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Large-scale neurochemical metabolomics analysis identifies multiple compounds associated with methamphetamine exposure

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Abstract

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Methamphetamine (MA) is an illegal stimulant drug of abuse with serious negative health consequences. The neurochemical effects of MA have been partially characterized, with a traditional focus on classical neurotransmitter systems. However, these directions have not yet led to novel drug treatments for MA abuse or toxicity. As an alternative approach, we describe here the first application of metabolomics to investigate the neurochemical consequences of MA exposure in the rodent brain. We examined single exposures at 3 mg/kg and repeated exposures at 3 mg/kg over 5 days in eight common inbred mouse strains. Brain tissue samples were assayed using high-throughput gas and liquid chromatography mass spectrometry, yielding quantitative data on >300 unique metabolites. Association testing and false discovery rate control yielded several metabolome-wide significant associations with acute MA exposure, including compounds such as lactate ($p = 4.4 \times 10^{-5}$, $q = 0.013$), tryptophan ($p = 7.0 \times 10^{-4}$, $q = 0.035$) and 2-hydroxyglutarate ($p = 1.1 \times 10^{-4}$, $q = 0.022$). Secondary analyses of MA-induced increase in locomotor activity showed associations with energy metabolites such as succinate ($p = 3.8 \times 10^{-7}$). Associations specific to repeated (5 day) MA exposure included phosphocholine ($p = 4.0 \times 10^{-4}$, $q = 0.087$) and ergothioneine ($p = 3.0 \times 10^{-4}$, $q = 0.087$). Our data appear to confirm and extend existing models of MA action in the brain, whereby an initial increase in energy metabolism, coupled with an increase in behavioral locomotion, gives way to disruption of mitochondria and phospholipid pathways and increased endogenous antioxidant response. Our study demonstrates the power of comprehensive MS-based metabolomics to identify drug-induced changes to brain metabolism and to develop neurochemical models of drug effects.

Keywords

Drugs of abuse; Psychostimulants; Inbred mice; Mass spectrometry; Neurotoxicity

1 Introduction

Methamphetamine (MA) is an illegal stimulant drug with over 500,000 individuals estimated to abuse MA in the United States each month (Substance Abuse and Mental Health Services Administration 2010). The negative health consequences to the individual and the costs to society of MA abuse are substantial. Long term users are at greatly increased risk for several health problems, including cardiovascular disease, psychosis, depression and dental pathology (Curtis 2006; Hendrickson et al. 2008; Kaye et al. 2007; Zweben et al. 2004). Over the last 2 decades, the number of MA users seeking, or being referred to, treatment programs has increased approximately threefold, with almost 40 % of state and local law enforcement agencies citing MA as their greatest drug threat (Gonzales et al. 2010). The total annual cost of MA use to the US was estimated to be $23.4 billion in 2005 (Nicosia et al. 2009).

In common with other psychostimulant drugs, previous research into the effects of MA on the brain has largely involved the monoamine neurotransmitters (Volz et al. 2007). The classical mechanism is that MA causes excess cytoplasmic dopamine, by increasing release and blocking re-uptake, leading to increased formation of reactive oxygen species that damage neurons (Carvalho et al. 2012). Additional research suggests that MA neurotoxicity involves not only oxidative stress, but also glutamate excitotoxicity and mitochondrial dysfunction (Kita et al. 2009; Krasnova and Cadet 2009; Yamamoto et al. 2010). Our lack of comprehensive understanding of how these mechanisms may work in concert, coupled with the fact that other mechanisms such as neuroinflammation are of emerging importance, suggests that our understanding of the neurochemical effects of MA is far from complete (Yamamoto et al. 2010). In addition, there are no approved pharmacotherapies for treating MA abuse currently available, and conventional, neurotransmitter receptor-mediated approaches to have not met with success (Karila et al. 2010). Therefore, novel

pharmacological directions will likely hold the greatest potential for future progress. Furthering our knowledge of MA's effects in the brain could potentially facilitate the design of novel treatments for psychostimulant toxicity and addiction.

