

The Methylazoxymethanol Acetate (MAM-E17) Rat Model: Molecular and Functional Effects in the Hippocampus

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Administration of the DNA-alkylating agent methylazoxymethanol acetate (MAM) on embryonic day 17 (E17) produces behavioral and anatomical brain abnormalities, which model some aspects of schizophrenia. This has led to the premise that MAM rats are a neurodevelopmental model for schizophrenia. However, the underlying molecular pathways affected in this model have not been elucidated. In this study, we investigated the molecular phenotype of adult MAM rats by focusing on the frontal cortex and hippocampal areas, as these are known to be affected in schizophrenia. Proteomic and metabolomic analyses showed that the MAM treatment on E17 resulted primarily in deficits in hippocampal glutamatergic neurotransmission, as seen in some schizophrenia patients. Most importantly, these results were consistent with our finding of functional deficits in glutamatergic neurotransmission, as identified using electrophysiological recordings. Thus, this study provides the first molecular evidence, combined with functional validation, that the MAM-E17 rat model reproduces hippocampal deficits relevant to the pathology of schizophrenia.

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INTRODUCTION

Administration of the short-acting DNA-alkylating agent methylazoxymethanol acetate (MAM; Cattabeni and Di Luca, 1997; Cattaneo *et al*, 1995; Meyn and Murray, 1984) on embryonic day 17 (E17) to rat dams interferes with the development of specific brain regions in the offspring (Bayer *et al*, 1993; Moore *et al*, 2006). Through selective disturbance of proliferation and migration of neuronal precursor cells undergoing their final mitosis, MAM induces morphological and cytological alterations modeling phenomena seen in schizophrenia post-mortem brains (for review see Lodge and Grace, 2009b). Behavioral studies of

offspring of MAM-treated rats (hereafter referred to as MAM rats) showed deficits in sensorimotor gating (Le Pen *et al*, 2006), an inability to ignore irrelevant stimuli (Flagstad *et al*, 2005), hypersensitivity to amphetamine and *N*-methyl-D-aspartate (NMDA) receptor antagonists (Penschuck *et al*, 2006), and social withdrawal (Flagstad *et al*, 2004) with onset in adolescence (Le Pen *et al*, 2006). Although direct evaluation of schizophrenia symptoms in animal models is inherently difficult, such effects have been related to neuropsychiatric deficits seen in schizophrenia (Braff *et al*, 1992; Hansen *et al*, 2009; Laruelle *et al*, 1996; Lubow and Gewirtz, 1995). Consequently, the MAM-E17 rat model is widely thought to represent a neurodevelopmental model of schizophrenia (Lodge and Grace, 2009b).

Utilization of any model for investigation of pathological mechanisms and novel drug discovery requires identification of which molecular components of a disease can be represented. Apart from studies which have used immunohistochemical staining for parvalbumin and reelin in the frontal cortex and hippocampus (Lodge *et al*, 2009a; Matricon *et al*, 2010; Penschuck *et al*, 2006), molecular characterization of the MAM-E17 rat model has not yet

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been reported. As most post-mortem and *in vivo* studies of schizophrenia patients have identified deficits in the prefrontal cortex and hippocampus (Bertolino *et al*, 1996; Harrison, 1999; Tsai *et al*, 1995), we have attempted to identify expression differences in the proteome and metabolome of the equivalent brain regions in MAM rats. Furthermore, we evaluated if the identified molecular changes translate into synaptic physiology. Such analyses may provide molecular and physiological correlates for some of the histological and behavioral deficits seen in MAM rats. The relevance of these findings to schizophrenia is discussed.

MATERIALS AND METHODS

Animals

All procedures were conducted in full compliance with the Home Office Guidance (UK Animals Scientific Procedures Act 1986) and the ethical policies of Eli Lilly & Co. Sprague Dawley rat dams (Charles River, Margate, UK) were injected intraperitoneally with either 22 mg/kg MAM (26 mg/ml concentration accounting for liquid density) or vehicle sham (0.9% saline) on gestational day 17. For proteomic and metabolomic experiments, rats were killed at 3 months by cervical dislocation. Brains were dissected on ice, frozen in liquid nitrogen and stored at -80°C until use. For electrophysiological recordings, samples were prepared as described below.

Tissue Fractionation

Frontal cortex and hippocampal tissues from 10 MAM and 10 sham rats were homogenized. For quality control purposes, control rat frontal cortex and hippocampus samples were homogenized, split into five aliquots and prepared in parallel with the MAM and sham samples. Two fractions were produced using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany). Both fractions were stored at -80°C until use. In an independent experiment, a frontal cortex homogenate was split into 12 aliquots, fractionated as above, and the resulting samples (fraction 2) were divided randomly into two groups of six for method validation. Each group was spiked with four non-homologous proteins (porcine albumin, bovine asialofetuin, bovine casein- β , and human lactogen) at the indicated concentrations (Figure 1a).

Liquid Chromatography

Proteins in fractions 1 and 2 (200 μg each) were precipitated using the ProteoExtract Precipitation Kit (Calbiochem). The resulting pellets were digested using the ProteoExtract All-in-one Trypsin Digestion Kit (Calbiochem). All further analyses were carried out in triplicate. Separation of the resulting peptides was achieved using a reverse-phase C18 column (inner diameter = 75 μm , length = 200 mm, particle size = 1.7 μm ; Waters Corporation, Milford, MA) on a nano Acquity system (Waters Corporation). Buffer A consisted of 0.1% formic acid in water, and buffer B was 0.1% formic acid in acetonitrile. The peptides were separated at a

a PROTEIN	Spiked concentration (ng/ μl)		Ratio expected	Ratio measured	Deviation of ratio
	group A	group B			
	Albumin (porcine)	1	1	1	0.80
Asialofetuin (bovine)	1	3	0.33	0.34	3%
Lactogen (human)	2	3	0.66	0.55	17%
Casein β (bovine)	4	2	0.5	0.31	39%

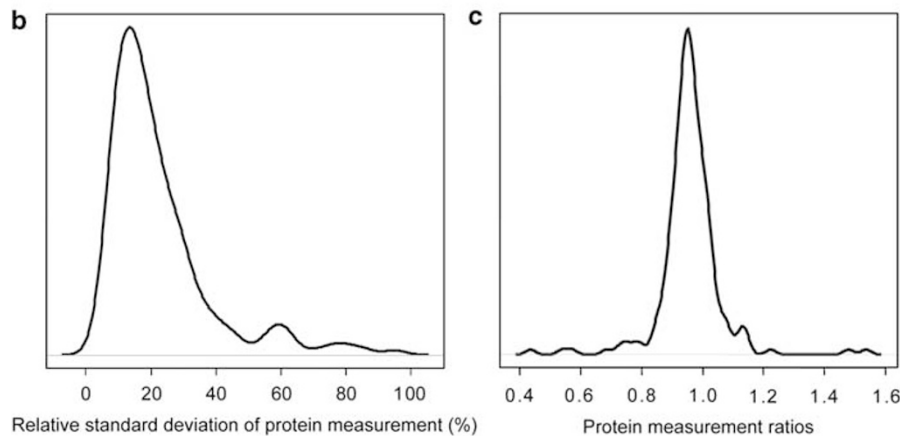


Figure 1 Technical validation of LC-MS^E proteomics using 12 spiked replicates of rat brain lysates. (a) Accuracy was assessed by measurement of four spiked non-rat proteins simulating different protein abundance ratios in the technical replicates. Reproducibility was assessed using density plots of (b) the RSD of the protein abundance measurements of 189 rat proteins in each technical replicate and (c) the relative protein abundance (ideal value = 1).

flow rate of 0.3 $\mu\text{l}/\text{min}$ using the following gradients: 0–90 min, 3–30% B; 90–115 min, 30–90% B; 115–125 min, 90% B; 125–126 min, 90–3% B; and 126–150 min, 3% B.

Mass Spectrometry

Eluting peptides were ionized at the electrospray interface and analyzed in positive ion mode using a Q-TOF Premier mass spectrometer (Waters Corporation), operated in alternate scanning data-independent acquisition configuration for label-free peptide quantification and identification (MS^E ; Silva *et al*, 2005). Precursor ion (MS , 0.6 s) and fragment ion (MS^E , 0.6 s) spectra were acquired continuously. External reference spectra of [Glu1]-fibrinopeptide B were recorded every 30 s for mass correction.

