The Mouse Brain Metabolome: Region-Specific Signatures and Response to Excitotoxic Neuronal Injury

Abbreviated title: Metabolic profile of normal and injured brain

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Number of text pages (including references and figure legends): 49

Number of tables: *2*; *Number of figures: 4*

Number of supplemental figures: 2; number of supplemental tables: 1

Number of words: Abstract: *219*; Introduction: *2016*; Discussion: *1849*.

Abstract

Neurodegeneration is a multistep process characterized by a multitude of molecular entities and their interactions. Systems' analyses, or "omics" approaches, have become an important tool in characterizing this process. While RNA and protein profiling have made their entry into this field a couple of decades ago, metabolite profiling is a more recent addition. The "metabolome" represents a large part or all metabolites in a tissue, and gives a snapshot of its physiology. Using gas-chromatography coupled to mass-spectometry, we have analyzed the metabolic profile of brain regions of the mouse, and found that each region is characterized by its own metabolic signature. We have then analyzed the metabolic profile of the mouse brain after excitotoxic injury, a mechanism of neurodegeneration implicated in numerous neurological diseases. Importantly, we have validated our findings by measuring, histologically and molecularly, actual neurodegeneration and glial response. We found that a specific global metabolic signature, best revealed by machine learning algorithms, rather than individual metabolites, was the most robust correlate of neuronal injury and the accompanying gliosis, and this signature could serve as a global biomarker for neurodegeneration. We also observed that brain lesioning induced a number of metabolites with neuroprotective properties. Our results deepen the understanding of metabolic changes accompanying neurodegeneration in disease models, and could help rapidly evaluate these changes in preclinical drug studies.

Introduction

Neurodegeneration, or the pathological loss of neuronal structure and function that culminates in neuron death, is typically assessed by histological and/or biochemical measurements of specific markers or mediators associated with neuronal demise and with the accompanying reaction of glial cells.

Over the last decades, to better understanding the multiple features of neurodegeneration, systems, or "omics", approaches have made their entry into the field. Transcriptomics in particular, have been used extensively to characterize, at the gene expression level, region-specific profiles [1] and degenerative processes in neurons affected in different diseases [2, 3]. More recently, metabolomics, or the comprehensive study of metabolites, has been added to the "omics" toolbox to study the brain [4]. The metabolome represents the collection of all or part of the molecules (metabolic intermediates, signaling molecules, secondary metabolites) that are the product of biochemical processes in a specimen. Metabolic profiling gives an instantaneous snapshot of the physiology of that specimen. The platforms used for such analyses include Nuclear Magnetic Resonance spectroscopy and Mass Spectrometry-based approaches[5-7].

Many metabolome studies on the brain and its diseases have been focusing on profiling on human's or rodent model's cerebrospinal fluid (CSF), in an effort to find biomarkers of disease severity and progression [4, 8]. Fewer are looking at the actual brain tissues. An integrative study mapping the metabolome of different brain regions, and the metabolic response to injury, with an eye on associations such changes have with neuropathological and molecular markers of neurodegeneration, is still lacking. Such studies are timely in view of the rapidly expanding populations of genetic rodent models for neurological diseases and their use in drug efficacy studies.

Therefore, we used gas chromatography coupled to mass spectrometry (GC-MS) to map the metabolome of different mouse brain regions. GC-MS allowed us to detect a large number of metabolites, and various neuronal populations that populate different regions of the mammalian, and that have very different responses and vulnerabilities to diseases, have not yet been characterized at this level. We then used that approach to detect metabolic changes in response to neurodegeneration induced by excitotoxic challenge, and compared these changes to pathological and molecular alterations that reflect neuronal injury and gliosis.

We found that, at baseline, each mouse brain region has its particular metabolic signature that could be distinguished from that of other regions via Principal Component Analysis, or, to a better extent, by supervised machine learning algorithms. We also found that, after excitotoxic injury, the metabolic signature of the cortex, a brain region that is severely affected by this process, shifts to a unique metabolic signature in affected animals. To validate these bioinformatical observations, we compared excitotoxicity-induced metabolic changes with histopathological and molecular measures of neurodegeneration and gliosis. Our results showed that a specific metabolite signature, revealed by machine learning, rather than individual metabolites, was the most robust correlate of neurodegeneration. Our data support the notion that metabolic profiling of brain regions is a valuable addition to the study of CNS in health and disease, and pave the way for linking metabolite changes to specific molecular CNS disease markers.

Materials and methods

Animals

All animal experimentation had been approved by the university and the appropriate Luxembourg governmental agencies (Ministry of Health, Ministry of Agriculture).

Four- to 6- month-old mice were of the FVB/N strain and purchased from Charles River, France. They were housed 1-2 weeks under a 12h light/dark cycle with *ad libitum* access to water and food until euthanasia, or treatment followed by euthanasia, in a conventional animal housing facility. For treatment, mice were randomly assigned to the kainic acid (KA) groups or the control group. Mice were injected peritoneally with 20mg/kg KA dissolved in sterile PBS or with PBS (vehicle control). Mice were euthanized either 2 days or 7 days after injection.

At euthanasia, mice were deeply anesthetized with a ketamine-methedomidine mix (100mg/kg and 1mg/kg, respectively), then transcardially perfused with PBS to remove the blood. The brains were quickly removed, and one hemibrain was drop-fixed in ice-cold phosphate-buffered 4% paraformaldehyde to be processed for histology. The other hemibrain was dissected within less than a minute using ultra-fine forceps on an ice-cold plate into the following brain regions: cortex, hippocampus, striatum, midbrain, cerebellum, and brainstem. The dissected parts were quickly weighted, then snap-frozen on crushed dry ice, and stored at -80^{o}C until use.

