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## **Persistently altered metabolic phenotype following perinatal excitotoxic brain injury**

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**Running head:** Metabolic Characterization in Perinatal Excitotoxic Brain Lesion

**Key words:** Ibotenate, Stroke, Hypoxic-ischemic Encephalopathy, Diffuse White Matter Injury, Metabolomics, Mass spectrometry, Metabonomics, Neonatal Encephalopathy

## Abstract

Excitotoxicity plays a key role during insults to the developing brain such as neonatal encephalopathy, stroke and encephalopathy of prematurity. Such insults affect many thousands of infants each year. Excitotoxicity causes frank lesions due to cell death and gliosis and disturbs normal developmental process, leading to deficits in learning, memory and social integration that persist into adulthood. Understanding the processes underlying the acute effects of excitotoxicity and its persistence during brain maturation provides an opportunity to identify mechanistic or diagnostic biomarkers, thus enabling and designing possible therapies. We applied mass spectrometry to provide metabolic profiles of brain tissue and plasma over time following an excitotoxic lesion (intracerebral ibotenate) to the neonatal (P5) mouse brain. We found no differences between the plasma from control (PBS injected) and excitotoxic (ibotenate injected) groups across time (P8, P9, P10, P30). In the brain, we found that variations in amino acids (arginine, glutamine, phenylalanine, proline) and glycerophospholipids were sustaining acute and delayed (tertiary) responses to injury. In particular, the effects of excitotoxic lesion on the normal profile of development was linked to alterations in a fingerprint of glycerophospholipids and amino acids. Specifically, we identified increases in the amino acids Glutamine, Proline, Serine, Threonine, Tryptophan, Valine and the sphingolipid SM C26:1 and decreases in the glycerophospholipids: arachidonic acid containing phosphatidylcholines (PC aa C30:2) and (PC aa C32:3). This study demonstrates that metabolic profiling is a useful approach to identify acute and tertiary effects in an excitotoxic lesion model generating a short list of targets with future potential in the hunt for identification, stratification and possibly therapy.

## Introduction

Excitotoxicity plays a key role in insults to the developing brain such as neonatal encephalopathy [1, 2], stroke [3, 4], encephalopathy of prematurity [5, 6] and traumatic brain injury [7]. Many thousands of infants suffer from these injuries every year in developed countries alone. Due to acute effects, such as cell death, and subsequent disturbances to normal development they are a leading cause of deficits in learning, memory and social integration that persist into adulthood [8-10]. During excitotoxicity, the excessive activation of glutamate receptors, (particularly N-methyl-D-aspartate (NMDA) receptors) leads to vast calcium influx, leading to cellular membrane damage and leakage of reactive oxygen species from mitochondria, inducing neural cell death and subsequent loss of neurites and connectivity [11-13]. However, the indiscriminate targeting of NMDA receptors induces localized neuronal death and behavioral deficits in adulthood [14, 15] as well as massive and diffuse neuronal death in the developing brain [16, 17] due to their crucial developmental and homeostatic functions. Preventing excitotoxic injury without directly interfering with NMDA receptors is therefore an appealing therapeutic strategy. Furthermore, lesions associated with excitotoxicity causes persisting micro- and astrogliosis, and this and other persisting processes may also represent viable neurotherapeutic targets [18]. A relatively novel approach in the search for therapeutic targets is metabolic phenotyping. Metabolites represent the functional endpoints of gene and protein expression and are also known as important modulators of cell signaling and cell survival. As such, metabolic phenotyping can be considered to encompass the overall physiologic status of an organism by capturing global biochemical events by assaying many hundreds of small molecules in cells, tissues and organs. This approach has been applied for acute injury profiling, and in the developing brain it is well known that hypoxia-ischemia induces major changes in metabolism. In 1984, Silverstein et. al. described the effects on monoamine metabolism in infants diagnosed with hypoxic-ischemic encephalopathy [19]. This work was extended in an experimental setting in fetal monkeys subjected to perinatal hypoxic insult [20] and changes in arachidonic acid metabolites was reported in 1989 in the fetal rabbit following brain hypoxia [21]. It took several decades until the importance of these metabolites was discovered [22] and recently, the effects (and altered levels) of endocannabinoids under ischemic and excitotoxic conditions has become of interest [23, 24]. More recently, the use of fluid metabolic phenotyping has been applied to clinical samples following perinatal brain injury for biomarkers discovery [25, 26], and improving our knowledge of injury specific and temporal components of the metabolic profile will improve our interpretation thereof.