A potentially powerful tool for understanding the global biological consequences of drug administration is meta-bolomics (Clayton et al. 2006; Kaddurah-Daouk et al. 2008; Lindon et al. 2006). In recent years, metabolomics has matured, with many applications pertinent to central nervous system (CNS) drug effects now possible (Kaddurah-Daouk and Krishnan 2009). While metabolomics analysis of human samples has been used to investigate drugs of abuse such as cocaine (Patkar et al. 2009), the greater experimental control afforded by model organisms has stimulated a larger body of research. Laboratory rodents have been used in metabolomics investigations of alcohol (Loftus et al. 2011; Shi et al. 2012), MDMA (or "ecstasy") (Perrine et al. 2009) and, of particular relevance to the current study, MA (Shima et al. 2011). A possible drawback of these studies from a CNS perspective is that they examined biofluids such as blood or urine, or tissues such as liver, and did not examine the brain directly. While several metabolomics studies have probed the rodent brain, these have typically focused on murine models of disease (Constantinou et al. 2011; Nomura et al. 2011), or the effects of genetic knock-outs (Kopp et al. 2010), rather than the effects of drugs. To our knowledge, only two studies to date have used metabolomics to probe the effects of drugs in the rodent brain. The first (McLoughlin et al. 2009) focused on therapeutic psychiatric compounds, while a very recent study (Lee et al. 2012) examined the effects of chronic ethanol administration.

The lack of previous metabolomics studies of MA in the rodent brain suggested that an exploratory neurochemical investigation could yield new insights and potentially inform new treatments. In planning our study, we incorporated several design features to maximize our chances of obtaining a comprehensive overview of MA effects in the brain. First, we used a combination of both gas and liquid mass spectrometry (MS)-based metabolomics, to enable detection of a wide range of metabolites. MS-based metabolomics is typically considered to be more sensitive than nuclear magnetic resonance (Pan and Raftery 2007), the main alternative technology to MS currently in use. Second, we employed a large sample size for a study of this type, examining a total of 150 brains. Third, in addition to larger scale, a further advance over previous studies is our use of focused beam microwave irradiation (FBMI) to instantaneously and permanently denature all enzymes in the brain at time of sacrifice (de Graaf et al. 2009). In a high-intensity microwave pulse lasting only milliseconds, brain metabolism is arrested completely and metabolites are preserved in the pre-sacrifice state (Ikarashi et al. 1985; Login and Dvorak 1994). The effectiveness of this study design is demonstrated by our detection of multiple highly significant MA-induced disruptions to murine neurochemistry.

2 Materials and methods

2.1 Subjects

Eight week old male mice representing eight genetically diverse (Petkov et al. 2004) inbred strains (129S1/SvImJ, A/J, C3H/HeJ, C57BL/6J, CAST/EiJ, DBA/2J, NOD/ShiLtJ, PWD/PhJ) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were allowed to acclimate to the vivarium (AAALAC-accredited) for 1 week prior to testing and were housed up to four per cage. Food (7012 Teklad LM-485 Sterilizable Diet, Harlan Laboratories, Indianapolis, IN) and water were available ad libitum under a 12-h/12-h light/dark cycle (lights on at 0700–1,900 h) with all testing occurring during the light phase. An overview of the study design is provided in Table 1. All procedures were approved by the Institutional Animal Care and Use Committee of VCU.

2.2 Drugs

(±)-Methamphetamine (MA), obtained from the National Institute on Drug Abuse (Rockville, MD), was prepared in 0.9 % saline stock solutions sterilized by filtration through 0.2 μm filtration disks. MA solutions were injected intra-peritoneally in a volume equivalent to 10 ml/kg body weight. A dose of 3 mg/kg was used in this study. This dose elicits maximal locomotor activity response, which persists significantly above baseline for over 90 min (Caligiuri and Buitenhuys 2005; Peachey et al. 1976).