Metabolites extraction and measurement in brain tissues: non-targeted metabolite analysis

For the analysis of metabolites of mouse brain regions at baseline, different dissected brain regions (see above) of a total of 6 mice (half males, half females) were used.

For the analysis of metabolites of mouse brain cortex after excitotoxic injury, the cortices of 16 vehicle-treated, and of 14 KA-treated mice (6 females, 8 males) were used. The cortex was used because it is one of the regions rich in receptors targeted by KA [9], and because, thanks to its relatively large size, the yield of sufficient material for this study was assured.

On the day of the extraction, three 7 mm grinding balls were added to each tissue frozen sample into the Eppendorf tube. The sample tubes were then put in liquid nitrogen for 15 sec. At the same time, a grinding block was cooled in liquid nitrogen for 15 sec. Sample tubes were then put into the grinding block and pulverized in the ball mill (Retsch) for 2 min at 20 s-1 to yield a fine powder. For homogenization, five small grinding balls (1mm) and the appropriate amount of extraction fluid (MeOH/H2O, 40/8.5 v/v) were added to the pulverized samples (485µl/100 mg tissue) and milled for 2 min at 20 s-1 leading to a homogeneous fluid.

A liquid-liquid extraction method was used for metabolite extraction form each brain tissue sample [10]. First chloroform (400 µl/100 mg tissue) was added to the homogenized tissue fluid, then H_2O (200 µl/100 mg tissue). The mixture was incubated on a Thermomixer (Eppendorf) for 20 min at 1300 rpm at 4 °C. After the incubation period, the suspension was centrifuged for 5 min at 5000xg at 4°C and 30 µl of the upper aqueous phase (containing polar metabolites) were transferred into a sample vial with micro insert for speed vacuum evaporation at -4 °C using a refrigerated CentriVap Concentrator (Labconco). The resulting dried samples were used for GC/MS analysis.

Metabolite measurements by Gas Chromatography/Mass Spectrometry (GC-MS)

Metabolite derivatization was performed by using a Gerstel Multiple Purpose Sampler (MPS). Dried polar metabolites were dissolved in 15 µl pyridine, containing 20 mg/ml methoxyamine

hydrochloride, at 40 °C for 30 min under shaking. After adding 15 µl N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) samples were incubated at 40 °C for 30 min under continuous shaking.

GC-MS analysis was performed by using an Agilent 7890A GC coupled to an Agilent 5975C inert XL MSD. A sample volume of 1 µl was injected into a Split/Splitless (S/SL) inlet in splitless mode at 270 °C. The gas chromatograph was equipped with a 30m Agilent J&W GC (DB-35MS) capillary column + 5 m DuraGuard capillary preceding the analytical column. Helium was used as carrier gas with a constant flow rate of 1 ml/min. For the analysis of brain regions, the GC oven temperature was held at 80 °C for 1 min and increased to 300 °C at 36 °C/min. After 10 min, the temperature was increased to 10 °C/min 325 °C, then held at that temperature for 4 min. The total run time for each samples was 59.167 min. After some in-house protocol simplifications, the procedure for the KA-treated brains and their vehicle controls was as follows: the GC oven temperature was held at 80 °C for 1 min and increased to 260 °C at 36 °C/min. After 1 min, the temperature was increased at 22 °C/min followed by an additional constant temperature period at 325 °C for 2 min. The total run time of one sample was 11.955 min. The transfer line temperature was set constantly to 280 °C. The MSD was operating under electron ionization at 70 eV. The MS source was held at 230 °C and the quadrupole at 150 °C. Full scan mass spectra were acquired from *m/z* 70 to 800. The total run time of one sample was 11.955 min.

All GC-MS chromatograms were processed using MetaboliteDetector [11] for non-targeted analysis. The software package supports automatic deconvolution of all mass spectra and calculates the retention indices based on a retention index marker. The obtained mass spectra were matched against a mass spectral library. Compounds were annotated by retention index and

mass spectrum. Selected fragment ions unique for each individual metabolite were used for quantification, and, finally, each compound was normalized by the summed sample signal measured for each individual samples. This approach, named Total Ion Current (TIC) normalization, has turned out to be a reliable way of comparing multiple samples and correct for possible variation in sample preparation and instrument drift[12, 13]. A total of 332 polar metabolites were measured, out of which 29% (95 metabolites) were identified.

RNA isolation and Reverse-Transcription PCR (RT-PCR)

To assess the molecular profile of reactive microglia after excitotoxic injury, transcripts for interleukin-1beta (Il-1β), chemokine (C-C motif) ligand 2 (Ccl2) were measured on extracted RNA [14]. To assess the molecular profile of reactive astroglia after excitotoxic injury, transcripts for tissue inhibitor of metalloproteinase 1 (Timp1), Vimentin (Vim), and serine peptidase inhibitor, clade A, member 3N (Serpina3n, Serp) were measured on extracted RNA [15].

For RNA isolation, the interphase resulting from the methanol-chloroform extraction (see above) was used. Previous studies have shown that RNA obtained with this protocol was of high quality and suitable for transcript level measurements [10, 16]. Total RNA was purified using the Qiagen RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. First strand cDNA was synthesized from 0.5 µg of total RNA using Superscript III (Invitrogen) with 1 µl (50µM) / reaction oligo$(dT)_{20}$ as primer. Individual 20 µl SYBR Green real-time PCR reactions consisted of 2 µl of diluted cDNA, 10 µl of 2X iQ™ SYBR Green Supermix (Bio-Rad), and 0.5 µl of each 10 µM optimized forward and reverse primers in 7 µL RNase-free water. Ribosomal protein L27 (Rpl27), Il-1β, Ccl2 primer sequences were designed using Beacon Designer software (Bio-Rad) and were provided by Eurogentec. Timp1, Vim, Serp3n primer sequences were designed using

the NCBI/Primer-BLAST tool available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/ and were provided by Eurogentec. All primer sequences are shown in Table 1.

