *et al.* 2003; Larvaron *et al.* 2006). The following 20 metabolites were included in the LCModel analysis: alanine (Ala), ascorbate (Asc), Asp, Cr, GABA, Gln, Glu, GSH, glycine, glycerophosphocholine, glucose (Glc), lactate (Lac), Ins, NAA, *N*-acetylaspartylglutamate (NAAG), phosphoethanolamine (PE), phosphocholine (PCho), phosphocreatine (PCr), *scyllo*-inositol, Tau. The Cramér-Rao lower bound (CRLB) was provided by

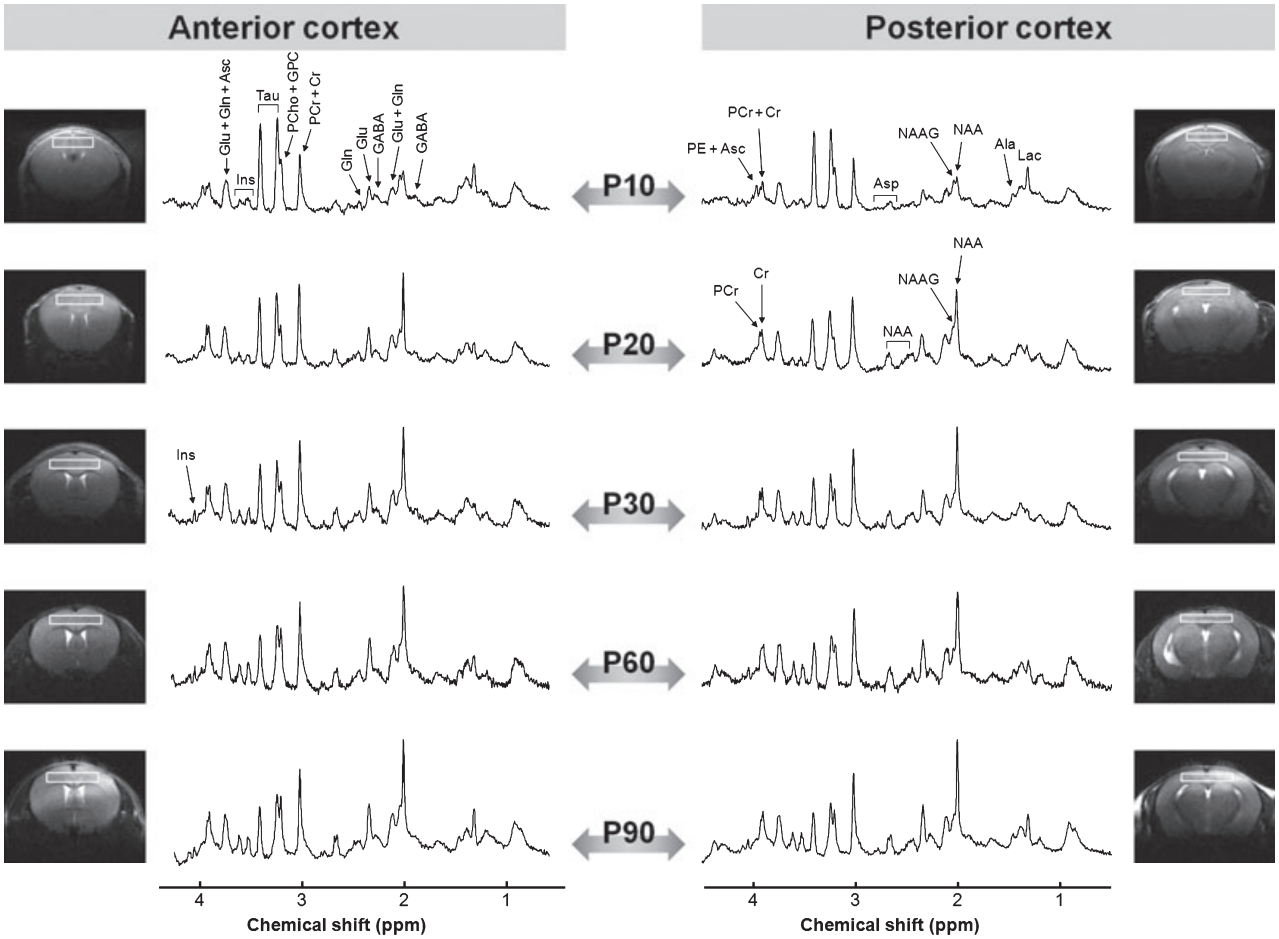

Fig. 1 Representative *in vivo* ^{1}H NMR spectra at 14.1 T in the mouse cortex during development and in adult. Spectra were acquired from volumes of 2.5–3.5 μL localized in anterior and posterior regions of the mouse cortex, as indicated in the respective T2-weighed images, using SPECIAL with TE = 2.8 ms, TR = 4s and typically 480 scans. Free induction decays were weighted with a shifted gausian function (gf = 0.12, gfs = 0.05) before Fourier transformation. The signals assigned in the spectra refer to the following metabolites: alanine (Ala), ascorbate (Asc), aspartate (Asp), creatine (Cr), phosphocreatine (PCr), γ-aminobutyrate (GABA), glycerophosphocholine (GPC), glutamate (Glu) glutamine (Gln), *N*-acetylaspartate (NAA), *N*-aceyl-aspartylglutamate (NAAG), lactate (Lac), *myo*-inositol (Ins), phosphocholine (PCho), phosphoethanolamine (PE), taurine (Tau).