2.3 Behavioral measurement

Locomotor activity tests were conducted in eight automated activity monitoring devices, each enclosed in sound-and light-attenuating chambers (AccuScan Instruments, Columbus OH). Sixteen photobeam sensors were spaced 2.5 cm apart along the walls of the chamber to detect movement. Behavioral variables (e.g., distance traveled in cm) were recorded in 10-min bins via computer. The interior of each device was divided into separate 20 × 20 × 30 cm arenas permitting the independent and simultaneous measurement of two mice.

2.4 Sample preparation

One hour after final drug or vehicle injection, mice were immediately sacrificed by microwave fixation (Muromachi 10 kW Microwave Fixation System, TMW-4012C, Tokyo). Brains were collected by dissection, flash frozen in liquid nitrogen and stored at −80 °C prior to overnight shipment on dry ice to the metabolomics facility (Metabolon Inc, Research Triangle Park, NC). Brain tissue was homogenized using a GenoGrinder (OPS Diagnostics, Lebanon, NJ) and extracted, using proprietary methods (Metabolon, Research Triangle Park, NC), into two fractions; one for analysis by liquid chromatography mass spectrometry and one for analysis by gas chromatography mass spectrometry. Samples were placed briefly on a TurboVap (Zymark, Hopkinton, MA) to remove organic solvent and then frozen and vacuum dried before preparation for the appropriate instrument. An aliquot of each experimental sample was pooled and aliquots of these “matrix” samples were injected throughout the platform day run and served as technical replicates. As such, variability in quantitation of the experimental samples was monitored.

2.5 Liquid chromatography/mass spectrometry (LC/MS, LC/MS^2)

The LC/MS component of our metabolomics approach has been described previously (Evans et al. 2009). Briefly, the LC/MS platform was based on a Waters (Milford, MA) Acquity ultra performance liquid chromatography (UPLC) and a Thermo-Finnigan (ThermoFisher Scientific, Waltham, MA) LTQ mass spectrometer, which consists of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contain eleven injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol, both containing 0.1 % formic acid, while the basic extracts, which also use water/methanol, contained 6.5 mM ammonium bicarbonate. The MS analysis was alternated between MS and data-dependent MS^{2}scans using dynamic exclusion.

The LC/MS mass accurate portion of the platform was based on a Surveyor high performance liquid chromatography (HPLC) and a Thermo-Finnigan LTQ-FT mass spectrometer, which has a LIT front end and a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer backend. For ions with counts >2 million, an accurate mass measurement was performed. Accurate mass measurements were made on the parent ion as

well as fragments and the typical mass error was <5 ppm. Ions with less than two million counts required a greater amount of effort to characterize. Fragmentation spectra (MS/MS) were typically generated in data dependent manner, but where necessary, targeted MS/MS was employed, such as in the case of lower level signals.

2.6 Gas chromatography/mass spectroscopy (GC/MS)

A previous implementation of the GC/MS platform can be found in (Ohta et al. 2009) The samples destined for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 h prior to being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). The GC column used was 5 % phenyl and the temperature ramp was from 40 to 300° C in a 16 min period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization.

2.7 Data pre-processing

Proprietary in-house software (Metabolon Inc) was used to perform detection and integration of MS peaks as described previously (Evans et al. 2009). Briefly, extracted ion chromatograms were binned by mass in a given range, baseline noise was determined, peaks areas were calculated, and various peak thresholds including minimum height, signal-to-noise, width, symmetry, and area were applied. MS peaks passing above threshold criteria were grouped based on peak apex retention time for ease of viewing similarly retained ion features. Deconvolution and identification of correlated ion features was performed as described previously (Dehaven et al. 2010), whereby correlated ion features from multiple platforms that belong to a given biochemical are organized into a single ion group that can be used to represent that metabolite in statistical analyses. Signatures for specific biochemicals were identified by comparison to library entries of purified standards (~ 1,500 for the GC and LC platforms at time of assay). Since MA is not an endogenous metabolite, it was added to the compound library so that it could be identified and quantified for validation purposes. The combination of chromatographic properties and mass spectra give an indication of a match to the specific compound or isobaric entity. Automated library matches for each compound were checked manually and corrected if necessary.