The PCR was carried out on a Light Cycler 480 (Roche Diagnostics), using a 3-stage program provided by the manufacturer: 10 min at 95°C and 40 cycles of 30 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C followed by 10 sec 70-95°C melting curves. All experiments included two no-template controls and were performed on the number of mice mentioned in the text with two technical replicates for each sample. For normalization, Ribosomal Protein L27 (Rpl27) was amplified simultaneously.

The threshold cycle of each gene was determined as PCR cycles at which an increase in reporter fluorescence above a baseline signal was measured. The difference in threshold cycles between the target gene and reference gene Rpl27 yielded the standardized expression level (dCt). The expression level of each gene was calculated with the formula 2^{-dCt}.

Generation of tissue sections, immunohistochemistry, and quantitation of neurodegeneration and gliosis

Hemibrains were left in fixative (phosphate-buffered 4% paraformaldehyde) at 4°C for 48h. They were then transferred to PBS containing 0.02% sodium azide as preservative and stored at 4°C until cutting. Forty μm free-floating sections were generated with a Leica VT1000 vibratome, and stored in anti-freeze medium (1% w/v polyvinyl pyrrolidone in a 1:1 v/v PBS/ Ethylene glycol mix) at -20°C until immunostaining.

The following antibodies were used: anti-Microtubule-Associated Protein 2 (MAP2, diluted 1:800, Millipore), anti-synaptophysin (SYN, clone SY38, diluted 1:900, Dakopatts), anti-Glial-

Fibrillary-Acid Protein (GFAP, diluted 1:4000, Dakopatts), and anti-Ionizing Calcium-Binding Adaptor Molecule 1(Iba1, diluted 1: 3000, Wako).

For the detection of neurodegeneration, fluorescence immunostaining on free-floating sections was performed based on a standard procedure [17]. Detection of anti-MAP2 and anti-SYN was done with 1:2000 diluted Alexa-488 coupled secondary antibody (Millipore) for MAP2 detection, and with 1:2000 diluted Alexa-594 coupled secondary antibody (Millipore). Two sections/mouse were stained for each marker, for a total of 16 vehicle-treated (6 females, 12 males), and 18 KA- treated mice (6 females, 12 males). MAP2 and SYN were imaged and analyzed mainly as described [17].

Briefly, MAP2 stained sections were viewed under a Zeiss Apotome 2.0 connected to an AxioImager Z1 microscope, and digital images (220 x 170 μm), 3 for the cortical area of each section, were collected with an Axiocam MRm3 camera, using a 40 x objective. SYN stained sections were viewed by a Zeiss LSM 710 laser scanning confocal microscope, using a 20x objective and a Zoom factor of 1.7., 3-4 images (180 x 180 μm) were collected from the cortical region of each section. Images were then transferred to a laptop PC and percent image area occupied by MAP2 positive dendrites or SYN positive presynaptic terminals was quantified using the Image J software. For each marker, values of individual animals were averaged. The analysis was performed blinded, and codes were broken once individual animal values had been obtained.

For the detection of micro- and astrogliosis, immunoperoxidase staining on free-floating sections was performed using standard procedures [18]. Three sections/mouse were stained for each marker, for a total of 16 vehicle-treated (6 females, 12 males), and 18 KA- treated mice (6 females, 12 males). The microglial marker anti-Iba was used at 1:3000 dilution, and the astroglial marker

anti-GFAP at 1:4000 dilution. Biotinylated goat anti-rabbit (Vector) secondary antibody was used at 1:200 dilution. The Vector "Elite" kit was used to visualize the signals, following manufacturer's instructions, except that "reagent A" and "reagent B" were both used at 1:200 dilution. The antibody binding was visualized with diamino-benizidine/H_2O_2 as peroxidase substrates, and the peroxidase reaction was stopped by transferring sections into 0.1M Tris buffer (pH 8.5-9). After washing, sections were mounted onto slides, air-dried, and coverslipped with NeoMount (Merck).

Bright field pictures were taken with a Zeiss AxioImager Z1 microscope coupled to an Axiocam MRm3 digital camera, using a 20X objective. Three images (450 x 335 μm each) were taken from the cortical area of each section. To quantify microglial and astroglial cells, the percent image area occupied by peroxidase reaction product was determined using the public domain ImageJ software. Values obtained for images from each animal were averaged, and then used to calculate group means. All sections were processed blinded and codes were broken once analysis was complete.

Principal Component Analysis and correlation analyses of metabolite profiles

Principal Component Analysis (PCA), in which the coordinate axes represent the uncorrelated maximum variance directions of the data, was applied to both the standardized brain region and kainic acid metabolome datasets and the resulting visualizations for the first two principal components were graphed.

Machine Learning

To investigate the region-specificity of the metabolite profiles, we applied two different machine learning approaches to the metabolic profiles obtained by GC-MS. First, we used a linear Support Vector Machine classification approach (SVM, [19]) with default parameters to train a predictive function relating metabolite measurements to the brain region they are derived from. To estimate the predictive accuracy of the resulting model for assigning samples to the correct brain regions, we used a standard *n*-fold cross-validation scheme as follows (with two settings: n = 5 and n = total number of samples): The samples were randomly partitioned into *n* matched-size groups, and each combination of *n-1* of these groups was used to train the machine learning model, while the accuracy of the brain region assignment was tested for the remaining hold-out group.

To enable a visual analysis of class separability, we applied Partial Least Squares Discriminant Analysis as a second machine learning algorithm (PLS-DA, [20]). PLS-DA is a statistical learning and data visualization approach designed specifically for datasets with high inter-correlation between the attributes (here: metabolites) and large numbers of attributes in relation to the numbers of samples.

Because feature selection (using only the most discriminative metabolites for the different brain regions) did not improve the cross-validated accuracy of the machine learning models for sample classification, the prediction models were built using the information from all metabolites.