Data Processing and Protein Identification

The data generated were processed and queried against the rat portion of the SwissProt database (release 55.6, 2008–06), using ProteinLynx Global Server v2.3 (Waters Corporation) as described (Geromanos *et al*, 2009; Li *et al*, 2009). Data filtering for peptides was performed using the free statistical software package R (<http://cran.r-project.org>). The filtering criteria required that peptides were (i) identified by database searching, (ii) present in at least two of the three liquid chromatography mass spectrometry in expression mode (LC- MS^E) replicates, and (iii) present in at least 80% of the samples of each treatment group. Quantitative peptide measurements for each replicate were normalized against the total ion volume of all de-convoluted spectra. Replicates were averaged and ratios of protein abundance for each protein were calculated based on the sum of peptides. Significance of protein expression differences was determined using Student's *t*-test with Welch's correction ($p < 0.05$). False discovery rate (FDR) was used to control for multiple hypothesis testing ($q < 0.5$; Benjamini and Hochberg, 1995; Storey, 2002).

In Silico Pathway Analysis

The most significant molecular functions, canonical pathways, and interactions represented by the integrated lists of significantly altered proteins or metabolites in the MAM-E17 rat model were determined *in silico* using the Ingenuity Pathway Analysis Knowledge Base (IPKB; Ingenuity Systems, <http://www.ingenuity.com>; Calvano *et al*, 2005). The IPKB contains molecular information available in the scientific literature. Lists of the significantly altered proteins or metabolites found in the MAM-E17 rat model were uploaded to the IPKB software (inclusion criterion for proteins: $p < 0.05$, $q < 0.05$, and metabolites: $p < 0.05$).

The functional and pathway analysis identified the biological functions that were most significant to the data set by overlaying the significantly altered molecules (inclusion criterion for proteins: $p < 0.05$, $q < 0.05$, and metabolites: $p < 0.05$) onto predefined maps containing functional and pathway information of the IPKB database. Right-tailed Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function or pathway assigned to that data set is due to chance alone. Furthermore, network analysis of significantly

altered proteins ($p < 0.05$, $q < 0.05$) was performed. Proteins were mapped to their corresponding objects in the IPKB. Networks were then generated algorithmically on the basis of the connectivity derived from molecular interaction information.

Targeted Analyte Cluster Analysis

Complementary to the *in silico* pathway analysis, we applied the targeted analyte cluster (TAC) algorithm, as described previously by Cheng *et al* (2010), as a means of identifying significantly affected pathways. Significantly changed proteins ($p < 0.05$, $q < 0.5$) identified by LC- MS^E screening of the MAM-E17 model were subjected to TAC analysis. This method identifies clusters of 2–4 molecules with correlated expression levels, which account for the main variance in the data. This is likely to occur for coordinately regulated molecules, such as those that are components of the same molecular complexes or pathways.

At the statistical level, data points that are not linearly separable in a two-dimensional (2D) space can be linearly separated in a higher dimensional (3D) space. TAC applies factor analysis to identify molecules with linear combinations of molecular expression levels, which are the most effective representation of the total variable space of the data set. On the basis of the representative molecules identified, the prediction power of the molecular combinations can then be evaluated by testing other data sets using kernel principal component analysis (kPCA). For each prediction, no more than 2–4 molecules are tested to avoid over-fitting in cases of small sample sizes. For example, if a specific combination of proteins A, B, and C gives a good separation of MAM and sham rat samples on 2D (planar) projections of kPCA, then the protein cluster comprised of A, B, and C is likely to be an effective marker for identifying MAM rats. Half of the MAM-E17 and sham rats were selected randomly as a training set. The trained model was applied to predict correct assignment as a MAM or sham rat from the remaining half. The training and prediction procedure was repeated 1000 times. The precision of each protein cluster was calculated finally as the average precision of the 1000 iterations. Precision was defined as the percentage of MAM rats projected correctly.

Immunoblot Analysis

Relative differences in abundance of the most significant proteins found using LC- MS^E profiling were tested by immunoblot analysis. Samples of fractions 1 and 2 (10 μg protein) from the same MAM ($n = 8$) and sham ($n = 8$) hippocampal extracts, used previously for LC- MS^E analysis, were loaded onto 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen, Paisley, UK) to separate the proteins by electrophoresis. The proteins were then transferred electrophoretically onto polyvinylidene fluoride membranes. Membranes were blocked, incubated with primary and secondary antibodies and then scanned with the Odyssey Infrared Imaging System following the provided protocol (LI-COR Bioscience; Lincoln, NE). The following primary antibodies were used (all from Abcam, Cambridge, UK; dilution indicated in parentheses): rabbit anti-AMPA1 (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic

acid; 1:200), mouse anti-ARP3 (1:10 000), rabbit anti-GAPDH (1:1000), mouse anti-GAPDH (1:1000), rabbit anti-HPCA (1:400), rabbit anti-MARCKS (1:5000), rabbit anti-PMCA1 (1:500), and rabbit anti-SYNJ1 (1:300). In addition, the following secondary antibodies were used (LI-COR Bioscience): donkey anti-mouse IRDye800CW and donkey anti-rabbit IRDye680 (both 1:15 000). Quantification of the infrared signals was performed with the analysis software provided. For normalization of the protein signals, GAPDH was used as a loading control. Only scanned blots with a relative standard deviation of less than 30% for the GAPDH signal were considered. The significance of protein band expression differences was determined using *t*-test statistics with Welch's correction ($p < 0.05$).

To identify the unpredicted band in the AMPA1 immunoblot of fraction 2, a pooled sample of fraction 2 was split into two aliquots. One aliquot was treated with λ -protein phosphatase, following the protocol provided (New England Biolabs, Ipswich, MA). Proteins of both aliquots were subjected to immunoblot analysis and probed with phospho-specific rabbit anti-phosphoSerin-845 and rabbit anti-phosphoSerin-831 AMPA1 antibodies (Abnova, Taipei City, Taiwan; both 1:1000) as described above.

¹H-Nuclear Magnetic Resonance Spectroscopy Analysis

Striatum (20 mg), hippocampus (20 mg), frontal cortex (30 mg), and cerebellum (65 mg) of 10 MAM and 10 sham samples were homogenized in 1-ml acetonitrile/deionised water (1:1). The homogenates were combined with an equal volume of 3:1 chloroform/methanol, forming aqueous and lipid phases. The phases were vortexed to homogeneity and centrifuged for 7 min at 1200g at 5°C. The aqueous phase was collected, left overnight at room temperature to remove residual acetonitrile by air drying and then freeze-dried at -70°C. The samples were reconstituted in 600 μ l of deuterium oxide. For each sample, a ¹H-nuclear magnetic resonance spectroscopy (NMR) spectrum was acquired at 600.13 MHz on a Bruker DRX-600 spectrometer (Bruker GmbH) using the first increment of the nuclear overhauser effect spectroscopy pulse sequence (relaxation delay, $\pi/2 - t1 - \pi/2 - tm - \pi/2$; repetition time) as described previously (Lan *et al*, 2009). For each spectrum, 256 transients were acquired to yield 32 000 data points, with a spectral width of 10 kHz at an ambient probe temperature of 25°C. The resulting free induction decays were multiplied by an exponential weight function corresponding to a line broadening of 0.3 Hz, prior to Fourier transformation. Complete spectra in the chemical shift range 0.20–10 p.p.m. (parts per million) were phased, baseline corrected, and calibrated according to the reference resonance at 1.33 p.p.m. (lactate doublet), using the Topspin v2.0 software (Bruker GmbH). Spectral variation effects due to presaturation of water were removed by zeroing intensity values between 4.5 and 4.9 p.p.m. To identify metabolites that differentiate MAM from sham animals, partial least squares discriminant analysis (PLS-DA) was performed on full-resolution ¹H-NMR spectral data using SIMCA-P v12 (Umetrics AB, Umeå, Sweden). To remove confounding variations, orthogonal projection on latent structures discriminant analyses (OPLS-DA) of full-resolution spectra was conducted as described previously (Cloarec *et al*, 2005). This method is

an improvement compared with the traditional supervised algorithm of PLS, and enhances the interpretability of spectral variation between classes (Eriksson *et al*, 2004; Lan *et al*, 2009). To confirm the differences identified by multivariate testing, univariate analyses on manually integrated single resonance groups were performed (Student's *t*-test, $p < 0.05$). Pathways and functions associated with significantly altered metabolites were determined using *in silico* pathway analysis, as described above.