We undertook a comprehensive analysis of the metabolic phenotype to both confirm and expand our knowledge about the expected mode of action of excitotoxicity. We hoped to

possibly pinpoint pathways outside those identified in non- metabolic phenotyping approaches or non-pediatric based analyses that could lead to the identification of new therapeutic targets [27]. Our hypothesis is that we that we could use metabolic phenotyping screening and the associated multivariate biostatistical analysis to provide new information on the effects of injury on and between multiple metabolites across time. We studied the well-validated and characterized excitotoxic lesion in the postnatal day (P) 5 mouse [28-30]. The lesion size following ibotenate injection is maximal by 24 hours, and remains stable for at least the following 4 weeks [28, 29]. The initial injury involves the death of neurons and astrocytes across all cortical and subcortical layers, and the almost immediate infiltration of microglia [28]. Although microglial and astrocyte number within the lesion return to normal (although the phenotype is unknown) there is a persistent injury to the periventricular white matter [28]. In this excitotoxic model we characterized the metabolic fingerprint occurring within the lesion and plasma at 4 time points, from the acute phase into young adulthood. We found that the excitotoxic lesion caused a substantial change away from profile observed in the control, PBS only injected group. A handful of metabolites related to amino acids and lipids were observed to be altered by the excitotoxic lesion.

## Materials and Methods:

Experimental protocols were approved by the Bichat and Robert Debré Hospital ethics committee (#2011-14/646-048) and all experiments adhered to the European Union Guidelines for the Care and Use of Animals. *In vivo* procedures were typically carried out between 10am and 1pm (light phase 7am-7pm daily); all animals were monitored daily during experimentation. Each experimental unit corresponds to a single animal, with groups spread between and across litters. Animals were housed (Plexiglas cages 30x18x15cm) together with littermates and their dam from birth to P20 and weaned into single sex groups at P21. Cages were supplied with wood-chip bedding and shredded paper for nesting (Pharmaserv, France) and animals had access to standard chow and water *ad libitum*.

### *Animals and drugs*

Male and female Swiss pups (Iffa Credo, L'Abresle, France) were used for *in vivo* experiments. Ibotenate was purchased from Sigma (St-Louis, MO, USA). Ibotenate was diluted in 1x phosphate buffered saline (PBS) containing 0.01% acetic acid.

### *Neonatal excitotoxic brain lesion and sample collection*

We induced excitotoxic brain lesions by injecting ibotenate into the developing mouse neopallial parenchyma on P5 as previously described [2, 31, 32]. In brief, using a stereotactic frame a 26 g syringe was inserted under the external surface of the scalp, 2 mm from the midline in the lateral medial plane and 3 mm from the junction between the sagittal and lamboid sutures in the rostro-caudal plane. One 1  $\mu$ l bolus of ibotenate (5 ng/ $\mu$ l in 0.01% Acetic acid in 1x PBS) was administered at 2  $\mu$ m depth (periventricular white matter placement), the needle was withdrawn 1  $\mu$ m (cortical placement) and a second 1  $\mu$ l bolus was administered. The needle was held in place for 20 seconds following the injections. Animals were euthanized by decapitation 24 (P6), 48 (P7), 72 (P8), 120 hours (P10) and 25 days (P30) after the injury. Blood was collected at decapitation using capillary tubes and plasma separated via centrifugation. The brain region corresponding to the site of injection was dissected out, and blood and plasma were stored at -80°C until further preparation as described below. Specifically, the area of the excitotoxic lesion was dissected by excising an area of 2mm wide and 2 mm thick of the whole depth of the cortex and underlying subcortical white matter from the injection site at each age. Although the necessity to take the entire lesion area for analysis precluded analysis of lesion size, animals performed in parallel for a separate study had the expected lesion size for this highly reproducible model [28, 33-35]. Each treatment group comprises 7 to 10 animals.