Table 1 Dimensions of the VOIs in anterior and posterior areas of the cortex was changed according to the brain size at different postnatal ages

		Height (mm)	Width (mm)	Length (mm)
P10	Anterior	0.8	3.5	1.0
	Posterior	0.7		
P20	Anterior	0.8	3.8	1.0
	Posterior	0.7		
P30	Anterior	0.8	4.0	1.0
	Posterior	0.7		
P60/P90	Anterior	0.8	4.0	1.2
	Posterior	0.7		

LCModel as a measure of the reliability of the apparent metabolite concentration quantification. At most of the measured ages, the present analysis was not able to reliably discern PCho from glycerophosphocholine. Therefore their sum was quantified as choline-containing compounds (GPC + PCho). As the relative content of Cr and PCr may depend on the energy status of the tissue, the total Cr concentration (Cr + PCr) was reported as a constitutive metabolic pool of the tissue.

Statistical analysis

Results are presented as mean ± SEM of *n* mice, unless otherwise mentioned. The concentration of each metabolite was analysed with a two-way ANOVA followed by the Bonferroni's multi-comparison post-test. Behavioural data were analysed with univariate ANOVA and significant interactions were followed up with independent sample *t*-tests. Significance was considered for $p < 0.05$.

Results

Effect of recurrent isoflurane anaesthesia on behaviour and neurochemical profile

In the present study, recurrent anaesthesia periods were required for *in vivo* determination of the cortical neurochemical profile evolution by ^{1}H NMR spectroscopy. Therefore, we first tested whether isoflurane anaesthesia during development has an effect on behaviour and the cortical neurochemical profile of adult mice.

Table 2 summarizes the behavioural data. Mice submitted to recurrent anaesthesia periods show similar performance as control mice in all behavioural tasks. We found a significant gender × group interaction for the latency to escape the hanging wire ($p = 0.008$). Follow-up *t*-tests for gender specific group differences were non-significant ($p = 0.061$ for males; $p = 0.126$ for females) suggesting that anesthetized and control animals of the same sex had a similarly good capacity to escape the hanging wire. This is in line with the fact that mice exposed to recurrent anaesthesia perform like control mice in all other measurements for general motor behaviour. Similarly, we found a significant gender x group interaction ($p = 0.047$) for the latency to float in the Porsolt swim test. Again, *post hoc t*-tests tests revealed no gender-specific impairments ($p = 0.077$ for males; $p = 0.246$ for females). Thus, overall our results suggest that repeated isoflurane anaesthesia during development does not affect adult mouse behaviour.

From the ^{1}H NMR spectra of recurrently anaesthetized and control mice (P90), we were able to estimate the concentration of 20 metabolites, in anterior and posterior regions of the cortex, with average CRLBs of 15 ± 4%. As the neurochemical profile in both cortical regions was similar in male and female mice, the data of both genders were combined. Table 3 shows that the neurochemical profile of both the anterior and posterior cortex was not generally affected by recurrent anaesthesia periods during development. Only Tau levels were consistently increased in posterior and anterior cortical areas by 15 ± 3% ($p < 0.01$) and 8 ± 3% ($p < 0.05$) respectively, in comparison to the unanaesthetized group. Glu content was significantly increased by recurrent anaesthesia in the posterior cortex (+ 9 ± 2%, $p < 0.05$).

Development of the neurochemical profile

Examples of typical *in vivo* ^{1}H NMR spectra of the developing mouse anterior and posterior cortical areas are shown in Fig. 1. A first visual examination of the spectra indicates that major metabolite signals were similar in anterior and posterior cortex. However, development over time had a great impact on the majority of metabolites.

Table 2 Behaviour in adult animals after recurrent isuflorane anaesthesia. Data are presented as mean ± SEM. Performance on the rotarod is evaluated as the latency to fall and is shown in seconds. Other measurements in seconds are: Escape latency from a hanging wire, time spent in the open field center and the latency to float in the forced swim test. Distance travelled in the open field and swim distance are represented in meters. Data were analysed with univariate ANOVA. Significant interactions were followed up with independent sample *t*-tests. Symbols: §§significant gender × group interaction, $p < 0.01$. Note: there were no significant gender-specific group effects