Electrophysiological Recordings

Electrophysiological recordings were made from transverse dorsal hippocampal slices (400 μ m) prepared from 3- to 4-month-old MAM and sham rats. The experimenter was blind to the treatment group. Animals were anesthetized using isoflurane and killed by cervical dislocation. Brains were removed and placed in ice-cold artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 10 mM MgSO₄, 10 mM glucose, 1 mM CaCl₂) aerated with 95% O₂ and 5% CO₂. Hippocampi were removed, sliced and maintained at 5°C in a 765-1 temperature-controlled chamber. The cornu ammonis 3 of hippocampus region was removed and the slices were transferred to aCSF containing 1 mM MgSO₄ and 2 mM CaCl₂. Slices were incubated at 32°C for 30 min to stimulate metabolism and then maintained at room temperature for at least 1 h before recordings were made.

Extracellular recordings were made in a Slicemate submerged chamber (Scientifica, Bedford, UK) maintained at 32°C and continuously perfused at 2 ml/min with aCSF modified to contain 1 mM MgSO₄ and 2 mM CaCl₂, and aerated as above. Standard extracellular recording techniques were used to measure the slope of field excitatory post-synaptic potentials (fEPSPs) from stratum radiatum in area CA1 (cornu ammonis 1 of hippocampus), evoked by stimulation of the Schaffer collateral by current injection using a concentric bipolar electrode. Recordings were made with an Axopatch 200B amplifier (Molecular devices, Silicon Valley, CA), filtered at 2 kHz, and digitized using a Digidata 1322A A/D board at a sampling rate of 10 kHz. In pharmacological experiments using GYKI53784, recordings were filtered at 10 kHz and digitized at 40 kHz, followed by post-filtering with a digital gaussian filter to an effective cutoff frequency of 1 kHz. Recordings were monitored and analyzed online and reanalyzed offline using WinLTP (Anderson and Collingridge, 2001). Pharmacological studies were performed to confirm the type of glutamate receptor responsible for synaptic transmission. Baseline responses were set at 5–10% of the maximal fEPSP obtained at 300 μ A stimulation. Following a stable baseline period of at least 20 min, 10 μ M GYKI53784 ($n = 5$ and $n = 5$ for MAM and sham rats, respectively) and 50 μ M 2-amino-5-phosphonopentanoic acid (AP5; $n = 9$ and $n = 10$ for MAM and sham rats, respectively) were applied in the bath solution for 20 min. To investigate the efficiency of synaptic transmission, 19 MAM slices and 11 sham slices from two batches were stimulated at a range of intensities that resulted in comparable fiber volley (FV) amplitudes. Four recordings were made at each stimulation strength at 0.033 Hz and the recordings were averaged. fEPSPs were related to the FV rather than the stimulation strength. This was carried out as

the FV is a measure of the action potentials generated in the Schaffer collateral by the stimulating electrode, and is therefore the most direct input to synapses in area CA1. Linear regression was performed on the data produced using Prism software v5 (GraphPad). Data are presented as mean \pm SEM. Significant differences between MAM and sham samples were assessed using paired Student's *t*-tests in the pharmacological experiments and an unpaired Student's *t*-test in the comparison of the slopes of the input-output relationships between groups ($p < 0.05$).

RESULTS

Technical Validation of LC-MS^E Brain Tissue Proteomics

The variation introduced by sample preparation and analysis can have a significant impact on the reliability of quantitative molecular profiling experiments. Protein quantification using LC-MS-based approaches has been challenged by physicochemical effects, which can obscure the linear relationship between the signal and abundance of an analyte (King *et al*, 2000). This is especially the case for complex samples such as tissue lysates. Therefore, we assessed the reproducibility and accuracy of the tissue extraction and LC-MS^E procedure in a validation experiment. A homogenized rat cortex sample was divided into 12 aliquots to generate technical replicates. After fractionation, the technical replicates were split into two equal groups (A and B) and spiked with non-rat proteins. Figure 1a shows the concentrations of the spiked proteins in both groups, simulating differential protein abundances between them. All 12 technical replicates were analyzed using LC-MS^E. To assess the reproducibility, the relative standard deviations (RSDs) and abundance ratios for 198 identified rat proteins were calculated and plotted (Figure 1b and c). Figure 1b shows that the original protein abundance in the technical replicates deviated by 22% on average, due to sample preparation and analysis. We found in a previous study that $\sim 10\%$ of the technical variation was introduced by the LC-MS^E analysis method (data not shown). This is comparable to or better than other proteomics methods, which show an average variation of 19–30% from the analysis phase alone (Molloy *et al*, 2003; Molloy *et al*, 2005). In addition, the protein abundance ratio distribution in Figure 1c illustrates that the relative protein measurements differed by only 4% from the ideal ratio of 1. Comparison of the simulated and measured fold changes of the four spiked non-rat proteins showed that the average deviation of the simulated expression difference was 20% (Figure 1a). The values for the individual proteins vary possibly due to different physicochemical properties of the proteins, resulting in different ionization efficiencies (King *et al*, 2000). We also obtained a similar result using 20 aliquots of serum samples, which required fewer sample preparation steps (Levin *et al*, 2007). Consequently, the data showed that the integration of quality controls for LC-MS^E analysis of brain tissue was necessary in order to assess the precision of the quantitative measurement for each protein. This guarantees a more accurate interpretation of the data.

LC-MS^E Analysis of MAM Rat Frontal Cortex and Hippocampus

Analysis of fractions 1 and 2 of frontal cortex from MAM rats by LC-MS^E resulted in identification of 743 proteins (data not shown). None of these proteins showed significant differences in expression between the MAM and sham rats ($q > 0.99$). LC-MS^E analysis of hippocampus from MAM rats resulted in identification of 673 proteins (data not shown), and 38 of these showed statistically significant differences in expression ($p < 0.05$, $q < 0.5$; Table 1). After controlling for multiple hypotheses testing, six proteins (AMPA1, ARP3, MARCKS, HPCA, PMCA1, and SYNJ1) showed FDR *q*-values of less than 0.05. The protein expression differences ranged between 6 and 25%, and could be measured with an average RSD of 7%. This low variability of the protein abundance (Figure 1a) provides confidence in the detection of subtle expression differences such as seen here, and thereby provides greater reliability of the candidate proteins.

Validation of LC-MS^E Hippocampal Candidate Proteins by Immunoblot Analysis

Six candidate proteins, showing the greatest significance after controlling for FDR ($q < 0.05$), were selected for validation. Owing to a lack of commercially available immunoassays for these proteins, we tested the candidate proteins using immunoblot analysis, which is considered to be a semiquantitative method (Mann, 2008). We used an infrared fluorescence detection system improving quantification (Schutz-Geschwender *et al*, 2004) and were able to confirm the altered expression of PMCA1, HPCA, and MARCKS ($p < 0.05$; MAM, $n = 8$; sham, $n = 8$) despite expression differences of only 7–21% (Figure 2a and b). We could not confirm expression differences for ARP3 and SYNJ1, most likely as these showed only 7–11% changes based on the LC-MS^E analysis. Interestingly, AMPA1 immunoblots of fraction 2 identified two bands. Further immunoblot experiments (Figure 2c) showed that the predicted 95 kDa band corresponds to the non-phosphorylated form of the protein, while the upper band represents a phosphorylated form (at Serine-845/Serine-838 (Mammen *et al*, 1997)). Neither of these bands was expressed at significantly different levels. However, the ratio between the upper and lower band did show a significant difference between MAM and sham rats ($p = 0.014$), suggesting a potential reduced level of phosphorylated AMPA1 receptor in MAM rats. Comparison of the measurements of each protein by LC-MS^E and immunoblot analysis showed significant correlations for PMCA1, HPCA, and the upper AMPA1 band (Figure 2b).