Following deconvolution and library matching, each identified compound was represented by a single, uncorrected quantitative variable. Metabolites with >50 % missing values were dropped from further analysis. Missing values for remaining metabolites were imputed as half the observed minimum value on the assumption that they were below the limits of detection. A data normalization step was also performed to correct variation resulting from instrument inter-day tuning differences to avoid confounding experimental variation with instrument sensitivity. Each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately (Dehaven et al. 2010; Evans et al. 2009).

2.8 Statistical analysis

There were four experimental groups: acute vehicle (AV), single acute dose of 3 mg/kg MA (AM), repeated vehicle (RV) and repeated 3 mg/kg MA (RM). Both acute groups had only a single test day, while the latter two groups received injections for 5 consecutive days. It should be noted that the RV group received vehicle injections for 5 days, but also received a single injection of 3 mg/kg MA on the final day 1 h before sacrifice (see Table 1).

In our main metabolomics analyses, linear mixed models (Searle 1971; Searle et al. 1992) were used to assess associations with the above experimental conditions by analyzing data from all conditions simultaneously. This method is superior to standard group-wise

comparisons for two reasons. First, mice from the same strain will be more similar to each other than mice from different strains. Mixed models allow us to effectively handle the resulting dependent observations and perform accurate significance tests. Second, it allows us to obtain maximum likelihood parameter estimates by fitting models to data from all conditions simultaneously. This means that our estimates have desirable properties such as being asymptotically unbiased with the highest possible precision. Specifically, the following model was estimated for each metabolite separately:

$$y_{ij} = b_0 + b_1 D1 + b_2 D2 + b_3 D3 + u_{0j} + e_{ij}$$

where y is metabolite concentration of mouse i from strain j, b_0 is the intercept, u_{0j} is the random effect of strain; and e_{ij} is the model residual. Parameters b_1, b_2 and b_3 are the effects of dummy variables D1, D2 and D3 that represent the experimental conditions that are coded as:

AV Group: D1 = 0, D2 = 0, D3 = 0;

AM Group: D1 = 1, D2 = 0, D3 = 0;

RV Group: D1 = 1, D2 = 1, D3 = 0;

RM Group: D1 = 1, D2 = 1, D3 = 1.

Thus, with this dummy coding:

- Parameter b_1 captures the difference: mean (AM Group)—mean (AV Group), which can be interpreted as the effects of being treated with acute MA at 3 mg/kg, compared to vehicle.
- Parameter b_2 captures the difference: mean (RV Group)—mean (AM Group), which can be interpreted as the effect of repeated injections, recalling that the RV group received a single dose of MA on the final test day.
- Parameter b_3 captures the difference: mean (RM Group)—mean (RV Group), which can be interpreted as effects of repeated MA treatment, net of the effects all other groups (acute vehicle, acute MA and repeated vehicle injections).

The p values of these parameters were then analyzed using false discovery rate (FDR) control (Benjamini and Hochberg 1995; Storey 2003; van den Oord and Sullivan 2003). An FDR threshold <0.1 was used for declaring metabolome-wide significance (van den Oord and Sullivan 2003). This ensures that, on average, only 10 % of associations declared significant are expected to be false discoveries. Operationally, these FDR levels were controlled using q values, which are FDRs calculated using the p value of the metabolite associations as thresholds for declaring significance (Storey 2003). Q values were estimated as outlined by (Bukszar et al. 2009).