	Males		Females	
	Control ($n = 8$)	Recurrent anaesthesia ($n = 10$)	Control ($n = 5$)	Recurrent anaesthesia ($n = 9$)
General motor behaviour				
Forepaw grip strength	0.217 ± 0.02	0.226 ± 0.02	0.213 ± 0.01	0.207 ± 0.02
Running on the rotarod	154.0 ± 21.8	133.5 ± 17.1	162.3 ± 14.5	195.2 ± 47.1
Escape from hanging wire	0.28 ± 0.05	0.16 ± 0.02§§	0.15 ± 0.02	0.24 ± 0.05
Open field exploratory activity				
Total distance	81.7 ± 2.8	92.1 ± 5.2	104.6 ± 4.7	93.9 ± 5.3
Time in center	163.3 ± 25.4	166.4 ± 14.1	194.7 ± 12.0	130.3 ± 10.7
Elevated plus maze				
Open arm entries	2.3 ± 0.5	1.8 ± 0.6	2.6 ± 0.6	2.8 ± 1.1
Forced swim test				
Total swim distance	11.1 ± 0.6	11.5 ± 0.6	10.5 ± 0.8	11.7 ± 1.1
Latency to float	109.0 ± 6.1	89.9 ± 8.2§	71.8 ± 14.8	92.8 ± 11.6
Spatial working memory				
Y-maze alternation ratio	0.60 ± 0.03	0.52 ± 0.02	0.53 ± 0.04	0.53 ± 0.05
Spatial memory				
Object exploration ratio	0.65 ± 0.02	0.63 ± 0.03	0.67 ± 0.03	0.63 ± 0.02
Object memory				
Object exploration ratio	0.68 ± 0.02	0.67 ± 0.02	0.71 ± 0.02	0.68 ± 0.04

Table 3 Adult neurochemical profile in the anterior and posterior cortex measured by *in vivo* ^{1}H NMR spectroscopy is not greatly affected by repeated isoflurame anaesthesia (2% in O_2 gas) for 90 min at P10, P20, P30, P60, and during scan at P90. Concentrations are shown as mean ± SD of nine male and eight female mice in the control group, and 10 male and nine female mice on the recurrent anaesthesia group. Data were analysed with the two-way ANOVA followed by the Bonferroni multi-comparison test (*$p < 0.05$, **$p < 0.01$ compared with unanaesthetized group)

	Anterior cortex		Posterior cortex	
	Control	Recurrent anaesthesia	Control	Recurrent anaesthesia
Alanine	0.90 ± 0.36	1.17 ± 0.57	0.88 ± 0.37	1.09 ± 0.39
Aspartate	1.84 ± 0.86	2.29 ± 0.48	1.85 ± 0.70	2.29 ± 0.87
PCho	0.48 ± 0.37	0.53 ± 0.35	0.56 ± 0.41	0.64 ± 0.30
Creatine	4.01 ± 0.57	3.85 ± 0.61	4.23 ± 0.74	4.50 ± 0.61
Phosphocreatine	3.59 ± 0.57	4.23 ± 0.70	3.94 ± 0.41	4.26 ± 0.83
GABA	1.80 ± 0.49	1.68 ± 0.26	1.99 ± 0.66	1.83 ± 0.26
Glutamine	3.57 ± 1.02	3.33 ± 0.83	3.85 ± 1.15	3.56 ± 0.91
Glutamate	10.4 ± 1.5	11.0 ± 0.8	10.3 ± 1.2	11.2 ± 0.9*
Glutathione	1.24 ± 0.41	1.36 ± 0.30	1.18 ± 0.41	1.19 ± 0.30
Glycine	0.92 ± 0.49	1.05 ± 0.44	1.53 ± 0.70	1.22 ± 0.44
myo-inositol	4.36 ± 0.86	4.51 ± 0.83	4.84 ± 0.90	4.62 ± 0.57
Lactate	1.69 ± 0.90	1.66 ± 0.87	2.05 ± 0.94	1.61 ± 0.87
N-Acetylaspartate	8.71 ± 0.98	8.54 ± 0.61	9.56 ± 0.98	9.73 ± 0.83
scyllo-inositol	0.18 ± 0.15	0.21 ± 0.73	0.18 ± 0.12	0.18 ± 0.09
Taurine	10.2 ± 1.8	11.0 ± 1.2*	8.90 ± 1.64	10.2 ± 1.3**
Ascorbate	1.49 ± 0.90	2.12 ± 1.04	1.21 ± 0.82	1.55 ± 0.83
Glucose	1.76 ± 1.68	1.60 ± 1.04	2.01 ± 1.35	1.83 ± 1.04
NAAG	0.82 ± 0.41	0.79 ± 0.22	0.85 ± 0.37	0.64 ± 0.26
GPC	0.70 ± 0.37	0.83 ± 0.35	0.52 ± 0.29	0.48 ± 0.30
Phosphoethanolamine	1.83 ± 0.62	1.94 ± 0.57	1.28 ± 0.62	1.72 ± 0.70
GPC + Pcho	1.11 ± 0.21	1.23 ± 0.22	1.03 ± 0.29	1.10 ± 0.22

GABA, γ-aminobutyrate; GPC, glycerophosphocholine; NAAG, *N*-aceylaspartylglutamate; Pcho, phosphocholine.

Clearly visible are gradual increases of the signals for Glu, NAA, Ins, Asp, total Cr and total choline. Simultaneously, signals corresponding to Tau, Asc and PE decreased with postnatal age.