In Silico Pathway Analysis of Differentially Expressed Hippocampal Proteins

We used the IPKB software for *in silico* characterization of the 38 differentially expressed hippocampal proteins ($p < 0.05$, $q < 0.5$; Table 1). The SwissProt accession number of each protein was uploaded into the software, and these were mapped onto the predefined IPKB global network. Global pathway analysis of all 38 differentially expressed

Table 1 Differentially Expressed Candidate Proteins in Hippocampal Tissue Derived From MAM and Sham Rats

	Swiss Prot ID	Gene name	Peptides quantified	Sequence coverage (%)	Average intensity, MAM ± SD	Average intensity, SHAM ± SD	Ratio, MAM/SHAM	p-value	RSD QCs (%)
(a)									
Protein fraction 1									
Guanine deaminase	Q9WTT6	GDA	41	76	31 338 ± 1839	28 491 ± 1490	1.09	0.0027	1
Hippocalcin	P84076	HPCA	21	86	49 947 ± 4522	62 606 ± 2655	0.80	0.0000	8
Lactate dehydrogenase A	P04642	LDHA	40	89	85 713 ± 5698	94 515 ± 6680	0.91	0.0058	3
Myristoylated alanine-rich protein kinase-C substrate	P30009	MARCKS	13	60	24 461 ± 1511	19 924 ± 2094	1.21	0.0001	2
(b)									
Protein fraction 2									
Amphiphysin	O08838	AMPH	22	49	19 898 ± 1111	21 274 ± 1369	0.94	0.0244	2
ARP3 actin-related protein 3	Q4V7C7	ACTR3	25	70	24 523 ± 837	27 415 ± 1753	0.89	0.0003	8
ATPase, Ca ⁺⁺ transporting, plasma membrane 1	P11505	PMCA1	69	62	26 650 ± 990	31 272 ± 1463	0.85	0.0000	3
ATPase, Na ⁺ /K ⁺ transporting	P06685	ATP1A1	60	51	81 921 ± 4513	77 393 ± 4361	1.06	0.0342	2
Basigin	P26453	BSG	8	30	10 443 ± 1056	12 180 ± 830	0.86	0.0014	12
Calcium channel, voltage-dependent, γ -subunit 8	Q8VHW5	CACNG8	3	14	20 737 ± 2923	24 053 ± 2917	0.86	0.0188	7
Calcium/calmodulin-dependent protein kinase II α	P11275	CAMK2A	31	65	107 485 ± 10 925	12 7287 ± 11 068	0.84	0.0010	6
Calcium/calmodulin-dependent protein kinase II δ	P15791	CAMK2D	17	44	18 553 ± 1273	20 051 ± 1139	0.93	0.0138	6
Calcium/calmodulin-dependent protein kinase II γ	P11730	CAMK2G	17	47	12 968 ± 670	11 998 ± 695	1.08	0.0058	7
Calmodulin	P62161	CAM	17	89	97 711 ± 12 227	10 9183 ± 6598	0.89	0.0257	9
Capping protein (actin filament) muscle Z-line, α 2	Q3TIK5	CAPZA2	8	42	16 601 ± 837	18 153 ± 1505	0.91	0.0124	7
Dipeptidyl-peptidase 6	P46101	DPP6	13	26	16 133 ± 770	17 054 ± 722	0.95	0.0118	7
Dynamin 1	P21575	DNM1	73	81	37 638 ± 1047	39 025 ± 1555	0.96	0.0313	2
FK506 binding protein 1A, 12 kDa	Q62658	FKBP1A	7	78	30 458 ± 3165	36 348 ± 2870	0.84	0.0009	7
Glial high-affinity glutamate transporter member 3	P24942	SLC1A3	21	46	61 487 ± 3811	57 770 ± 3472	1.06	0.0335	6
Glutamate receptor, ionotropic, and AMPA-1	P19490	AMPA1	27	42	14 177 ± 870	15w938 ± 659	0.89	0.0001	7
Glutamate receptor, ionotropic, and AMPA 2	P19491	AMPA2	52	62	13 719 ± 588	14 760 ± 888	0.93	0.0064	6
Glutaminase	P13264	GLS	46	70	22 286 ± 1140	24 418 ± 1362	0.91	0.0013	5
Hemoglobin, β	P02091	HBB	15	83	87 376 ± 20 210	69 534 ± 12 326	1.26	0.0217	10
Hippocalcin	P84076	HPCA	15	70	32 618 ± 2910	38 319 ± 2639	0.85	0.0003	9
Hydroxyprostaglandin dehydrogenase 15-(NAD)	O08699	HPGD	5	32	14 214 ± 853	15 848 ± 1917	0.90	0.0354	5
Limbic system-associated membrane protein	Q62813	LSAMP	4	9	11 014 ± 1105	12 013 ± 659	0.92	0.0231	12
Myosin, heavy chain 4	Q29RW1	MYH4	27	20	16 105 ± 510	16 803 ± 555	0.96	0.0093	6
Myristoylated alanine-rich protein kinase-C substrate	P30009	MARCKS	16	63	18 180 ± 954	14 564 ± 3383	1.25	0.0218	9
Neural cell-adhesion molecule 1	P13596	NCAM1	46	66	33 399 ± 1662	30 806 ± 1661	1.08	0.0029	5
Peroxisoredoxin 6	O35244	PRDX6	20	74	22 516 ± 1223	20 572 ± 2199	1.09	0.0294	9
Protein phosphatase 2, catalytic subunit, α isoform	P63331	PPP2CA	3	21	4523 ± 815	5932 ± 1049	0.76	0.0033	29
Protein phosphatase 3, catalytic subunit, α isoform	P63329	PPP3CA	31	66	37 842 ± 1688	41 973 ± 3455	0.90	0.0042	5
RAB14	P61107	RAB14	23	74	19 017 ± 1391	20 161 ± 707	0.94	0.0382	9
RAB3C	P62824	RAB3C	12	49	40 694 ± 3487	36 178 ± 3076	1.12	0.0076	6
RAB8B	P70550	RAB8B	7	51	11 284 ± 837	10 442 ± 851	1.08	0.0393	11
Solute carrier family 4, sodium bicarbonate cotransporter, member 4	Q9J166	SLC4A4	18	29	11 815 ± 832	11 013 ± 650	1.07	0.0286	4
Synaptic vesicle glycoprotein 2B	Q63564	SV2B	3	8	11 568 ± 1315	13 008 ± 1322	0.89	0.0261	17
Synaptojanin 1	Q62910	SYNJ1	33	27	26 469 ± 1194	29 115 ± 1604	0.91	0.0006	4
Tubulin, α 8	Q6AY56	TUBA8	12	36	14 945 ± 2221	17 439 ± 1984	0.86	0.0268	10
Tubulin, β 2B	Q3KRE8	TUBB2B	4	16	22 304 ± 1794	20 001 ± 2580	1.12	0.0331	5

The proteins in (a) fraction 1 (10 MAM and 10 sham rats) and (b) fraction 2 (10 MAM and 10 sham rats) indicating averaged protein intensities (rounded to integers) and the standard deviation (SD), MAM/sham protein abundance ratios, p-values and relative standard deviation (RSD) for the measurement of each protein (determined by analysis of five quality controls that have been prepared and analyzed with the MAM and sham samples).