Statistical analyses

For comparison of means between controls and treated mice on neuropathology/histology or molecular marker endpoints, statistical analyses was done by ANOVA followed by Tukey's post-hoc test, using the PRISM (Graphpad) software. A p-value smaller than 0.05 was considered significant. Mann-Withney and Kolmogorov-Smirnov tests were used to confirm the comparability of metabolite profiles for the two genders. Correlation analysis was performed with Pearson's test, and two-group comparisons with unpaired t-test.

To obtain a ranking of the most discriminative metabolites in terms of their differential abundance across brain regions and between treatment for the kainic acid dataset (kainic acid vs. vehicle), the empirical Bayes moderated t-statistic [21] was used. This approach tests whether all pairwise contrasts between different outcome-classes are zero and uses an empirical Bayes method to shrink the probe-wise sample-variances towards a common value.

To reduce the influence of signal-dependent noise, the variance stabilizing transformation [22] was applied to the metabolite abundance data prior to computing the differential abundance statistics. For problems with more than two outcome classes (here for the comparison of different brain regions), the F-statistic was computed as an overall test from the t-statistics for every metabolite. The p-value significance scores were adjusted for multiple hypothesis testing [23]. This allowed to determine the set of significant metabolites as those with a false-discovery rate below 0.05 (adjusted $p < 0.05$ considered significant). Statistical analyses other than ANOVA, t-test, and correlations, and all bioinformatical analyses were performed using the programming language R.

Results

The metabolic signature of mouse brain regions

To investigate whether the metabolite signature differed between anatomically distinct brain regions, we analyzed the metabolite profile of the following mouse brain regions by GC-MS: cortex, hippocampus, striatum, midbrain (with the main populations of dopaminergic neurons, such as Substantia Nigra, Ventral Tegmental Area, and Retrorubral Nucleus, but excluding the colliculi), cerebellum, and brainstem (pons and medulla). We found that 191 out of a total of 332 measured metabolites showed significantly differential levels across brain regions (false-discovery rate: 0.05); out of these 85 were identified, and 10 were derivatives of identified ones. A qualitative listing of the differential abundance for each of these 85 metabolites in the analyzed brain regions is shown in Table 2.

To characterize the differential abundance of key identified metabolites quantitatively across brain regions, we used differential abundance statistics (see Materials and Methods). The levels of the 9 identified metabolites that showed the most differences across brain regions are shown in Figure 1. These 9 metabolites were: pyrophosphoric acid, taurine, 2-hydroxypyridine, phosphoethanolamine, glycine, 5-oxoproline, hydrogen sulfide, dopamine, and glycerol. The differential abundance of the neurotransmitters dopamine and glycine showed an expected profile across brain regions. Dopamine, and its metabolite homovanillic acid, were highly abundant in the striatum, which receives the projections of dopaminergic neurons of the Substantia Nigra, and the cortex, which receives projections of dopaminergic neurons primarily from the Ventral Tegmental Area [24]. The inhibitory neurotransmitter glycine was highly abundant in the brain stem (pons and medulla), where it is known to be the major inhibitory neurotransmitter [25]. Two other metabolites with a strikingly different abundance

across regions were also neurotransmitters: Gamma-aminobutyric acid (GABA), which had the highest level in striatum, and midbrain [25]; and glutamate, which had the highest level in the cortex, hippocampus, and striatum [9]. These results support the robustness of our GC-MS methodology to analyze differentially abundant metabolites in various brain regions. The primary GC-MS data obtained for all the metabolites that were measured are listed in supplemental Table 1. Finally, there were also a total of 96 unknown metabolites that showed significantly differential distribution across brain regions, the most prominent of them are shown in supplemental Figure 1. This finding points to a vast area that still awaits investigation.

To characterize and visualize the global metabolic signatures of brain regions, by taking into account all measured metabolites, we used two approaches: Principal Component Analysis (PCA), a classical unsupervised visualization approach for multidimensional datasets, and supervised machine learning algorithms for quantitative predictive analysis as well as visualization.

To generate a two-dimensional PCA plot, we choose the first two principal components that were at the basis of most of the variation between the metabolite data: 18.5% (PC1) and 12.7% (PC2), respectively. Using this approach, we obtained a first visual representation of the metabolic signatures of the different brain regions forming largely distinct clusters (Figure 2A, left panel). There was, however, some overlap between the higher brain regions such as cortex, hippocampus, and striatum, that was possibly due to dimension reduction inherent to PCA.

To investigate if the brain regions can be quantitatively differentiated based on their metabolite signature, and to evaluate the utility of multidimensional brain metabolite data for predictive sample classification, we applied two different machine learning algorithms to our metabolite

measurement datasets. Supervised machine learning algorithms are computer algorithms that can learn from concrete, measured data to classify objects and predict the classification of objects of similar nature (here: sample groups representing brain regions), and that can be used to help interpret large amounts of biological data, such as those typical for "omics" studies [26]. We used two different machine learning algorithms to classify our brain metabolite data (see Material and Methods): (**1**) A support vector machine (SVM) algorithm, which separates the samples for different brain regions in high-dimensional space (with number of dimensions equal to the number of metabolites), by finding optimally separating hyperplanes; (**2**) A Partial Least Squares Discriminant Analysis (PLS-DA), which maps the data onto a lower-dimensional space, used for graphic visualization.