The spectra allowed determining a neurochemical profile of 18 metabolites which were quantified with CRLB generally below 25%. Exceptionally, some metabolites that occur at low concentration in the cortex were quantified with CRLB above 25% at some postnatal ages, like Glc and NAAG, and Asp. The spectrum of *scyllo*-inositol was included in the linear combination analysis but since it was not detected in a large part of the analysed spectra, its concentration was excluded from the reported neurochemical profile during development.

Figures 2, 3 and 4 show the evolution of the concentration of each metabolite for both cortical areas. The neurochemical profile of the mouse cortex changed gradually from P10 to P90, with the most prominent modifications occurring between P10 and P20. Exception was GABA (Fig. 2) that remained nearly constant over time. While the cortical concentrations of NAA, Asp, Glu, Gln, Ins, Cr, choline compounds and glutathione increased with age, the content of glycine, NAAG, Tau, PE and Asc decreased in the developing mouse cortex ($p < 0.01$ for each of the metabolites, $n = 12$). As shown in Fig. 4, Glc concentration in the cortex increased with age ($p < 0.05$, $n = 12$); however, its glycolytic products displayed a different trend. Although Ala concentration reduced in the developing mouse cortex ($p < 0.001$, $n = 12$), Lac content was variable between 2.0 ± 0.2 and 1.3 ± 0.1 μg/mol with a maximum concentration at P60 ($p < 0.05$, $n = 12$).

The determination of absolute metabolite concentrations and respective analysis of variance revealed that the development of the neurochemical profile was distinct in the two analysed cortical areas. The metabolites that developed differently in anterior and posterior cortex include NAA, Asp, Gln, Ins, Cr and choline-containing compounds. More specifically, the concentrations of NAA, Ins and total Cr were similar in both regions from P10 to P60, but increased to a greater extent in the posterior than the anterior cortex at P90 ($p < 0.01$ for the three metabolites, $n = 12$). Gln concentrations were significantly higher in the posterior as compared to the anterior cortex at early development ($p < 0.001$, $n = 12$). In addition, the posterior cortex displayed lower

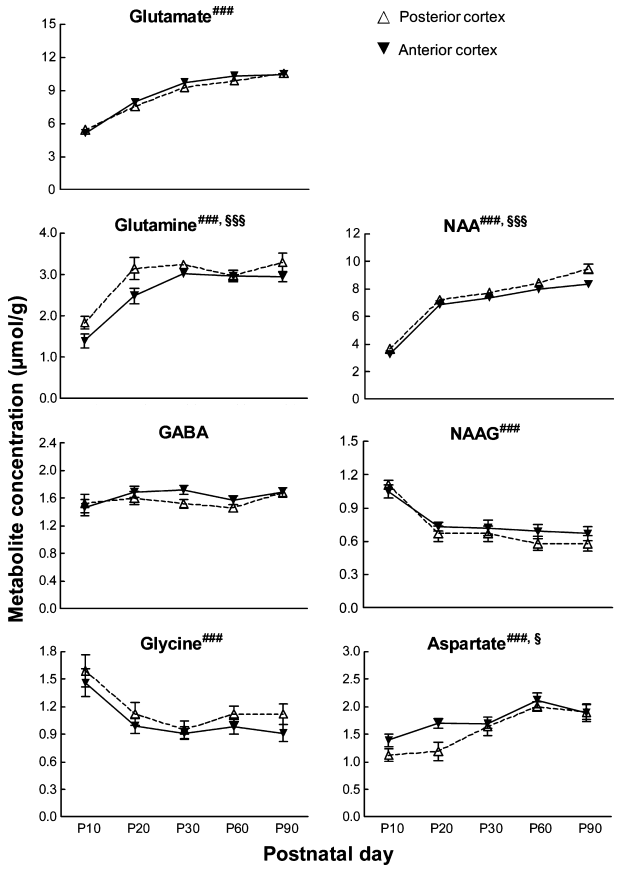

Fig. 2 Concentration of metabolites in the anterior (open symbols) and posterior (filled symbols) cortical areas of the developing mouse. Data are shown as mean ± SEM of 18 mice (five males and 13 females) and compared with two-way ANOVA. With exception of GABA, the concentrations of the metabolites in the figure were modified during development ($^{###}p < 0.001$); concentrations of glutamate, glutamine, NAA and aspartate develop differently in each cortical area ($^{§}p < 0.05$, $^{§§§}p < 0.001$).