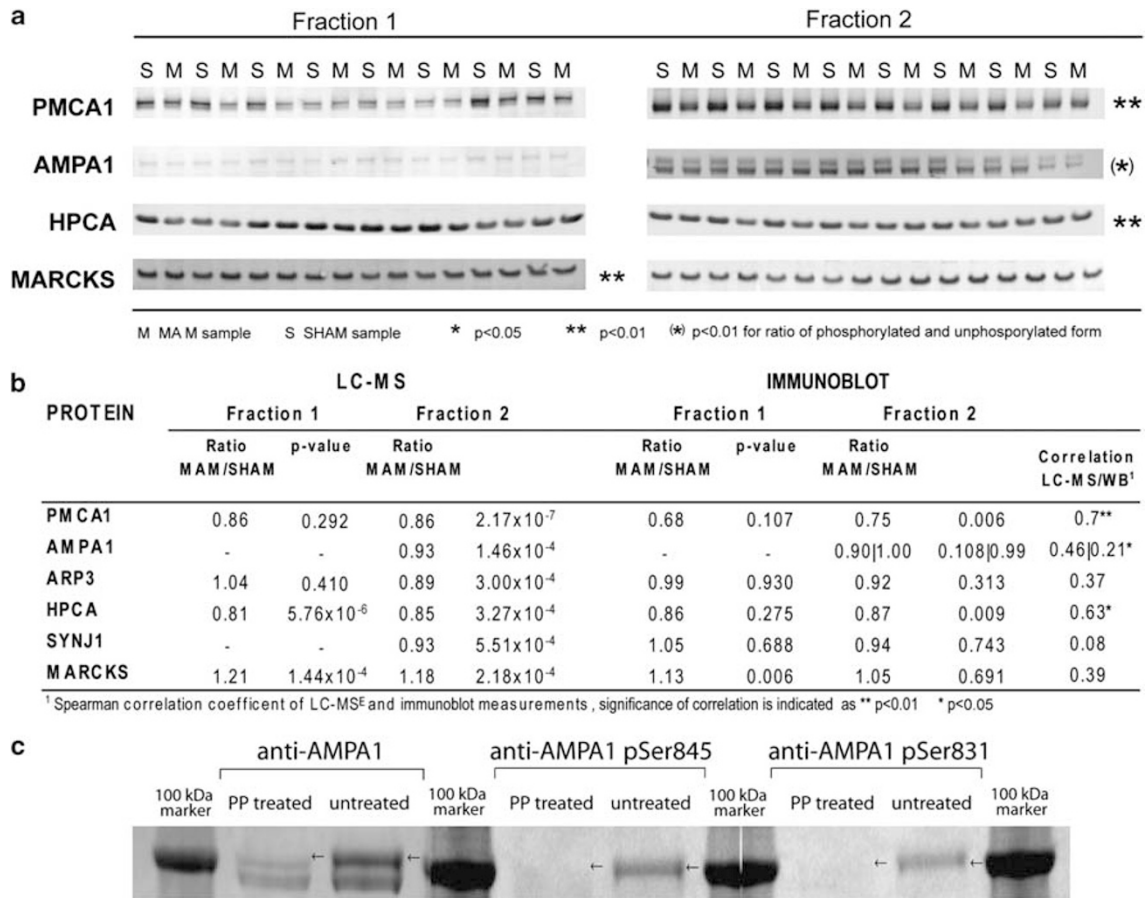


Figure 2 Immunoblot analysis of differentially expressed hippocampal candidate proteins identified by LC-MS^E. (a) Western blot images of candidate proteins identified in fractions 1 and 2. MAM and sham samples were loaded alternately to reduce any potential artifactual effects of differential electrotransfer. (b) Expression ratios and p -values are listed for each fraction. Correlation values are given for the LC-MS^E and immunoblot signals. (c) Immunoblot characterization of the two AMPA1 receptor bands in rat hippocampal tissue of fraction 2. Anti-AMPA1 receptor immunoblot analysis of MAM and sham hippocampus shows the predicted 95 kDa band and an additional band at ~ 110 kDa (indicated by \leftarrow). Phosphatase treated (protein phosphatase treated) and untreated samples were tested by western blot analysis with phospho-specific AMPA1 antibodies, demonstrating that the 110 kDa band is reduced in intensity after dephosphorylation. These results indicated that the ~ 110 kDa band is a phosphorylated form of the AMPA1 receptor. Lanes 1, 4, 7, and 10 show the position of a 100-kDa protein marker.

hippocampal proteins ($p < 0.05$, $q < 0.5$; Table 1) showed that the most significant function associated with the altered proteins were related to cell-to-cell signaling and neuronal function ($n = 23$ proteins, associated with function, Figure 3a). At the level of canonical pathways, the three most significant associations with the 38 differentially expressed proteins ($p < 0.05$, $q < 0.5$; Table 1) were calcium signaling ($n = 8$ proteins, Fisher's exact test p -value = 9.34×10^{-9}), synaptic long-term potentiation ($n = 6$ proteins, Fisher's exact test p -value = 2.65×10^{-7}), and glutamate receptor signaling ($n = 4$ proteins, Fisher's exact test p -value = 1.19×10^{-5}) (Figure 3b). The proteins assigned to these pathways are subsets of the functional network previously identified by functional analysis (Figure 3a and b).

Furthermore, the IPKB software detected significant molecular interaction of 22 of the 38 differentially expressed hippocampal proteins ($p < 0.05$, $q < 0.5$; Table 1) using network analysis (Fisher's exact test p -value = 1×10^{-53} , Figure 3c). Proteins of the network are involved in nervous system development and function. Ten of the network

proteins (AMPH, ATP1A1, CAMK2A, CAMK2D, AMPA1, AMPA2, NCAM1, PPP3CA, SYNJ1, and TUBA8) have been associated previously with psychological disorders, such as bipolar affective disorder ($n = 8$ proteins), schizophrenia ($n = 6$ proteins), and major depressive disorder ($n = 4$ proteins; see IPKB database for further details).

Cluster Analysis of Differentially Expressed Proteins in the MAM Rat Hippocampus

We next attempted to identify clusters of co-behaving proteins among the 38 significantly altered proteins ($p < 0.05$, $q < 0.5$; Table 1), which contribute most to the differences observed in the hippocampus between MAM and sham rats. co-behavior was examined by TAC analysis (Cheng *et al*, 2010), as described in the methods. This led to the identification of four clusters of proteins in fraction 2, which gave 100% precision for correct classification of MAM and sham hippocampi after iterative testing (precision was defined as the proportion of MAM samples predicted correctly). The proteins forming the four

a FUNCTIONAL ANALYSIS

CELL-TO-CELL SIGNALING AND NERVOUS SYSTEM FUNCTION

Predominantly processes of synaptic transmission and plasticity

Significance of functional associations:
Fisher's exact test $p=4.05 \times 10^{-7}$ - 4.47×10^{-2}

Actin-related protein 3 (ACTR3), amphiphysin (AMPH), Na⁺/K⁺-transporting ATPase (ATP1A1), **Ca²⁺ transporting ATPase (PMCA1)**, basigin (BSG), voltage-dependent calcium channel (CACNG8), calcium/calmodulin-dependent protein kinases (CAMK2A, CAMK2D, CAMK2G), dynamin 1 (DNM1), FK506 binding protein 1A (FKBP1A), glutamylase (GLS), **glutamate receptors AMPA (AMPA1, AMPA2)**, myosin (MYH4), neural cell adhesion molecule 1 (NCAM1), **protein phosphatase 3 (PPP3CA)**, peroxiredoxin 6 (PRDX6), RAB14 protein (RAB14), glial high affinity glutamate transporter (SLC1A3), synaptic vesicle glycoprotein 2B (SV2B), **synaptojanin 1 (SYNJ1)**, tubulin (TUBA8)