We found that, using the first approach, SVM, the average predictive accuracy of the metabolite signature for any of the analyzed brain regions was between 94.29% (estimated by 5-fold cross-validation) and 97.06% (estimated by leave-one-out cross-validation). To visualize, in a graph, the separability of the metabolite signature of different brain regions, we applied a second machine learning algorithm, PLS-DA. The result of this analysis, for which we used the entire datasets and obtained a training set accuracy of 97.06%, is shown in Figure 2A (right panel). We observed only one misclassified sample: one cerebellum sample overlapped with the metabolite profile of the brainstem. It is unclear whether this particular sample contained a contamination, or whether PLS-DA has some limitations in separating brain regions with somewhat related metabolite profiles. Overall though, machine learning approaches that don't use dimension reduction, in particular SVM, provided the most robust separability of brain region's metabolic profiles.

The metabolic response to excitotoxic brain injury reflects neurodegeneration and gliosis

To investigate the brain's metabolic response to a disease challenge, we used peripheral injections of kainic acid (KA) to induce excitotoxic injury. KA, a non-degradable analog of the transmitter glutamate, is an agonist for a subtype of ionotropic glutamate receptors, and a potent epileptic and neurotoxic agent [27, 28]. Upon entering brain regions rich in glutamate receptors, such as the hippocampus and cortex, KA induces a series of cellular events leading to neurodegeneration and gliosis[27-29]. Systemic injection of KA in rodent is widely used as a model to study the pathology and mechanism of neurodegenerative processes involving excitotoxicity.

We injected mice peripherally with KA or vehicle (PBS), and euthanized them 2 and 7 days after treatments. To enable detection of metabolic correlates of neurodegeneration, one hemibrain of each mouse was dissected and frozen for metabolite measurements and RNA extractions, the other one was post-fixed in paraformaldehyde for histology (see Materials and Methods). We focused, in this study, on the cortex, since it is a rather large region that provides sufficient amounts of material for metabolite extraction, and allows for ample and reliable sampling in histological measures. The cortex is also a region that is sensitive to excitotoxicity in mice [17].

We measured the metabolic signature of the mouse cortex in KA- and vehicle treated mice. We observed that there was no significant difference between the response to KA or vehicle at 2 days and at 7 days (by Mann-Withney U to check for differences in distribution center, and by Kolmogorov-Smirnov to check for differences in distribution shape). This observation was confirmed by the neuropathology findings (see below), and justified combining the groups of the two treatment time points for a sufficiently powered statistical analysis.

To examine the separability of KA-induced metabolic changes versus vehicle controls, and obtain a 2D visualization of the brain injury associated multi-dimensional metabolome data set, we first applied PCA, and then the two previously used machine learning algorithms SVM and PLS-DA. The results of these analyses are shown in Figure 2B.

We observed no clear grouping pattern using PCA, with the vehicle- versus KA-associated graph points showed extensive overlap (Figure 2B). Thus, PCA, did not highlight any biologically relevant grouping patterns between non-injured versus injured brain, and we assume that PCA-associated data dimension reduction had led to that outcome.

Therefore, to determine the separability of normal versus injured cortex metabolic signatures quantitatively and visualize it, we again applied the supervised machine learning algorithms, SVM and PLS-DA (see above). With the SVM approach, we obtained an average cross-validated accuracy of 82.7% (with 5-fold cross-validation) and 82.1% (with leave-one-out cross-validation). In other words, applying supervised machine learning to the metabolite signature of mouse cortex, without any other information on its origin or treatment (injured or control mouse), nor its pathological alterations, enables to predict whether it originates from an injured brain with an average accuracy of over 80%.

Using the second machine learning algorithm for visualization, PLS-DA, we graphed the data as shown in Figure 2B. We observed an obvious separation of the metabolic signatures of injured versus non-injured brain, with the exception that 3 out of 28 graph points of KA samples were part of the overlapping region with vehicle samples, and could not be separated out.

To confirm the metabolite measurements, we set out to quantify, histologically, the extent of KA-induced neurodegeneration and gliosis after KA injection. For that purpose, we measured a set of markers: two for neuronal integrity, MAP2 and synaptophysin, which have been

reported to decrease after an excitotoxic challenge [17], GFAP as a marker for astrocytosis [30], and IBA1 as a marker for microgliosis [31].

We observed that the extent of KA- induced neurodegeneration was 35% for MAP-2, and 28% for synaptophysin at 2 d as well as at 7 d after injection (Figure 3A, and supplemental Figure 2). We also observed that the extent of KA-induced gliosis was, both at 2 d and 7 d post injection, about 2-fold for microglia, and 5-fold for astroglia (Figure 3A). The changes induced by KA were similar at 2 d and at 7 d, an observation reflecting the great sensitivity of the FVB/N mouse strain to excitotoxicity [32]. Thus, we combined the values of both time points for the investigation of metabolome changes induced by KA (see above). To characterize the glial-cell mediated inflammatory response to KA-induced injury, we performed a series of RNA quantitations for specific inflammatory/glial markers (see Materials and Methods). The results of this analysis are shown in Figure 3B. As expected, the expression of molecular markers of glial activity were elevated after KA administration.

Interestingly, 2 out of these 3 points corresponding to KA-treated mice whose metabolite profile overlapped with that of control mice (PLS-DA graph, Figure 2B, right panel) were from mice that showed only a very mitigated neurodegenerative response to KA injection (less than 5% loss of the two neuronal markers MAP-2 and synaptophysin), thus being essentially non-injured. Therefore, our neuropathogical analyses showed that both machine learning algorithm, SVM and PLS-DA, provided, respectively, a clear separation and visualization of the metabolite profile of injured versus non-injured brain.