### *Sample preparation*

Frozen brain samples were weighted into 2 ml Precellys tubes (Peqlab Biotechnologie GmbH, Erlangen, Germany) equipped with ceramic beads. Homogenates were prepared by adding ethanol/10 mM phosphate buffer (85/15) solvent mixtures to the tissue sample, ratio 3:1 (v/w). The Precellys-24 homogeniser (Peqlab Biotechnologie GmbH, Erlangen, Germany) uses a figure-eight motion to rapidly gyrate beads to grind up to 24 samples temperature controlled (0-4°C) at once with the following program: 3 cycles of 30 s at a frequency of 5800 rpm with a 25 s pause between cycles. Homogenates and plasma samples were centrifuged at 18000g at 2°C for 5 min, the resulting supernatant was pipetted into a cryovial (1.5 ml, Biozym GmbH, Oldendorf, Germany) and analysed immediately to avoid degradation of the analytes.

### *Analytical procedures*

The flow injection analysis (FIA-MS/MS) based platform was employed for the simultaneous quantification of a broad range of endogenous intermediates namely acylcarnitines, amino acids, sphingomyelins, glycerophospholipids. A chromatographic step (LC-MS/MS) was applied for the additional quantification of series of amino acids and biogenic amines, small organic acids and other members from the energy metabolism pathway, oxysterols and eicosanoids. Analytical protocols can be found in Urban et. al. [36] and are validated according to the FDA bioanalytical method validation recommendation for industry [37]. Samples were randomized within and across the two analytical batches. All pre- and post-analytical procedures were performed by analysts blinded to the experimental groups. In total, 215 unique compounds were quantified. Analytes that did not meet quality control criteria or that were not detected in at approximately >70% of the samples were not selected for further analyses resulting in 182 unique metabolites retained for analysis and reporting. A list of excluded compounds for the plasma analysis was OH-Kynurenine, PEA, Nitro-Tyr, Dopamine, OH-Pro, and for the brain analysis was OH-Kynurine, Nito-Tyr, OH-Pro, 9-HODE, 12(15)-EpETE, 15S-HpETE, 5S-HpETE, LTD4, 8-iso PGF2, PEP, alpha-KGA, Tetra-P, 22ROHC, 20aOHC, 22SOHC, THC, 7aOHC, Desmosterol, 7DHC, Cholesteroe, 24DHLan. A quantitative (or semi quantitative) targeted approach was employed resulting in the generation of normalized concentration value for specific compound. Data preprocessing is therefore limited to two activities: zero values imputation and data transformation. A detailed list of all metabolites that are considered for interpretation is given in Supplementary Table 1. In short, it is comprised of 25 acylcarnitines, 93 lipids derivatives (11 sphingomyelins, 72 glycerophosphatidylcholines and 10 lysophospholipids), 21 amino acids, 11 biogenic amines, 12 eicosanoids and prostaglandins, 13 oxidised products of cholesterol

(oxysterols) and 6 members of the energy metabolism pathway. Where the limit of detection was not reached for a given variable then the value was set to 0 and columns or rows comprising of only zeros were removed prior to analysis. Data were formatted as a data matrix comprising 80 lines (samples) and 147 columns (metabolic variables).