b PATHWAY ANALYSIS

CALCIUM SIGNALING

Fisher's exact test Fisher's exact test $p=9.34 \times 10^{-9}$

AMPA1, AMPA2, CAMK2A, CAMK2D, CAMK2G, PMCA1, MYH4, PPP3CA

LONG TERM POTENTIATION

Fisher's exact test Fisher's exact test $p=2.65 \times 10^{-7}$

AMPA1, AMPA2, CAMK2A, CAMK2D, CAMK2G, PPP3CA

GLUTAMATE RECEPTOR SIGNALING

Fisher's exact test Fisher's exact test $p=1.19 \times 10^{-5}$

GLS, AMPA1, AMPA2, MYH4, PPP3CA, SLC1A3

c NETWORK ANALYSIS

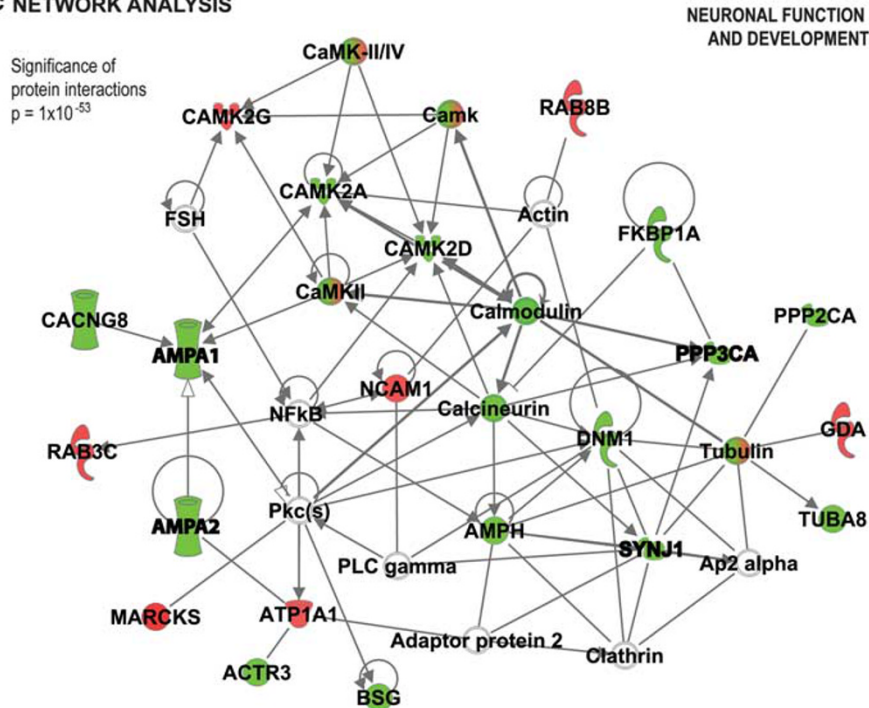


Figure 3 *In silico* functional, pathway and network analysis of the 38 differentially expressed candidate proteins in the hippocampus of MAM rats ($p < 0.05$, $q < 0.5$; Table 1): The differentially expressed proteins were overlaid onto predefined functional networks and pathways of the IPKB database. Significance of the association with a function and pathway, or protein interaction was calculated using Fisher's exact test. (a) The table lists the proteins sharing the most significant function. Proteins in bold font are part of a protein cluster identified by TAC analysis. (b) The three most significant pathways identified are listed with the associated proteins. (c) Overall, 22 of the 38 proteins are connected in an interaction network. Green = MAM/sham expression ratio < 1 , red = MAM/sham expression ratio > 1 , and mixed colors = subunits/isoforms differentially expressed. Non-colored symbols = not significant in LC-MS^E analysis, although incorporated as an interacting protein by the IPKB software.

clusters (#1 (AMPA2 + PPP3CA + SYNJ1), #2 (AMPA1 + AMPA2 + PPP3CA), #3 (AMPA1 + PMCA1 + PPP3CA), and #4 (AMPA1 + AMPA2 + HPCA1)) have been previously mapped to cell-cell signaling and neuronal function by functional analysis using IPKB (Figure 3a). Interestingly, the clusters describe proteins involved in calcium and glutamate receptor signaling, as previously identified using IPKB (Figure 3b). This highlighted the importance of these pathways for the hippocampal pathology in the MAM-E17 model, as each of these clusters predicted MAM-E17 and

sham rats correctly based only on the expression patterns of the three cluster proteins.

¹H-NMR Spectroscopic Analysis

To study the effect of MAM treatment on the brain metabolites, ¹H-NMR spectra were acquired for frontal cortex, hippocampus, striatum, and cerebellum. The PLS-DA analysis indicated differences in the metabolic profiles of the hippocampus ($Q^2(\text{cum})=0.62$, Figure 4a). No

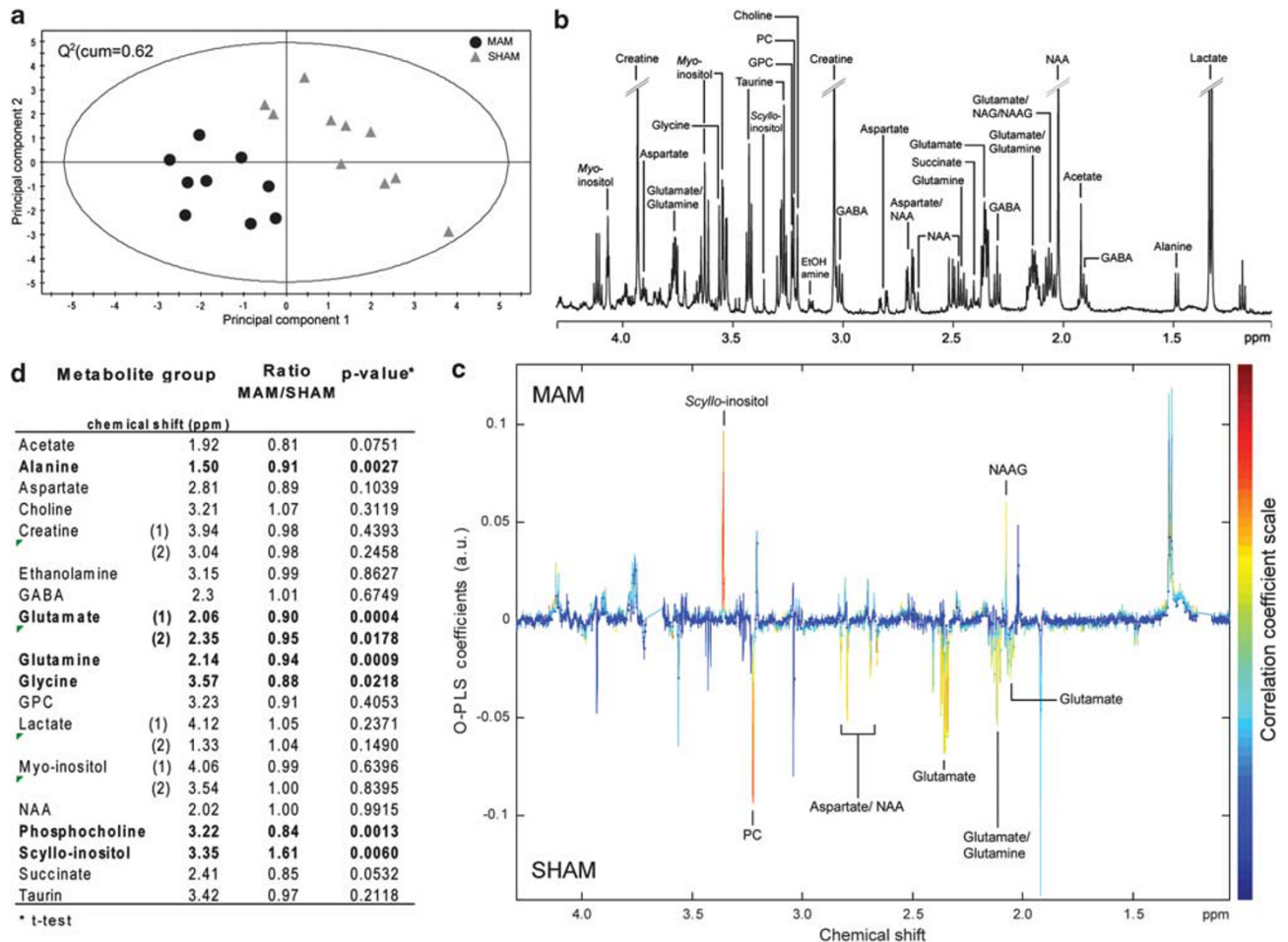


Figure 4 Multivariate and univariate analysis of full-resolution $^1\text{H-NMR}$ spectral data of MAM and sham hippocampi. (a) The scores plot of the PLS-DA model shows a sharp separation between MAM and the sham group ($Q^2=0.62$). (b) Representative sample spectrum of a sham rat. The partially overlapping resonance regions of the 20 identified metabolites are indicated. (c) The coefficients plot of the OPLS-DA analysis indicates significantly different metabolic profiles for MAM and sham animals ($Q^2=0.35$). Labeled spectral regions show metabolites contributing to the difference between the two groups. The direction of the peak indicates the direction of metabolite level differences (up = higher measurements in MAM rats, down = higher measurements in sham rats). The importance of the contribution to the difference between the groups is color-coded according to the correlation coefficient heat map (red = high contribution, dark blue = low contribution). (d) Univariate analysis of manually integrated $^1\text{H-NMR}$ spectral data. The table presents the non-overlapping metabolic resonance groups and the ratio of the signal means of MAM and sham rats. Analytes with a significant difference ($p < 0.05$) of the integrated signal are highlighted.