Behavioral metabolomics analyses, in which we tested metabolites for association with locomotor activity, were also conducted using linear mixed models. Here, however, we examined only animals receiving a single MA exposure and our dependent variable was acute MA-induced change in total distance traveled. This was calculated by subtracting the mouse-specific mean baseline total distance traveled in the 1 h baseline session from total distance traveled in the final 10 min period in the test session, i.e. the period most proximal to sacrifice (see Table 1). MA-induced change in total distance traveled was regressed on metabolite levels, with mouse strain once again included as a random effect to account for within-strain similarity. FDR control and q values were calculated as described above. All data management, plots and analysis were conducted using R (www.r-project.org) or Stata (www.stata.com).

Metabolite set enrichment analysis (Xia and Wishart 2010), implemented using the MetaboAnalyst package (Xia and Wishart 2011), was used to test metabolites for enrichment within known biological pathways. Enrichment was calculated using overrepresentation analysis (ORA), where the p value from ORA indicates the probability of seeing at least a particular number of metabolites from a known pathway in the selected compound list. In our analysis, we tested three lists of selected compounds, the first being all metabolites showing nominal association (p values <0.05) with the D1 variable (acute MA), the second was all metabolites showing nominal significance with the D3 variable (repeated MA-specific), while the third was all metabolites showing nominal significance with MA-induced change in locomotor activity.

3 Results and discussion

Animals receiving acute 3 mg/kg MA demonstrated greatly elevated locomotor activity compared to baseline over the entire 1 h period following the injection (see Supplementary Material, Figure S1), consistent with long-standing observations (Caligiuri and Buitenhuys 2005; Peachey et al. 1976; Wise and Bozarth 1987). Specifically, for the 10 min period immediately preceding sacrifice, locomotor activity remained elevated ($p = 5.76 \times 10^{-5}$), and highly significant levels of MA were detectable in brain material of MA-treated animals compared to controls ($p = 6.9 \times 10^{-15}$). These observations indicate that the mice were under the influence of MA at time of biomaterial collection. Besides MA, which was added specifically to our compound library for this study, we were able to identify 347 unique compounds in our mass spectra of the homogenized brain tissue samples. Following quality control, 301 metabolite variables remained with which to perform association testing. While the complete murine metabolome is not yet fully elucidated, the Human Metabolome Database version 2.5 (www.hmdb.ca) has data on ~ 700–1,000 compounds per biofluid or tissue type, on average. We anticipate the murine brain metabolome to comprise of a similar number, so our assessment of over 300 in this study likely represents a sizeable portion of all relevant metabolites.

3.1 Main analysis

Our primary metabolomics analysis tested for metabolite associations with the different treatment conditions. Results are displayed in a quantile–quantile (QQ) plot (Fig. 1). In a QQ plot, the ordered observed p values, on a $-\log_{10}$ (p values) scale, are plotted against those expected under the null hypothesis represented by the straight line and assuming that none of null hypotheses can be rejected (no “true” effects). The QQ plot shows that many of the observed p values deviated systematically from this straight line and were outside the 95 % confidence intervals. The implication is that many of the metabolites differed between experimental conditions.

We observed 12 metabolome-wide significant associations using our pre-defined FDR significance criterion ($q < 0.1$) (Table 2). Of these, seven were with the D1 variable, which captures the effects of being treated with a single acute dose of MA at 3 mg/kg. Many of these compounds are associated with energy metabolism (e.g. lactate, malate and succinate). A further three compounds were associated with the D2 variable, which captures the effects of repeated injections. Associations with D2 can be considered the effects of simply being in the 5 day arm of the study, over and above the effects of acute vehicle or acute MA. Finally, two compounds were associated with the D3 variable, which can be considered to be specific to repeated MA only.

The three compounds associated with D2 (repeated injections) are not of substantive interest in relation to the biology of MA. However, by identifying these compounds we can drop them from further analyses (see below). Furthermore, while we observed 12 significant

associations overall, these were accounted for by only 11 unique compounds. This is because fructose was associated with both D1 (acute 3 mg/kg MA) and D2 (repeated injections). Our analysis was designed such that metabolite associations were with the marginal (i.e. net) effects of each treatment condition. The association of a single compound with two conditions can be explained because the effect of fructose is opposite in each group. That is, it was lower in concentration following acute MA, whereas it was increased following repeated injections. Nevertheless, because of its association with repeated injections, we do not give further consideration to fructose in this study.