#### *Multivariate statistical analysis*

Data were pareto scaled before analysis. Unsupervised principal component analysis was run in both sub-populations to check sample homogeneity and exclude outliers. An O-PLS analysis (orthogonal projections to latent structures) [38, 39] was carried out to investigate the information shared by the mass spectral matrix X and the descriptive variable matrix Y. Spectra are visualized in scores plots and the metabolic variations sustaining the discrimination are presented in loadings plots. The O-PLS analysis was run to discriminate populations by regressing a supplementary data matrix Y, containing information about the membership of samples to each group. Model performances were assessed by goodness-of-fit parameters  $R^2$  and  $Q^2$ , related, respectively, to the explained and predicted variance. We performed model validation by resampling the model 1000 times under the null hypothesis (i.e. by random permutation). Observed and predicted Y matrixes were computed using an O-PLS-DA MATLAB routine developed in the lab. Receiver Operating Characteristic (ROC) curves were plotted using MATLAB. These represent the true positive rate as a function of the false positive rate and determine the best threshold for a given test to define the membership of a class. The diagonal stands for random prediction, whereas the more the curve moves to the top left corner, the better the prediction ability of the model is. Scaled to maximum aligned and reduced trajectories (SMART) analysis [40] was used to cancel out the differences between the metabolic starting positions of the different groups and evaluate the metabolic trajectories with respect to time. O-PLS-VIPs (variable influence on projection) allow the identification of variables that are important in the multivariate signature discriminating the 2 groups. They were computed with a MATLAB routine based on the published algorithm [41]. VIP values above 2 were chosen as variables of interest.



**Results:**

Our first analysis evaluated if metabolic phenotyping could discriminate between samples from brain tissues from mice injected with PBS (controls) and the samples of brain injected with ibotenate (model of focal lesion induced via excitotoxicity). Subsequent to this, as a post-hoc analysis, we then investigated the metabolic changes occurring between the different time points to monitor the metabolic evolution induced by the excitotoxic lesion.

*Between group discrimination analysis*

Brain and plasma samples were analyzed with the aim of identifying metabolic differences between PBS injected (control) animals and ibotenate injected (lesioned) animals and between 7-10 samples were generated for each data point. Plasma samples did not exhibit significant metabolic variations. However, the PBS and Ibotenate brain samples could be robustly separated via a computed single predictive component from the 1+7 component O-PLS analysis (Figure 1A). This is a dimension reduction technique that allows regression analysis where we know that some of our independent variable (metabolites) can co-vary. Goodness-of-fit parameters of the model were high, with an explanation capacity  $R^2$  of 0.830 and a prediction capacity  $Q^2$  of 0.677 (maximum = 1).

We used two strategies to test the strength of our model to explain the difference between the control and ibotenate groups in this study, which was exploratory for the utility of metabolic phenotyping to discriminate these 2 groups. First we used resampling under the null hypothesis. We identified, in comparison to our real data, that there was a clear decrease of the goodness-of-fit parameters (Figure 1B) when the model is based on a random class information matrix (decrease in  $R^2$  and  $Q^2$  with the correlation between the original and permuted sample identifications). An empirical  $p$ -value was computed to measure the number of random models outperforming our initial real data model. The  $p$ -value value was 0, demonstrating that no random model achieved better quality coefficients than the original real data model, thus validating its robustness. Secondly, discrimination capacity was assessed using a cross-validated receiver-operating characteristic (ROC: Figure 1C) showing an area under the curve (AUC) of 0.98 further validating the strength of the model to explain the difference between the control and ibotenate groups.

To identify statistically significant variation of a single metabolite that would explain the observed difference we used a univariate statistical analysis (analysis of variance) coupled with multiple hypotheses testing correction (Benjamini-Yekutieli correction). This failed to identify a single discriminatory metabolite. Instead, we implemented variable importance on projection (VIP) for O-PLS to identify, in a multivariate way, the metabolic changes that were

important to explain the difference between the groups. VIPs were only derived from the predictive component of the model. Variables exhibiting a stringent O-PLS-VIP (above 2[41]) were kept and are shown in Figure 1D. We observed an increase of many amino acids (glutamine, proline, serine, threonine, tryptophan, valine) and the sphingolipid SM C26:1 (sphingomyelin, 26 carbon residues, 1 double bond) associated with the ibotenate lesion compared with PBS and a decrease in glycerophospholipids (arachidonic acid-containing phosphatidylcholines, PC aa 30:2 & PC aa C32:3).