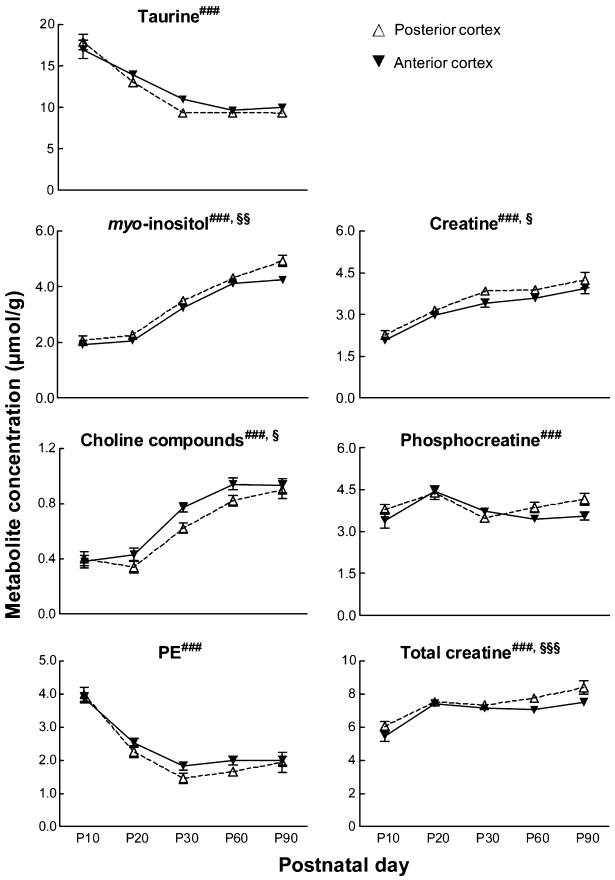

Fig. 3 Concentration of metabolites in the anterior (open symbols) and posterior (filled symbols) cortical areas of the developing mouse. Total creatine represents the sum of creatine and phosphocreatine concentrations. As at most ages the LCModel analysis failed to distinguish phosphocholine and glycerophosphocholine resonances, their sum is here displayed as the total choline-containing compounds. Data are shown as mean ± SEM of 18 mice (five males and 13 females) and compared with two-way ANOVA. The concentrations of all the metabolites in the figure were modified during development $^{###}p < 0.001$); the development of *myo*-inositol, creatine (and total creatine) and choline compounds is different the anterior and posterior cortex ($^{§}p < 0.05$, $^{§§}p < 0.01$, $^{§§§}p < 0.001$).

concentrations of choline-containing compounds than the anterior cortex at P30 and P60 ($p < 0.05$, $n = 12$).

Discussion

Recurrent anaesthesia

This is the first report of an extended neurochemical profile of 18 metabolites determined in the mouse cortex during development until adult age. The neurochemical profile was determined in a non-invasive manner using ^{1}H NMR spectroscopy under isoflurane anaesthesia. Previous studies have shown that isoflurane exposure during early development can lead to dose-dependent abnormal neuronal development, abnormal synaptic plasticity, neurodegeneration (reviewed in Wang and Slikker 2008), and behavioural and cognitive impairments (Rothstein *et al.* 2008; Valentim *et al.* 2008). Here, we demonstrate that recurrent 2% isoflurane anaesthesia during mouse development leaves cortical function intact as suggested by the generally unaltered neurochemical profile and normal behavioural performance. Tau was significantly changed in both anterior and posterior cortex following anaesthesia. It is known that isoflurane can modulate Tau release and tissue content (e.g. Ritz *et al.* 2006). The mechanism, however, remains unclear. We also observed a minor, although significant, elevation of Glu levels in the posterior cortex. Previous studies have shown that isoflurane can exert effects on the glutamatergic synapse by reducing pre-synaptic Glu release and increasing its uptake from the synaptic cleft (e.g. Larsen and Langmoen

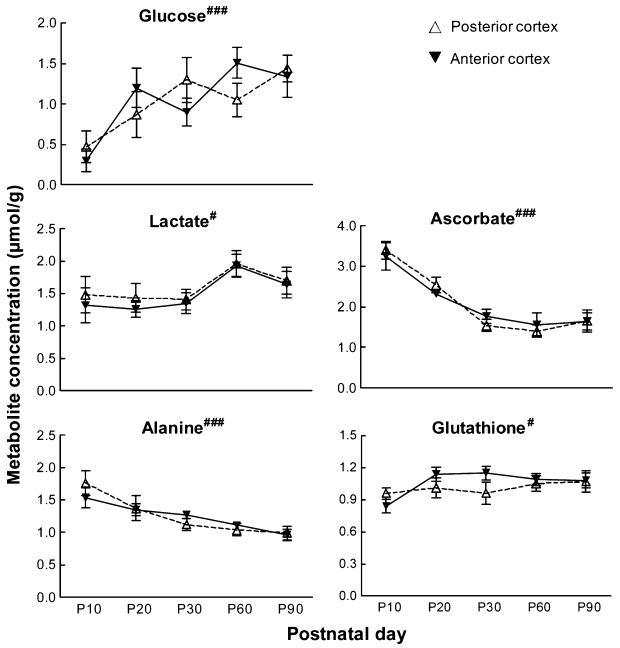

Fig. 4 Concentration of metabolites in the anterior (open symbols) and posterior (filled symbols) cortical areas of the developing mouse. Data are shown as mean ± SEM of 18 mice (five males and 13 females) and compared with two-way ANOVA. The concentrations of glucose, lactate alanine glutathione and ascorbate were modified during development ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$); the development of all the metabolites presented in the figure was not significantly different between anterior and posterior cortical areas.