statistically robust PLS-DA model could be constructed for the other brain regions. A representative and annotated spectrum of a sham animal is presented in Figure 4b. OPLS-DA analysis was conducted to identify the metabolites responsible for the differences between MAM and sham hippocampi. The coefficients plot of the model indicated disparities in the spectral regions of *scyllo*-inositol, phosphocholine, glutamate, *N*-acetyl aspartyl glutamate (NAAG), overlapping spectral regions of *N*-acetyl aspartate (NAA)/aspartate and glutamate/glutamine (Figure 4c). Manual integration of spectra was conducted to verify and quantify the differences found by multivariate analysis (Figure 4d). Only spectral regions showing resonances of single metabolites were considered. The metabolic profile changes seen in the OPLS-DA model were confirmed with exception of the aspartate/NAA signal. These analytes showed signal changes only in an overlapping region of the spectrum. Confirmation of the difference in NAAG levels identified by multivariate statistics was not possible, as other metabolites

interfere with the signal of all NAAG resonance groups. However, the integration of the alanine and the glycine region showed significantly lower levels in MAM compared with sham rats.

Pathway analysis of the metabolites responsible for the differences between MAM and sham showed that glutamate receptor signaling was the most significant canonical pathway affected ($p = 0.000002$). Furthermore, enzymes and transporters of the glutamate–glutamine cycle, which were found to be changed in the LC-MS^E analysis, are known to facilitate interconversion of glutamate and glutamine in astrocytes and neurons. In addition, many significantly changed metabolites seem to be connected in an interaction network. Neuropeptide and NMDA receptor antagonist NAAG is cleaved by neuropeptidase glutamate carboxypeptidase II (GCPII) to NAA and glutamate (Slusher *et al*, 1990), and NMDA agonists glycine, alanine, and glutamine can be reversibly converted by transaminase reactions.

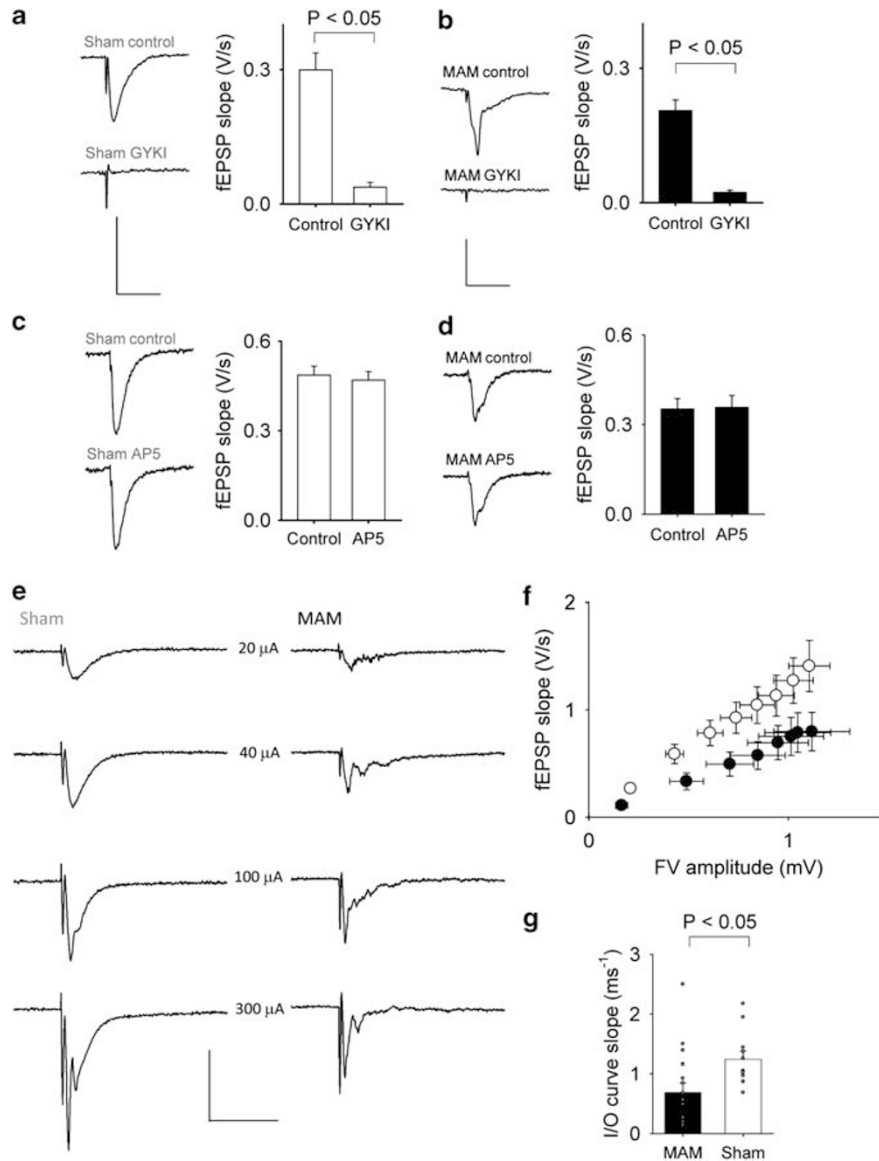


Figure 5 MAM treatment on E17 resulted in reduced synaptic transmission in transverse hippocampal slices. (a) fEPSPs in sham slices are blocked by GYKI53784. (b) fEPSPs in MAM slices are blocked by GYKI53784. (c) fEPSPs in sham slices are unaffected by AP5. (d) fEPSPs in MAM slices are unaffected by AP5. Scale bar for (a) and (c, sham traces) represents 0.5 mV and 20 ms. Scale bar for (b) and (d, MAM traces) represents 0.3 mV and 20 ms. (e) Example traces at increasing stimulation strengths showing FVs (the first fast peak) and fEPSPs (the second slow peak). Scale bars represent 1 mV and 20 ms. (f) Comparison of the FV and the fEPSP in MAM (filled points) and sham (open points) hippocampal slices. (g) Linear regression analysis of input–output relation from Figure 5f.

Electrophysiological Analysis of MAM Rat Hippocampal Slices

The proteomic and metabolomic results indicated changes of molecules involved in neuronal transmission in the hippocampus of MAM-E17 rats. In particular, proteins and metabolites of the glutamate receptor signaling pathway, as identified by *in silico* pathway and TAC analysis, seem to be affected in both studies. To test this, we investigated glutamatergic synaptic transmission using electrophysiological recordings in the CA1 area of the hippocampus. Previous studies have shown that synaptic transmission in this area is mediated by AMPA receptors (Andreasen *et al*, 1989; Davies and Collingridge, 1989). To investigate if

AMPA receptor changes alter excitatory synaptic function in MAM rats, synaptic transmission was investigated. In order to confirm that synaptic transmission is mediated by AMPA receptors in our preparation, the AMPA receptor antagonist GYKI53784 (10 μM) was applied and found to block synaptic transmission in both sham and MAM slices. Average fEPSPs in sham slices were 0.04 ± 0.01 and 0.30 ± 0.04 V/s in the presence and absence of GYKI53784, respectively (Figure 5a, $p < 0.05$). Average fEPSPs in MAM slices were 0.02 ± 0.01 and 0.21 ± 0.02 V/s in the presence and absence of GYKI53784, respectively (Figure 5b, $p < 0.05$). Conversely, the NMDA receptor antagonist AP5 (50 μM) did not affect synaptic transmission in either sham or MAM slices. Average fEPSPs in sham slices were

0.47 ± 0.03 and 0.49 ± 0.03 V/s in the presence and absence of AP5, respectively (Figure 5c, $p > 0.05$). Average fEPSPs in MAM slices were 0.36 ± 0.04 and 0.35 ± 0.03 V/s in the presence and absence of AP5, respectively (Figure 5d, $p > 0.05$). FVs and fEPSPs were then recorded at a range of stimulation strengths. Comparative analysis showed that for any given FV, the fEPSP generated was significantly reduced in slices from MAM rats compared with those from sham rats (Figure 5e and f). Quantification of the FV vs fEPSP relationship by linear regression analysis of the MAM data set gave a slope of $0.68 \pm 0.16 \text{ ms}^{-1}$, compared with $1.24 \pm 0.15 \text{ ms}^{-1}$ in sham slices ($p < 0.05$; Figure 5g). This suggested a deficit in AMPA receptor-mediated synaptic transmission.