### *Time-series analysis*

To evaluate the metabolic changes associated with time after the injection of either PBS or ibotenate within the 2 groups we ran a time-series analysis. SMART analysis was performed (Figure 2A), showing 2 different metabolic trajectories (after PBS injection in black or ibotenate injection in red) across our time points (1, 2, 3, 5 and 25 days after the injury). Note that the 2 trajectories do not have the same starting point because we do not have a 0-time sample (pre-injury) in the analysis and the first samples were collected after exposure. The SMART analysis produced a first component that is associated with 69% of the explained variance and a second component associated with 21% of variance. It drops below 5% for the third principal component. Metabolites and their direction of change collectively responsible for the variance within the 1<sup>st</sup> and 2<sup>nd</sup> principal components were identified (Figure 2B) and the values for each metabolite for each time point are presented in Figure 3. It is worth noting that the model captures both the direction and magnitude of change and as such a metabolite moving in opposing directions over time or due to treatment can appear in two principal components.

We considered how the data changes with respect to time within the PBS and Ibotenate groups. Initially, PBS and ibotenate samples have different positions along the 1<sup>st</sup> component, but similar positions along the 2<sup>nd</sup> component. Across time points, the 2 metabolic trajectories have similar shapes (moving right to left within PC1 and top to bottom within PC2). However, the PBS trajectory has a greater range in the metabolic space for both PC1 and PC2. After 25 days, the 2 groups have close positions along the 1<sup>st</sup> component but clearly different positions along the 2<sup>nd</sup> component. Furthermore, the final position of the ibotenate signal along the 2<sup>nd</sup> component is equivalent to its initial position. It thus seems that injury effects are translated in the metabolic space along the 1<sup>st</sup> component (variations in acylcarnitine, arginine, glutamine, phenylalanine, proline, serine, tyrosine, valine, glycerophospholipids and sphingolipids) but that this difference is corrected after 25 days of development. However, in the PBS group only another metabolic difference appears

(variations in arginine, glutamine, valine and glycerophospholipids) showing a clear change over time in the 2<sup>nd</sup> component. This component was associated with an up-regulation of arginine, glutamine and glycerophospholipids and a down-regulation in valine.

## Discussion

In the present study, a mass spectrometry based metabolic phenotyping strategy was applied to evaluate the impact over time of an excitotoxic lesion in the developing mouse brain. We used an O-PLS-based analysis to discriminate between the two groups at the 4 timepoints (1+7 components,  $R^2=0.830$ ,  $Q^2=0.677$ ). From this analysis, we observed changes in concentrations of the amino acid and lipid biochemical classes were associated with ibotenate injury. We also performed a time-series analysis (SMART), generating a model in which the first two principal components accounted for 90% of the data variance (69% and 21% respectively). Investigating time dependent changes was not the primary goal of this study. However, it is noted that early time points were more associated with changes in the 1<sup>st</sup> component (including serine and sphingolipid SM C26:1), and the later (tertiary) time points more associated with changes in the 2<sup>nd</sup> component (including the glycerophospholipids [arachidonic acid-containing phosphatidylcholines] PC aa 36:3)

In comparison to NMR (nuclear magnetic resonance spectroscopy), mass-spectrometry based strategies are justified for several reasons. The small amount of immature and/or specific brain regions in rodent models typically requires sample pooling for analysis via NMR. However, mass spectrometry can be performed on single animal samples, maintaining the optimal balance between statistical power and number of experimental animals. In combination with the improved metabolite discrimination, the modularity of MS-based platforms allows a wider coverage of not only the metabolome but also of specific class of metabolites by including their members present a lower concentration. In the scope of this study, our targeted MS-based metabolic phenotyping enabled the simultaneous coverage of pathways that were hypothesized to represent intermediary metabolism involved in excitotoxic injury [42], namely phospholipids, sphingomyelins, eicosanoids, oxidised products of cholesterol, acylcarnitines and several members of the TCA cycle. In addition, the whole range of amino acids and several biogenic amines (e.g., serotonin, dopamine) central to brain research were also included.