3.2 Secondary analyses—behavioral associations, PLS-DA and pathways

In a secondary analysis, we examined metabolite associations with individual differences in acute MA-induced change in locomotor activity. Our hypothesis was that many of the metabolites that were associated with receiving acute MA would also be associated with increased locomotor activity. The outcome of this analysis is shown in Table 3. Here we also see a highly significant association with the energy-related metabolite succinate ($p = 3.9 \times 10^{-7}$) and a nominally significant association ($p < 0.05$) with malate. Since hyperlocomotion is a primary consequence of MA exposure, these associations indicate a consistency of findings across domains.

Multivariate statistical tools are frequently employed to examine metabolomics data and a typical method of this type is partial least squares-discriminant analysis (PLS-DA) (Wishart 2007). This supervised classification method provides an alternative, although theoretically less optimal, approach to our primary mixed model analysis. We first applied PLS-DA to distinguish acute MA from acute vehicle (for a complete description of methods, see Supplementary Material Sect. 2). The most important variables separating acute MA versus acute vehicle were 2-hydroxyglutarate, tryptophan, lactate and succinate (see Supplementary Figure S6). These were among the top findings in our primary analysis (Table 2), suggesting that differences in the levels of these metabolites are robust and not dependent on the analytical strategy used. We also performed PLS-DA between acute and repeated MA. However, in this analysis, the model was indicated non-significant in permutation testing ($p = 0.404$). Hence, our PLS-DA analysis of acute versus repeated MA administration was not informative.

In Fig. 1, we observe a general inflation of small p values, suggesting that there are multiple small effects that are insufficiently large to be detected individually, even in a sample of this size. Nevertheless, if we can aggregate these effects into cohesive, higher order units, we may be able to detect their combined effects. Metabolites are ordered into discrete pathways that are interconnected and display conservation of mass. It therefore makes theoretical sense to look for disruptions of multiple metabolites within pathways. To carry this out, we performed metabolite set enrichment analysis (MSEA) (Xia and Wishart 2010) on all metabolites showing association with the D1 (acute 3 mg/kg MA) or D3 (repeated 3 mg/kg MA) variables with p values < 0.05 in our primary analysis (see Supplementary materials for complete lists), in addition to all metabolites nominally associated ($p < 0.05$) with MA-induced change in locomotor activity (Table 3). However, we dropped all compounds significantly associated with repeated injections (fructose, sorbitol, and putrescine). The MSEA method tests for enrichment, or over-representation, of multiple compounds in the same pathway, beyond what would be expected by chance. All pathways showing significant enrichment in the MSEA are shown in Table 3. For D1 (acute 3 mg/kg MA), we find three significant pathways: alpha linolenic and linoleic acid metabolism, the citric acid cycle and mitochondrial electron transport. For D3 (repeated 3 mg/kg MA), we detect enrichment among metabolites involved in the oxidation of branched chain fatty acids. Our MSEA on metabolites associated with MA-induced change in locomotor activity was not

significant because no reference pathway contained more than one behaviorally-associated metabolite.

3.3 Acute MA associations—disturbances to energy metabolism

In this study, we identified several specific metabolites in the murine brain that were altered following MA administration. Primarily, the effects of acute MA were observed in changes to energy metabolism. Compounds related to the citric acid cycle that we found to be associated with acute MA administration include succinate, fumarate and malate. Fumarate is formed by the oxidation of succinate by succinate dehydrogenase and fumarate is then converted by fumarase to malate. In addition to these compounds, citrate was nominally significant ($p < 0.05$) but not metabolome-wide significant ($q < 0.1$). Citrate was, however, one of the most important metabolites in distinguishing acute MA treated animals versus vehicle controls in our PLS-DA analysis (Supplementary Material Figure S6). Together, these findings provide strong evidence for disruption of the citric acid cycle by MA, confirming long-standing observations in this area (Carvalho et al. 2012; Krasnova and Cadet 2009; Sylvia et al. 1977).