DISCUSSION

This study describes the first molecular characterization, combined with a functional validation, of the MAM-E17 rat model for schizophrenia. The combined use of two molecular profiling platforms demonstrated that MAM administration on E17 had a significant impact on protein and metabolite levels in the hippocampus. The frontal cortex did not show any significant molecular effects of prenatal MAM exposure, even though neuroanatomical changes have been reported for this brain region (Moore *et al*, 2006). However, previous studies have shown that the frontal cortex has milder morphological effects in response to MAM treatment compared with those reported in the hippocampus (Flagstad *et al*, 2004; Lodge *et al*, 2009a; Matricon *et al*, 2010; Moore *et al*, 2006; Penschuck *et al*, 2006). Therefore, it is possible that such subtle changes might not lead to detectable molecular correlates in whole-tissue lysates using the current methodologies.

Many of the differentially expressed proteins and metabolites in the hippocampus appeared to be the components of an *in silico*-determined interaction network, suggesting significant effects of embryonic MAM treatment on neuronal signal transduction. MAM treatment may affect neurotransmission by altering the levels of specific neurotransmitters, glutamate receptor agonists/antagonists, glutamate–glutamine cycle proteins, or synaptic vesicle controlling proteins. The data showed that MAM treatment may alter synaptic neurotransmission by affecting the extent or innervation and/or post-synaptic signal-transduction mechanisms through alterations in AMPA receptor subunit expression or phosphorylation, clathrin-mediated receptor internalization, and calcium signaling, as identified by *in silico* pathway analysis and supported by TAC analysis. As a variety of molecules involved in neurotransmission were found to be altered, this may indicate that multiples of these possibilities might have a synergistic role. The potential functional consequences of the molecular changes identified in the hippocampus were analyzed by *in vitro* electrophysiology in hippocampal slices. The fEPSP recordings in slices from MAM-treated rats showed a reduction in glutamatergic synaptic transmission. In particular, they are compatible with less expression of phosphorylated AMPA1 subunits, as preliminary biochemical data showed. Phosphorylation of AMPA1 is known to have dramatic effects on both AMPA receptor trafficking

and gating. Not only highly relevant synaptic plasticity events, such as long-term potentiation, long-term depression, and depotentiation, depend on AMPA1 phosphorylation, but in AMPA1 'phosphorylation-deficient' transgenic mice, specific cognitive deficits relevant to schizophrenia were also found (Lee *et al*, 2003). Finally, it is worth mentioning that while the recordings suggested a reduction in AMPA receptor-mediated synaptic transmission of potential post-synaptic origin, we cannot exclude at this stage that some of the functional deficits and molecular alterations could also be caused by further changes in innervation, total number of synapses formed, or functional alteration in the interplay between neurons and glia cells at synapses in the hippocampi of MAM rats (Matricon *et al*, 2010). Further studies to identify and confirm the molecular basis of the synaptic deficits, investigating more complex aspects of synaptic plasticity and combining morphometric and immunohistological methods, are warranted given the potential relevance of this model for schizophrenia research and drug development.

The importance of the hippocampus in the pathology of schizophrenia has been the focus of several studies. Most of the evidence derives from post-mortem or neuropsychological studies, and many of the findings are consistent with those found in the MAM-E17 model. The most reproducible effect in patients with schizophrenia appeared to be a reduction of hippocampal size (Bogerts *et al*, 1990; Lawrie *et al*, 1999; Shenton *et al*, 2001), which is evident in MAM rats (Featherstone *et al*, 2007; Moore *et al*, 2006). Similarly, post-mortem studies of schizophrenia patients show disarray of pyramidal cells and a decrease in parvalbumin-expressing interneurons in the hippocampus (Kovelman and Scheibel, 1984; Zhang and Reynolds, 2002), as reported for MAM rats (Flagstad *et al*, 2004; Moore *et al*, 2006; Penschuck *et al*, 2006). The assignment of neuropsychological deficits in mental illnesses to specific brain structures has been a controversial subject. However, there have been suggestions that the hippocampus is involved in sensorimotor gating and latent inhibition (Gothelf *et al*, 2000; Gray *et al*, 1995; Swerdlow *et al*, 2001), both of which are deficient in schizophrenia patients (Braff, 1993; Braff *et al*, 2001; Braff *et al*, 1992; Gray, 1998) and adult MAM rats (Flagstad *et al*, 2005; Le Pen *et al*, 2006; Lodge *et al*, 2009a; Moore *et al*, 2006).

Functional and post-mortem studies of schizophrenia patients have shown a reduced neuronal activity and altered synaptic markers in the hippocampus, implying disrupted neuronal function (Gao *et al*, 2000; Medoff *et al*, 2001; Nesvaderani *et al*, 2009; Tamminga *et al*, 1992; Tsai *et al*, 1995). The most consistent findings point toward a glutamatergic synaptic pathology in schizophrenia. Various studies have shown that glutamate levels, glutamate receptors, glutamate transport, and neurotransmitter vesicle molecules are altered in the hippocampus of patients with schizophrenia (Eastwood *et al*, 1995b; Gao *et al*, 2000; Harrison *et al*, 2003; Hemby *et al*, 2002; Knable *et al*, 2004; Tsai *et al*, 1995; Webster *et al*, 2001). Similar effects have been demonstrated in this study for MAM rats at both the molecular and functional levels, reflecting some aspects of the glutamatergic hypothesis of schizophrenia (Kim *et al*, 1980). Not all of the molecular changes observed in hippocampi of schizophrenia patients have been detected

in the MAM rats (Eastwood *et al*, 1995a; Eastwood and Harrison, 1998; Fatemi *et al*, 2000), although the same pathways and cellular functions were found to be affected, as identified by *in silico* pathway analysis and supported by TAC analysis. Such molecular changes may result from a decreased number of total synapses, defective synaptic coupling, or a change in the neuropil formation, as suggested by Matricon *et al* (2010) for the MAM-E17 model and demonstrated in cases of human schizophrenia subjects (Garey, 2010).

Future Directions and Conclusion

The combined use of a proteomics approach with complementary molecular and functional studies has resulted in identification and validation of a significant effect on glutamate neurotransmission in the offspring of MAM-treated rats. Nevertheless, the origin of these changes in the hippocampus is unclear. LC-MS^E profiling of whole hippocampal tissue as used here does not take into account cell morphological and subfield-specific expression differences as seen in schizophrenia studies (Benes *et al*, 1991; Eastwood *et al*, 1995b; Gao *et al*, 2000; Garey, 2010; Knable *et al*, 2004). Therefore, we suggest that future studies should investigate subcellular and subregional expression and phosphorylation patterns of synaptic markers and glutamatergic indices, as identified in this study, in combination with synaptic structure and density measurements in hippocampal subfields of the MAM-E17 rat model. In addition, further investigation of the functional markers NAA (Simmons *et al*, 1991), aspartate, and related molecules is of interest as these have also been found to be reduced in hippocampi of schizophrenia patients (Bertolino *et al*, 1996; Deicken *et al*, 1998; Maier *et al*, 1995; Tsai *et al*, 1995). A reduction in NAA might result from reduced GCPII activity as seen in schizophrenia (Tsai *et al*, 1995). This enzyme is known to cleave NAAG to produce NAA and glutamate, which were both changed significantly in this study.

It is now accepted that animal models *per se* can recapitulate only certain aspects of neuropsychiatric disease. The present studies showed that MAM treatment of rats may lead to an altered glutamatergic neurotransmission in the hippocampus, although the exact cause of this deficit remains to be elucidated. Nevertheless, this study provides the first molecular evidence, combined with functional validation, that the MAM-E17 rat model reproduces hippocampal deficits relevant to the pathology of schizophrenia. Further investigation of these deficits using this model may lead to further insights into the hypoglutamatergic hypothesis of schizophrenia and demonstrate its potential utility for drug discovery and development.

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