Our O-PLS analysis comparing control and excitotoxic lesion metabolites produced a robust model that we validated to explain the vast majority of variance in the data. A limitation of the model validation is that we did not have access to an independent data set. The benefits of the O-PLS analysis are its supervised nature, allowing a focus on the specific effects of the study. However, the model displays information along predictive and orthogonal axes that both should be investigated. Although we were not able to identify a single metabolite responsible for a significant amount of variation in this model we did identify a fingerprint of 9

metabolites belonging to the amino acid, glycerophospholipid and sphingolipid families. It is of course likely that change in these key factors is due to metabolic and cellular dysfunction including changes in the cellular composition of the injured brain.

Regarding the cellular composition of the brain and how this corresponds to the metabolic results, we have previously characterized this model at 4h, 8h, and 1d, 2d, 3d, 4d, 5d and 20d post lesion [2, 28, 43]. We observed that neuronal death is occurring at +12 hours after ibotenate, and becomes maximal at +24 hours but that there is no more neuronal loss at +48 hours. Microglia increase substantially in number between +2 and +4 hours, and their numbers remain elevated at +5 days. Regarding astrocytes, we observe that there is an initial reduction in number to a nadir at +24 hours and that numbers reach above control by +48 hours and normalize between +5 and +25 days. We have previously demonstrated that microglia are the chief mediators of injury in this model, using pharmacological and antibody mediated approaches [43]. Regarding the role of altered amino acids as revealed by our current analysis, microglial activation dynamics have been studied in depth with metabolomics approaches and these reveal specialized expression patterns of amino acids tied to activation states [44]. However, specific links cannot be made between amino acid changes in our data, given the heterogeneity in the cell types in this lesion. Nevertheless, we can mention that high levels of branch chain amino acids such as valine favor astrocyte-dependent neurotoxicity by NMDA receptor activation and influence gene expression and immune properties of microglial cells [45]. In addition, we could associate the increases in glutamine with the higher numbers of astrocytes within the lesion, as this amino acid is enriched in these glial cells [46]. Regarding lipid class metabolites, the membranes of neural cells are composed of glycerophospholipids, sphingolipids, and assorted proteins asymmetrically distributed between the two lipid bilayers. Glycerophospholipids consist of four primary phospholipids; phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. Glycerophospholipids and sphingolipids play a role in the structural integrity of these neural membranes. Also, neuronal membrane phospholipids are important reservoirs for second messengers, such as prostaglandins, eicosanoids, platelet activating factor and diacylglycerol. As such, we could suppose that there is a plausible link between increases in glial cell numbers and neuronal death profiles and the observations of metabolites. Supporting a facet of this, is that, a recent study reports that arachidonic acid-containing phosphatidylcholines (PC aa metabolites) are enriched in microglia, specifically in the spinal

cord microglia following peripheral nerve injury [47]. In addition, the death of oligodendrocytes and the formation of a white matter cyst is a key component of this model [35]. As such, changes in sphingolipids are logical as sphingolipids are found in animal cell membranes, especially in the membranous myelin sheath which surrounds some nerve cell axons.