Metabolic correlates of neurodegeneration and gliosis

To investigate whether specific metabolite changes correlated with the extent of neurodegeneration and/or of gliosis, and thus could provide more in-depth information about the molecular processes of excitotoxic disease process, we performed case-by-case correlation of known metabolite levels with histology and molecular marker data (see Materials and Methods). Perhaps not surprisingly, we observed that the decrease in one well known correlate of neurodegeneration, N-Acetyl-Aspartate (NAA) [33], correlated significantly with the decrease in neuronal degeneration marker MAP-2 ($p = 0.042$), and with the increase of astrogliosis measured by GFAP ($p=0.037$) (Figure 4A). We detected a possibly interesting metabolic correlate of markers of astroglial reactivity, 3-hydroxybutyric acid (3-HBA), in mice 2 days after KA injection (n=6). Its increase correlated tightly with Timp1 ($p = 0.008$), Vim ($p = 0.017$), and Serpina3n ($p= 0.008$) expression increases. It was not observed 7 days after KA injection, indicating that its level is regulated. It will be interesting, in future studies, to look more closely at the regulation of the level of this metabolite after brain injury. Three-HBA is a ketone body produced primarily by hepatocytes, and, in the brain, by astrocytes [34], as an alternative source of energy and neuroprotectant[35, 36] .

We also applied a differential abundance test statistic to reveal the most prominent metabolic changes induced by excitotoxic injury. The four top-ranked known metabolites that displayed the most robust KA-induced increases were Lysine, Glutamine, Inosine, and Ribitol (median fold changes of 1.49, 1.39, 1.55, and 1.18 respectively; Figure 4B). L-glutamine, another astroglial marker, is a metabolite of L-glutamate, whose release by neurons in enhanced after injury[37, 38]. Two of the other top elevated metabolites, inosine and lysine, have been reported to have neuroprotectant properties [39-42].

Thus, by investigating the brain's metabolic response to excitotoxicity, we uncovered a number of metabolites that, in addition to their potential use as metabolic markers for neuronal or glial response to injury, likely play an active role in the mitigation or repair of neuronal damage.

Discussion

In this study, we have characterized, using GC-MS, the metabolic signature of different mouse brain regions, and followed the changes in this signature in the mouse cortex after excitotoxic neuronal injury. We have demonstrated that, at baseline, each brain region has a distinct metabolic signature that can be best drawn out by using supervised machine learning algorithms. These algorithms provide high accuracy tools for the predictive assessment of metabolic profiles of brain regions, and can serve as a baseline to investigate the metabolic response to different kinds of neurological disease and injury. . Importantly, we have also demonstrated that excitotoxic neuronal injury in the mouse cortex induces a metabolic signature that reflects neuronal injury and the accompanying gliosis. Finally, we found that some metabolites that change after injury might have a role in neuronal protection and repair.

Mammalian brain regions have typically one or more unique neuronal populations and connectivity patterns. Hence, their molecular profiles, and therefore function, do not overlap. Large scale gene expression studies have provided insights into the molecular heterogeneity of brain regions [43, 44], showing for instance that the cerebellum had an unique gene expression signature, whereas forebrain regions tended to show more overlap. Interestingly, this is also reflected in the metabolic profile of brain regions revealed in this study: lower regions, such as the cerebellum and brainstem, have a clearly different metabolic profile than the hippocampus, cortex, and striatum, which show some overlap.

Our study provides a map of metabolic signatures of mouse brain regions. Some known metabolites, such as dopamine or glycine, were clearly enriched in the region one would expect them to be (striatum and brainstem, respectively). Many of the detected metabolites still await identification, and it is likely that many of these metabolites have essential

biological functions. For instance, we have recently identified, in activated microglial cells, the immune effectors of the brain, the metabolite itaconic acid, which has anti-microbial properties [16]. We have also detected enhanced message levels of immunoresponsive gene 1 (Irg1), the mRNA coding for the enzyme catalyzing the production of that metabolite, in the brain of KA-treated mice (data not shown), indicating that it is also induced in the absence of infection.

A study on rat brain [45] , has analyzed the metabolic profile using ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR), and has also revealed region-specific signatures, with at least some overlap to our study, in particular high levels of taurine and N-Acetyl-Aspartate (NAA) in the cortex.

Mammalian brain regions, with their different neuronal subpopulations, are also characterized by differential susceptibilities to different neurodegenerative disease processes and inducing agents [46-53]. Our analysis of region-specific metabolic profiles provides the basis for the investigation of how specific neurodegenerative diseases change those profiles. Excitoxicity for instance, a neurodegenerative process that can be experimentally induced by the administration of KA, and affects brain regions rich in glutaminergic receptors, such as hippocampus and cortex [17, 28]. Excitotoxic injury involves Ca^{2+} overload, endoplasmatic reticulum stress, disruption of cell and organelle membrane, C-jun-terminal kinase 3 activation, lipid peroxidation, DNA fragmentation, mitochondrial potential breakdown [28], neuroinflammation[27, 29, 54] and, based on our findings, a specific set of metabolites that also participate in this process. Excitotoxitic injury has been implicated in a range of neurological disorders, ranging from acute CNS injury and stroke, to chronic afflictions such as Alzheimer's disease, Huntington's disease, and motor neuron disease [55, 56].

Together with other endpoints, such as quantitative histology, metabolic profiling can be of practical use not only from a biomarker perspective, for the assessment of neuronal injury in disease models, but also to better understand the effect of experimental therapies and evaluate their efficacy. Because metabolic profiling can comparatively easily be scaled up and automated, it could ultimately end up being more widely used early in the lead optimization and selection process of pharmacological compounds, before more time-consuming steps, such as quantitative histology, are used to follow-up on further compound selection.

Metabolic profiling has already made its entry into the field of biomarker discovery for neurodegenerative disease [4]. Many clinical studies are using NMR for brain imaging studies, or for the analysis of cerebrospinal fluid (CSF), to study metabolite changes associated with neurological diseases [8]. In contrast to NMR, GC-MS provides good resolution for thermally stable compounds that can be made volatile through derivatization [57]. However, although GC-MS, in contrast to NMR-based techniques, can resolve hundreds of metabolites, compound derivatization and fragmentation during the procedure precludes direct metabolite identification. Thus, GC-MS leaves a number of metabolites for which no standard exists unidentified. While GC-MS has this limitation, ^{1}H-NMR platforms are often restricted to a limited number of highly abundant metabolites, such as NAA, choline, creatine, or lactate, and are also to some degree limited in their spatial resolution [57]. Thus, the precise approach to choose for metabolic profiling will depend on specific questions and needs of each study. We opted to use GC-MS, because it would allow us to capture a large population of metabolites in the mouse brain, and follow their global alterations in a disease condition.