1998). However, with our protocol, Glu alteration caused by recurrent anaesthesia was below 10% and was only significant in one of the measured cortical areas. Recently, it was shown that isoflurane anaesthesia during development may lead to neuronal loss and reduced neurogenesis in the hippocampus, propitiating memory impairment (Zhu *et al.* 2010). In this study by Zhu *et al.* (2010), like others, mice were submitted to anaesthesia sessions for four consecutive days, while in this study sessions occurred with intervals of 10 days. Interestingly, although not significant, PCr/Cr tended to increase in recurrently anaesthetized mice, supporting the lack of metabolic deterioration when compared to controls. In conclusion, recurrent isoflurane exposure during development might have small effects on Tau and Glu concentrations, which were detected as a result of the high reproducibility of the methodology used. All other metabolites remained unaltered, indicating that longitudinal study designs with C57BL/6 mice involving recurrent isoflurane anaesthesia during development can provide valuable data generally devoid of unspecific effects introduced by the anaesthesia procedure itself.

Developmental neurochemical profile of the mouse cortex

Two previous spectroscopy studies of mouse brain development investigated the neurochemical profile of the thalamus, the olfactory bulb, cerebellum (Larvaron *et al.* 2006), midbrain, and the basal ganglia (Weiss *et al.* 2009). This is the first study that evaluates the developing neurochemical profile of the mouse cortex. Our findings are in general agreement with results in rat cortex obtained *in vivo* (Tkáč *et al.* 2003) or from brain extracts (e.g. Burri *et al.* 1990). The concentrations of most brain metabolites comprising the neurochemical profile changed with development reflecting the structural and functional evolution inherent to the differentiation of cortical networks.

Neurotransmitter metabolism

Amino acid neurotransmitters are the most abundant transmitters in the CNS playing an important role in the wiring of neuronal networks and organizing brain cytoarchitecture during development. Glu and Asp are the dominant excitatory amino acids. Our spectra revealed that the concentrations of both increased steadily from P10 to P60 and reached a steady state thereafter, as previously demonstrated in cortical extracts (Benítez-Diaz *et al.* 2003). The fast early concentration rise especially of Glu closely parallels the time course of glutamatergic synaptogenesis and marks a period of ongoing refinement of cortical circuitry. As glutamatergic synapses develop and mature, energy demand rises. Glutamatergic neuronal activity requires 60–70% of total Glc (Chowdhury *et al.* 2007). Thus, this early period is also associated with a major step in the maturation of energy metabolism, demonstrated by the concomitant increase in Glc levels.

Glutamine concentrations rise within the first weeks of life, similar to those of Glu. The same pattern has been observed in the developing rat cortex (Tkáč *et al.* 2003). It is expected that Glu and Gln concentrations develop similarly as both are functionally coupled through the Glu–Gln cycle (for review see e.g. Zwingmann and Butterworth 2005).

GABA concentrations remained stable throughout the investigated period. This is in accordance with previous reports in the rat brain (e.g. Tkáč *et al.* 2003). Changes in GABA concentrations would be expected before P10, at the time of GABAergic inhibitory synapse formation (Anderson *et al.* 1995) and the increment of cortical GABA-binding sites (Skerritt and Johnston 1982).

NAAG is a highly abundant neuromodulatory peptide in the brain that is expressed in neuronal terminals (Shave *et al.* 2001) and to a lesser extend may also be expressed in glial cells (Cassidy and Neale 1993). It acts on pre-synaptic metabotropic Glu receptors (mGluR2/3) to down-regulate neurotransmitter release via negative feedback (Zhao *et al.* 2001) and it was shown to act on NMDA either as direct agonist (e.g. Westbrook *et al.* 1986) or antagonist (e.g. Burlina *et al.* 1994). We found that NAAG levels are highest at P10 and decline thereafter to reach a stable plateau at adult age. This exactly mimics early results obtained with tissue extracts (Koller and Coyle 1984), reporting a substantial

NAAG increase from mid-gestation to P8 and followed by a steady decline.

Glycine has a dual role as inhibitory neurotransmitter, activating glycine receptors, and as co-agonist for Glu excitatory transmission through NMDA receptors (Betz and Laube 2006). We observed highest glycine concentrations at P10. Others have reported that the rate of glycine synthesis peaks around 10–15 days after birth (Lahoya *et al.* 1980; Benítez-Diaz *et al.* 2003) and that likewise, during this period, glycine receptors undergo a major switch of the relative expression of glycinergic subunits (Lynch 2004). It is interesting to note that the increase of levels of excitatory neurotransmitters, Glu and Asp (NMDA receptor agonists), is paralleled by a reduction in the concentration of its modulator NAAG and co-agonist glycine.

Among other functions, Tau is also a neuromodulator (Gupta *et al.* 2009). Tau released from glial cells may be an important endogenous ligand for the glycine receptor during the period of cortical synaptogenesis (Flint *et al.* 1998). Accordingly, like glycine, Tau concentration in the cortex was maximal at P10 and decreased to adult age.