There are also previous reports on the effects of excitotoxicity on brain metabolites. Specifically, He et al. [49] identified variations in ceramide metabolism in a rat model of hippocampal injury, induced by excitotoxic lesion using kainate acid. We observed variations in amino acids (and in particularly serine) and sphingolipid metabolism that can be connected to ceramide metabolism. However, in our analysis changes in these metabolites did not reach the level of statistical significance

Using the SMART analysis to assess the metabolic trajectories over time, at the first-time point (+24 h) we noted there was considerable difference in the 1<sup>st</sup> component metabolites (such as C9 acylcarnitine and the sphingolipid SM C26:1). We speculate this variance is due to the significant excitotoxicity induced changes in the brain tissue at this first time point of analysis, namely cell death and gliosis [28]. Also, at the latest time point, 25 days after the lesion (in the tertiary phase), it has previously been observed in this model that gliosis has subsided but that there remains a considerable lesion in the periventricular white matter. At this tertiary time point the greatest variation identified by the SMART analysis was due to increases in metabolites from the 2<sup>nd</sup> component, such as the glycerophospholipids (C36:3). A disturbance to normal developmental processes are considered a major contributor to brain damage observed in preterm born infants, the encephalopathy of prematurity [48]. We speculate that this change in PC2 represents a developmental disturbance caused by the excitotoxic lesion. Although the study of normal development was outside the planned scope of this study, it is of interest to speculate that the PC2 related changes in the PBS (control) group likely reflect normal changes in cellular composition and cellular maturation.

Metabolic phenotyping has been shown to be useful in discriminating in human cohorts between variables as diverse as aging and viral infection, see [27]. Fluid based metabolomics is an increasingly popular area of research in perinatal medicine, with studies related to neonatal encephalopathy and chorioamnionitis [26, 50-52]. Attempts have even been made to correlate metabolic changes with neuropathology in a sheep model of inflammation driven perinatal brain injury [53]. This sheep study also demonstrated changes in the mid-term caused by injury, out to 10 days post-insult, although this injury is more severe and it is likely to have also persisted out into the tertiary phase as in this study We

did not observe the multitude of changes in the metabolic phenotypes as observed in the sheep study and it is likely due to the more moderate nature of our injury that explains the limited effects in this study.

Analysis of injury in this model revealed that at least by 24 hours that there is a compartmentalization of response to injury in this model (no plasma changes). We can assume that there is little systemic-central crosstalk as cell death and gliosis peak at 48 hours onwards in this model and as such that the metabolic changes are driven primarily by the events within the brain. This is an interesting facet of the model and will allow us to further refine brain specific metabolic phenotypes for biomarker discovery.

In conclusion, this is the first assessment of the metabolic phenotype in a model of perinatal excitotoxicity including an analysis of the effects into young adulthood. We found that a short list of amino acids and glycerophospholipids explained the effects of the excitotoxic lesion. Furthermore, we found that there were differences in the normal processes of development that could be visualized with this approach. These targets may be useful starting points for studies into acute or tertiary phase biomarkers specifically for excitotoxic processes.

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**Figure 1.** (A) 1+7 component OPLS score plot discriminating PBS injected (control as plain black squares) and ibotenate injection (excitotoxic lesion in empty red circles) brain tissue samples. (B) Resampling under the null hypothesis for model validation. (C) Cross-validated receiver operating characteristics curve. (D) Metabolites identified by high OPLS-VIP (above 2) as valuable for the discrimination between the 2 groups. PC = phosphatidylcholines, aa= arachidonic acid, C = carbon number, SM = sphingomyelin.

**Figure 2.** (A) SMART-scaled analysis of the PBS (black) and ibotenate (red) injected groups. Each dot represents a time point in the analysis in both PBS and ibotenate trajectories. Samples were taken at day 1, 2, 3, 5 and 25-post injection. Note that the 2 trajectories do not start on the same point because there is no pre-exposure sample. (B) Metabolites involved in the first and second components of the SMART model. PC = phosphatidylcholines, aa= arachidonic acid, C = carbon number, SM = sphingomyelin.

**Figure 3.** Values for each of the metabolites varying in the SMART analysis. Metabolites are listed for each time and both treatments in arbitrary units (A.U). Values are color coded within each metabolite across time for PBS and Ibotenate with higher values in green and lower values in red. PC = phosphatidylcholines, aa= arachidonic acid, C = carbon number, SM = sphingomyelin.

**Supplementary Table 1:** A detailed list of all metabolites and raw data used for analysis