The use of mouse models allows for longitudinal studies and the direct analysis of extracted tissue targeted by the disease or therapeutic process, thus opening the possibility of identifying

and following through on biomarker candidates in a way that isn't always feasible or practical in the clinic. For instance, a number of studies have analyzed the CNS metabolite profile of transgenic mouse models for Alzheimer's disease [58-60]. These mice express a mutated form of the human Amyloid Precursor Protein (hAPP) under the control of a neuronal promoter, and develop, as they age, a number of typical AD-like features, such as amyloid deposits, and synaptic and neuronal degeneration and dysfunction [61]. While NAA appeared consistently decreased across these models, only one study attempts to correlate metabolite levels with histology measures [60], and shows that NAA correlates negatively with amyloid-beta plaque load. In our study, we also observed, as we had expected, a decrease in NAA in the cortex of mice after excitotic injury. NAA, an amino acid that is present in high concentration in the brain, is a neuron-specific marker of unclear function, and a general marker for neuronal demise, in animal models as well as in the clinic [33]. Its prominent signal in NMR studies has led to its wide us as an indicator of neuronal pathology and disease progression in a variety of CNS diseases and animal models of these diseases[33]. In humans, it has been reported to be decreased in a variety of neurological afflictions, such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, and Huntington's disease [33]. In animal models, it has been one of the most robustly affected metabolites in models of Huntington's disease [62], Parkinson's disease [63], and Fragile-X syndrome [64]. While very informative as a general biomarker for neuronal demise, NAA is, diagnostically, an unspecific marker. Thus, NAA is a very informative marker for monitoring neuronal damage associated with CNS diseases, even though it lacks specificity for any particular disease.

Other notable changes we have observed were metabolites that were elevated after brain injury, some of them of astroglial origin, and that are likely play a role in neuronal protection and repair.

Tree-HBA was elevated 2 days after KA injection, and correlated with markers of astroglial activation. It is produced in the brain when glucose supply is limited, such as during starvation or injury [65]. *In vitro*, it prevents neuronal damage after glucose deprivation [66] or mitochondrial toxin exposure [35], and, *in vivo*, it protects neurons from the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) a neurotoxin that induces Parkinson's disease-like symptoms [36]. Our observation that is was only elevated at a certain time point after injury indicates that its role after excitotoxicity may be very restricted, and this may require further investigation in follow-up studies.

The other metabolites that were elevated were L-glutamine, inosine, lysine, and ribitol. L-glutamine results from the conversion of neuronal glutamate after it is taken up by astrocytes form the synaptic cleft. Under non-pathological conditions, it is returned to neurons where it is converted back into glutamate, but under pathological conditions, when neurons die, it ends up being elevated in injured tissue[37, 38]. Inosine and lysine are of unknown cellular source in the injured brain, but both have been reported to have neuroprotective properties when administered after stroke[39-41]. Therefore, a number of metabolites that are elevated after injury might reflect the attempt of surviving brain cells to limit neuronal damage, and thus they may be biomarkers of the brain's ability to protect itself. The pathological role of the polyol sugar ribitol, which is thought to be a metabolic end-product in humans[67], is unknown. Pathological accumulation of polyols might play a role in secondary neuronal damage associated with metabolic diseases[68, 69], even though a direct neurotoxic effect could not be demonstrated[70].

It is ultimately more informative though to complement individual metabolite measurements with assessing metabolite signatures that reflect a wider spectrum of neuronal dysfunction and degeneration, such as we have performed in this study. A population of biomarkers is far more

likely than a single or even just a few molecules to capture the essence of a disease process, and to monitor the effects of therapies. In our study, the widely used unsupervised PCA, a technique that facilitates the analysis of multi-dimensional datasets by providing a low-dimensional data representation and retains as much as possible of the variance information of the input data, was sufficient to separate the metabolite profile of different mouse brain regions at baseline, but not optimal to distinguish between the metabolite profiles of injured *versus* non-injured brain tissue. PCA does not retain the distances between the original untransformed data points, and therefore can only provide a limited visual impression of the separability between metabolite signatures. Supervised machine learning, on the other hand, turned out to be better for the analysis and interpretation of metabolic profiles of brain regions and the alterations of such profiles by injury or disease. The application of machine learning to analyze the injured mouse brain suggests that a global change in metabolic profile is an intrinsic part of the response of brain cells to injury, and that this profile could be used as a global, reliable biomarker in preclinical pharmacological efficacy studies. Machine learning, even though based on solid theoretical foundations, is still only rarely used in the assessment of large multidimensional biological datasets, and our study shows that it can be applied successfully to the study of such datasets.

Thus, we suggest that supervised machine learning algorithms can discover latent patterns in multidimensional datasets, discriminate between different types of biological samples, and thus could help with the interpretation of highly regulated and dynamic biological processes.

In conclusion, we have shown that, at least preclinically, a metabolic profiling approach of the mouse brain and its response to injury, guided by adequate bioinformatical evaluation, and validated by quantitative neuropathology and molecular analysis, provides novel insights into brain biology and pathology. These insights could help pave the way for similar studies of

biomarker candidate population identification and assessment in various models of neurodegenerative disease, and possibly their human counterparts.

Acknowledgements: The authors wish to thank Dr. Karsten Hiller (Luxembourg Centre for Systems Biomedicine) for help and support with Gas Chromatography-Mass Spectrometry measurements and evaluation.