Associations with fumarate, malate and succinate were also found in a previous metabolomics study of the urine of MA-treated rats (Shima et al. 2011). However, levels of these compounds were *reduced* compared to controls, suggestive of a reduction in energy metabolism, whereas in our study we found levels of these metabolites to be *increased*. The explanation for this discrepancy is in the timing, dose and duration of administration in the two studies. Here, we observed increased levels of citric acid cycle metabolites 1 h after a single dose of MA at 3 mg/kg. At this time point, locomotor activity was still highly elevated in our test animals (Supplementary material Figure S1). Indeed, the increase in locomotor activity was directly associated with higher levels of energy metabolites (Table 3). Furthermore, our association with the alpha linolenic acid and linoleic acid metabolism pathway (Table 4), whereby levels of long chain metabolites such as docosahexaenoic acid and dihomo-linolenic acid were elevated (see Supplementary material), suggests a concomitant increase in fatty acid metabolism. These findings are consistent with in vivo imaging observations of rats administered a single dose of MA at 5 mg/kg, which resulted in an increase in brain metabolism and mitochondrial overactivation (Shiba et al. 2011). In the previous metabolomics study mentioned above, rats received MA at 10 mg/kg once per hour for 24 h. After this time, energy stores are likely depleted. Furthermore, after the initial increase in energy consumption, MA subsequently disrupts energy metabolism by inhibiting mitochondria (Yamamoto and Bankson 2005).

Energy consumption and supply in the brain are tightly linked to neuronal activity. Neuronal depolarization causes opening of voltage-gated sodium channels and activation of the Na+/K+ ATPases, leading to an increased demand for energy (ATP). In response, the mitochondrial respiratory chain is rapidly activated (Kasischke et al. 2004). We observed metabolites in the mitochondrial electron transport chain pathway to be significantly associated with MA in our study (Table 4) and the effect of MA on mitochondria unifies many of our observations. The pathway by which excess 2-hydroxyglutarate builds in the mammalian brain following MA exposure is probably as a by-product of disrupted mitochondrial activity. The formation of 2-hydroxyglutarate results from a side-activity of mitochondrial malate dehydrogenase, the enzyme that inter-converts oxaloacetate and malate, but which also catalyses, usually very slowly, the NADH-dependent conversion of alpha-ketoglutarate to 2-hydroxyglutarate (Van Schaftingen et al. 2009). However, under conditions of excess mitochondrial activity, 2-hydroxyglutarate may accumulate via this mechanism. Excessive accumulation of this compound in the human brain causes 2-hydroxyglutaric aciduria, a rare inherited neurological disorder (Van Schaftingen et al. 2009). In addition, this compound has been shown to cause oxidative apoptosis in the

zebrafish brain (Parng et al. 2006). It is therefore conceivable that increased 2-hydroxyglutarate following MA administration may be damaging.

3.4 Associations with repeated MA

The initial mitochondrial overactivation following MA administration is soon accompanied by disruption of the mitochondrial electron transport chain by inhibition of complexes II and IV (Yamamoto and Bankson 2005). This disruption involves increased production of reactive oxygen and nitrogen species (Yamamoto et al. 2010). Via this mechanism, MA can cause nerve terminal degeneration and may lead to apoptosis (Krasnova and Cadet 2009). This disruption to mitochondrial activity may also lead to decreased levels of N-acetylaspartate (NAA), a marker of neuronal health and viability that is synthesized in mitochondria (Krasnova and Cadet 2009; Moffett et al. 2007). Decreased NAA has been repeatedly recorded in MA abusers (Ariyannur et al. 2010; Salo et al. 2011) and we also found NAA to be decreased at a nominal level of significance with repeated MA administration (D3 variable—see Supplementary Material Table S2).