Myelination/cell proliferation/membrane metabolism

Phosphorylethanolamine (PE) is precursor for phosphatidylethanolamine, which is a major phospholipid in the brain (Quarles *et al.* 2006). PE concentration decreased during mouse cortical development, consistent with observations in rats and humans, parallel to the progression of myelination (Blüml *et al.* 1999; Rao *et al.* 2003). Developmental increase of NAA content in cortical regions may be associated with its role in lipid and myelin synthesis (D'Adamo and Yatsu 1966; Burri *et al.* 1991). In fact, although NAA is mainly synthesized and stored in neurons (e.g. Baslow 2003), its deacetylation is required by oligodendrocytes for myelin synthesis (Kirmani *et al.* 2002). The significant reduction in PE and increase in NAA concentrations in the cerebral cortex from P20 onwards may reflect the start of deposition of myelin that is very rapid between P14 and P30 yet continues afterwards (Costantino-Ceccarini and Morell 1972; Muse *et al.* 2001).

We observed an increase in choline concentration between P20 and P60 in both the anterior and posterior cortex. Previous *in vivo* or *in vitro* spectroscopy studies with mice have disregarded cortical areas (e.g. Yao *et al.* 1999; Larvaron *et al.* 2006; Weiss *et al.* 2009) and, therefore, we could not find a comparison for these data. Interestingly, in the developing rat cortex, choline concentration did not change substantially during development (Tkáč *et al.* 2003). Choline compounds are essential for membrane lipid synthesis and act as precursor for the biosynthesis of the neurotransmitter acetylcholine. Specially during embryonic brain development, choline availability seems crucial for the onset of GABAergic neuronal differentiation (Albright *et al.* 2003) and progenitor cell proliferation and apoptosis in mouse brain (Craciunescu *et al.* 2003).

Energy metabolism

Depending on its physiological state and maturity, the brain utilizes different energy substrates including Glc, ketone bodies and Lac (e.g. Lust *et al.* 2003). We report very low levels of cortical Glc at P10 and a gradual increase thereafter, suggesting that energy metabolism of the immature neonatal rodent brain may rely to a large extend on energy sources different from Glc. In fact, in the brain of suckling rodents, ketone bodies can represent up to 70% of the total energy substrate pool (Nehlig 2004). Rheims *et al.* (2009) have shown that, during early postnatal development (P3–P19), ketone body availability modulates GABA signalling and efficiently controls the excitability of neonatal cortical neurons. Cortical development was characterized by increasing Glc concentrations which result from larger increase in the density of Glc transporters (e.g. Vannucci *et al.* 1994) rather than the augment in the cerebral Glc consumption rate (Nehlig 2004).

We found that Lac concentrations are relatively unaltered during early development and increase at adult age. Data from developing rats are controversial. Tkáč *et al.* (2003) failed to find changes in Lac concentration during rat cortical development, whereas Lust *et al.* (2003) found high lacate in neonate rats and a strong decrease in adults. The developmental expression pattern of Lac transporters within the brain is complex. Some Lac transporters (e.g. monocarboxylate transporter 1) are highly expressed during early postnatal development and less so in the adult brain. Other transporters (e.g. monocarboxylate transporter 2) increase with neuronal maturation and synaptogenesis (e.g. reviewed in Castro *et al.* 2009). The fluctuation in Lac levels found in the present study may, therefore, be a reflection of this complexity. The developmental time course of Lac utilization remains unclear.

Recent studies suggest that Lac generated and released by astrocytes serves as energy substrate for neurons, especially during synaptic activity (Pellerin and Magistretti 1994), and that Asc plays a role in this process (Castro *et al.* 2009). Here, we found that cortical Asc concentration decreases from P10 to adult age, therefore suggesting that Asc-induced Glc transport inhibition and Lac transport stimulation may be higher in early developmental stages than in adulthood. This corroborates the preferential use of other substrates rather than Glc by the developing brain (Lust *et al.* 2003).

Alanine is an amino acid with close links to metabolic pathways such as glycolysis, tricarboxylic acid cycle and protein synthesis. Like for Lac, with which it is in equilibrium although pyruvate, conflicting observations were reported for Ala concentrations in the developing rat brain (Bayer and McMurray 1967; Burri *et al.* 1990; Tkáč *et al.* 2003). We found that cortical Ala content decreases during

development, as previously shown in mouse brain extracts (Yao *et al.* 1999), reflecting the high protein turnover in the developing brain as result of cell proliferation and tissue expansion. These events require high availability of stored energy, typified in the larger concentration of PCr relative to Cr at early developmental ages. Cr that was found to increase in the cortex during development follows the modification in brain Cr kinase activity (Manos *et al.* 1991), which is a critical component in maintaining cellular energy homeostasis by buffering brain energy demands.

Finally, it is important to stress that the brain energy status depends on brain activity and, therefore, on the anaesthesia used for the NMR study (Lei *et al.* 2010). Thus, much care should be taken when comparing the present observations to other reports found in the literature.