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Figure legends

Figure 1 Box-plots of 9 known metabolites that were the most significantly differentially abundant across brain regions. As expected, the inhibitory neurotransmitter glycine was the most abundant in the brainstem, and the neurotransmitter dopamine was the most abundant in the striatum, and relatively high in the cortex even though the variation was extremely high in that region. Dopamine was barely detectable in the other regions, which explains the negative log values. These observations validate the GC- MS technology used. For details, see text.

Figure 2 Visualization of metabolite profiles of different mouse brain regions (**A**), and after excitotoxic brain lesioning with KA (**B**). To visualize the region-specific metabolic signatures of different mouse brain structures and of the brain's response to injury, PCA (left panels) and PLS-DA (right panels) were performed as described in Materials and Methods. **A.** At baseline (unmanipulated mice), the profile of different brain regions showed a clear separation. Some overlap was observed between the hippocampus, striatum, and cortex, in the PCA graph. The supervised machine learning algorithm PLS-DA provided a better separation, in 2 D, between the metabolic signatures of different brain regions. **B**. After excitotoxic injury, important changes in the metabolic profile of the mouse cortex were observed. There was significant overlap between the sample points of the two groups (vehicle versus KA) in the PCA graph (lower left panel). A better separation between the KA- and vehicle treated mice was achieved by using the supervised machine learning algorithm PLS-DA (lower right panel). In this graph, 3 points of KA-treated groups overlapped with the vehicle group, and 2 out of these 3 corresponded to mouse cortices that didn't show signs of neurodegeneration by quantitative histology (Figure 3). This indicates

that machine learning analysis of metabolite population datasets mirrors the pathological injury status of a brain sample with high accuracy, and predict whether a given metabolite profile originates from an injured or a control brain.

Figure 3 Neurodegeneration and gliosis induced by kainic acid (KA). Mice were injected with 20mg/kg KA or vehicle (VEH), and euthanized 2 days, or 7 days later. **A.** Histological quantitation of neurodegeneration was performed on fluorescently labeled MAP2 (neuron dendrites and cell bodies, first row) sections. Quantitation of gliosis was performed on immunoperoxidase GFAP (reactive astrocytes, second row), and Iba1 (microglia, third row) labeled sections (see Materials and Methods). Qualitative microphotographs (middle and right panels) show examples of MAP-2 (first row), GFAP (second row), IBA1 (third row) immunostains on vehicle or KA-treated mice. Results obtained for the synaptic markers synaptophysin were similar to those of MAP-2 (Suppl. Fig.1). The extent of neurodegeneration and gliosis was similar at 2 d and at 7 d, probably a reflection of the high sensitivity of the FVB/N mouse strain to excitotoxicity. * $p<0.05$, *** $p<0.01$. Scale bars: 50 μm for the first row (MAP-2), 120 μm for the second and third row (GFAP and IBA1). **B.** Gene expression of microglial cells and astrocytes molecular markers in KA-lesioned mouse cortex (quantitative PCR). Levels of mRNAs were measured in cortical tissues harvested injected with vehicle (VEH) or kainic acid (KA, 20 mg/kg) at 2 days or 7 days. The levels of mRNA were determined by real-time RT-PCR and normalized using Rpl27 as housekeeping gene. Each bar represents the average expression of six mice (±SEM). Timp 1 = Tissue metallopeptidase inhibitor 1, Vim = Vimentin, IL-1β = interleukin 1 beta, Ccl2 = chemokine (C-C motif) ligand 2. Results obtained for the astroglial marker Serpina3n (not shown) were similar to those obtained for Timp1 and Vim. All

markers were significant by ANOVA, but only intergroup comparisons that showed significant increases are indicated by (*p< 0.05, **p<0.01, ***p<0.001).

Figure 4 Metabolic correlates of excitotoxic neurodegeneration. For metabolites shown, GC-MC signal values were calculated as % of the means of vehicle control. **A**: The neuronal metabolite N-Acetyl-Aspartate (NAA) was significantly reduced after kainic acid (KA) injection (*p<0.05, unpaired t-test), and correlated positively with the loss of MAP-2 (p = 0.045, Pearson r = 0.47), and negatively with the increase of GFAP (p=0.006. Pearson r = 0.64). **B**: Glutamine, lysine, inosine, and ribitol were significantly increase after KA treatment (*p< 0.05, **p<0.01, ***p<0.001, unpaired t-test). The increase glutamine most likely resulted from excess glutamate released by injured neurons, which take up by astrocytes and metabolized to glutamine. The increase in lysine and inosine, which have neuroprotectant properties, may be the brain's attempt at limiting and repairing neuronal damage. The function of ribitol is unknown.

Supplemental figure 1 Box-plots of 18 unknown metabolites that were significantly differentially abundant across brain regions, shown for illustration. Since many, if not all, of these metabolites have a biological role, this observation points to a vastly unexplored field. It is also tempting to speculate that these differentially abundant metabolites play a role in the differential susceptibility to injury and disease of brain region-specific neuronal populations.

Supplemental figure 2 Synaptic degeneration induced by kainic acid (KA). Mice were injected with 20mg/kg KA or vehicle, and euthanized 2 days, or 7 days later. Left panel: Quantitation of

synaptic degeneration was performed on sections fluorescently labeled for the synaptic marker synaptophysin (SYN, see Materials and Methods). Middle and right panels: Qualitative microphotographs of examples SYN immunostains on vehicle- (VEH, middle panel) or KA-treated (right panel) mice. The extent of synaptic degeneration was similar at 2 d and at 7 d (*** $p<0.01$, ANOVA and Tukey post-hoc), probably a reflection of the high sensitivity of the mouse strain used (FVB/N) to excitotoxicity. Scale bar: 50 μm.

Supplemental table 1 Primary GC-MS data for all measured metabolites.