Osmoregulation

Osmolarity regulation in the brain is considered to occur, among others, via the concentrations of Tau and Ins. Tau is known to have three main roles in the brain, namely acting as neuromodulator (discussd above), antioxidant and osmoregulator (Gupta *et al.* 2009). Accordingly, it is one of the most concentrated metabolites in the cortical tissue, decreasing from 17 mM at P10 to 10 mM in adult mice. Similar observations were reported for other brain regions (Weiss *et al.* 2009). Although these *in vivo* studies did not follow the mice until adult age, *in vitro* determination of Tau lead to similar findings (Yao *et al.* 1999). However, to our knowledge this is the first determination of Ins concentrations *in vivo* during mouse brain development. During development, Ins content increased in the mouse cortex, as was shown to occur in the rat (Tkáč *et al.* 2003). As osmolyte, brain Ins increases in compensation to Tau reduction. On the other hand, this increase of Ins concentration may be related to the demand for synthesis of inositol-containing phospholipids during synaptogenesis, axonal growth and myelination. In line with this, Yao *et al.* (1999) reported increase of phosphatidylinositol that may be incorporated into lipid membranes (Quarles *et al.* 2006) during mouse brain development.

Antioxidant defense

Glutathione, a major redox regulator and antioxidant, slightly increased before P20 and remained unaltered through the following development similar to reports in rats (Tkáč *et al.* 2003). Glutathione acts in concert with Asc (vitamin C) which is a potent antioxidant and enzyme co-factor in the brain. As such Asc is involved in antioxidant protection, but also plays a role in myelin formation (Eldridge *et al.* 1987), enhancement of synaptic activity (Rebec and Pierce 1994), regulation of Glc metabolism (Castro *et al.* 2009) and protection from excitotoxicity (Qiu *et al.* 2007). Our spectra showed high Asc concentrations in young (P10) animals and a subsequent steady decline until adulthood. In line with this are observations that young rodents are more prone to oxidative stress than are mature animals (Lykkesfeldt 2002) and that the developing rodent brain is particularly susceptible to vitamin C deficiency because of rapid growth and immature antioxidant defence systems (Lykkesfeldt *et al.* 2007). Indeed, vitamin C deficiency in early postnatal life results in impaired neuronal development and spatial memory deficits (Tveden-Nyborg *et al.* 2009). Mice, in contrast to humans, are able to endogenously synthesize vitamin C. Thus, one possible explanation for our results is that mice adapt vitamin C synthesis according to demand, and produce higher levels during the particularly sensitive early postnatal period. As the organism and its antioxidant systems mature, less vitamin C is needed and synthesis is down-regulated. Also the osmolite Tau that has an antioxidant function was more concentrated at early development.

Anterior and posterior cortical regions

The fine quality of the spectra in this study revealed differences in the neurochemical profile of anterior and posterior regions of the cortex. The anterior VOI covered parts of the anterior cingulate cortex, primary and secondary motor cortex as well as primary somato-sensory cortex. The posterior VOI contained parts of the retrosplenial granular and dysgranular as well as secondary visual cortices and associative areas. The time course of maturation is considerably different across diverse cortical areas and generally parallels the time course of cognitive milestones. Thus, primary sensory-motor areas mature earliest, followed by posterior parietal and temporal cortices associated with spatial attention. Anterior prefrontal areas modulating higher cognitive functions such as planning mature latest (for review see Casey *et al.* 2005). In this study, both the anterior and the posterior VOIs encompass parts of sensory as well as higher cognitive areas.

We observed that the concentrations of Gln, Asp, NAA, Cr, Ins and choline-containing compounds were significantly different in anterior and posterior regions of the mouse cortex. As discussed above, NAA and Ins are both related to membrane phospholipid synthesis. Since the posterior cortex VOI is in the proximity of corpus calosum, which contains high density of myelinated fibers, the higher concentration of NAA and Ins may relate to larger phospholipid synthesis in the posterior compared with anterior cortex of the adult mouse (see Kirmani *et al.* 2002). This requires extra energy and it is of interest to note that Cr concentration was also increased in that region. Although the concentration of choline-containing compounds evolved differently between the two regions, reflecting this difference in phospholipid metabolism, it reached similar levels in the adult mouse. Gln, mainly present in glia, occurred at higher concentration in posterior than anterior cortex at P10 and P20 and the opposite was observed for Asp. This may be related to higher density of oligodendrocytes in the posterior cortex, being

responsible for myelin synthesis for the fibers of the adjacent corpus callosum (see Quarles *et al.* 2006).

Conclusions

This is the first report of *in vivo* simultaneous quantification of 18 metabolites in the developing mouse brain. The reliability and reproducibility of ^{1}H NMR spectroscopy allowed detecting significant metabolic differences at different time points corresponding to stages of cortical development. It also allowed detection of regional differences in the anterior and posterior cortex that we attribute to different phospholipid metabolism related to myelin deposition. Moreover, this work allowed concluding that the anaesthesia protocol here employed is devoid of adverse effects on the development of the mouse cortex. As such, high resolution NMR in mice may qualify as method of choice for *in vivo* translational investigations of pre-clinical animal models of human developmental neurological and psychiatric pathologies.

Acknowledgements

The study was supported by Centre d'Imagerie BioMédicale (CIBM) of the UNIL, UNIGE, HUG, CHUV, EPFL and the Leenaards and Jeantet Foundations, the Swiss National Science Foundation #31-116689 and the 'Loterie Romande'.

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