In addition to lower levels of NAA, a consistent observation in the brains of MA abusers is an increase in levels of choline compounds (Chang et al. 2005; Ernst et al. 2000). In the current study, we found increased phosphocholine to be associated with repeated MA administration (D3 variable) at a metabolome-wide level of significance, consistent with observations in humans. Notably, phosphocholine levels have also been shown to be increased following chronic ethanol administration in rats (Lee et al. 2012). Phosphocholine is a common phospholipid in the brain and is an intermediate in the synthesis of biological membranes. It is formed by the action of choline kinase on choline and the fate of most cellular phosphocholine is conversion to CDP choline by phosphocholine cytidylyltransferase (PCCT) (Li and Vance 2008). While reduced PCCT has been observed in the brains of human cocaine abusers, no difference in PCCT activity could be detected between MA abusers and controls (Ross et al. 2002). However, the sample size in this study was small (10 MA abusers), so the lack of association with MA may be a Type II error. Notably, administration of CDP choline to MA addicts was associated with abstinence from the drug and an increase NAA levels in the brain, indicating improved neuronal viability (Yoon et al. 2010). The potential efficacy of CDP-choline in treating MA addicts, plus the increase in phosphocholine levels seen with MA administration, suggest that PCCT may indeed be inhibited by MA in some way. Further exploration of this pathway in the context of developing treatments for MA abuse seems warranted.

Ergothioneine was also associated with repeated MA administration in our study, yet the function of ergothioineine has remained unclear since its discovery in the early twentieth century (Grundemann et al. 2005). It is sourced from the diet, present in most tissues and is notable for its strong antioxidant activity (Akanmu et al. 1991). Ergothioneine has been demonstrated to protect against glutamatergic excitotoxicity (Moncaster et al. 2002) and its dynamic response to oxidative stress has been measured in the intact erythrocyte (Reglinski et al. 1988). In our study, ergothioneine levels increased following repeated MA treatment. Given the lack of known endogenous synthesis pathways for this compound, but the presence of a specific transporter (Grundemann et al. 2005), a plausible hypothesis is that ergothioneine uptake from the diet increases in response to oxidative stress, in our case from MA exposure. While requiring more research, this presents the possibility of administering this dietary compound to MA abusers, to increase endogenous levels and perhaps to attenuate MA-induced neurotoxicity.

3.5 Limitations

Classical neurotransmitters such as dopamine, serotonin, etc. were identified in our samples, but were not associated with MA at metabolome-wide levels of significance. This would appear contrary to established findings, given the well-characterized increase in dopamine release associated with MA exposure. However, metabolomics can only detect changes affecting the overall metabolite pool (Goodacre et al. 2004). Highly localized effects, such as synaptic release of neurotransmitters, will not be detectable in the tissue homogenate because it is impossible to distinguish between, for example, vesicular and synaptic neurotransmitter molecules. Furthermore, low relative abundance of many neurotransmitters may mean changes in their levels are often insufficient in magnitude to be detected reliably. Refinement of our method, for example by incorporating additional fractionation steps that enrich for important, low abundance compounds, could improve this situation. However, as a screening tool to detect the largest changes in metabolite levels following drug administration, our method already appears to be successful.

4 Concluding remarks

Overall, our study has taken some known aspects of MA action, such as increased energy metabolism, but has linked these with very specific metabolites. In addition, we observed novel metabolite associations, such as 2-hydroxyglutarate, that are likely due to disrupted mitochondrial activity. Finally, with sustained MA exposure, we see altered phosphocholine levels which, in conjunction with lowered N-acetyl-aspartate, has been associated with neuronal damage (Salo et al. 2007; Yoon et al. 2010). We also see increases in ergothioneine, a product with strong antioxidant properties. Thus, we are able to synthesize several aspects of the neurochemical effects of MA in a single experiment. Future studies could extend to different classes of psychoactive drugs, in addition to examining multiple doses and time points, in order to further our understanding of neurochemical processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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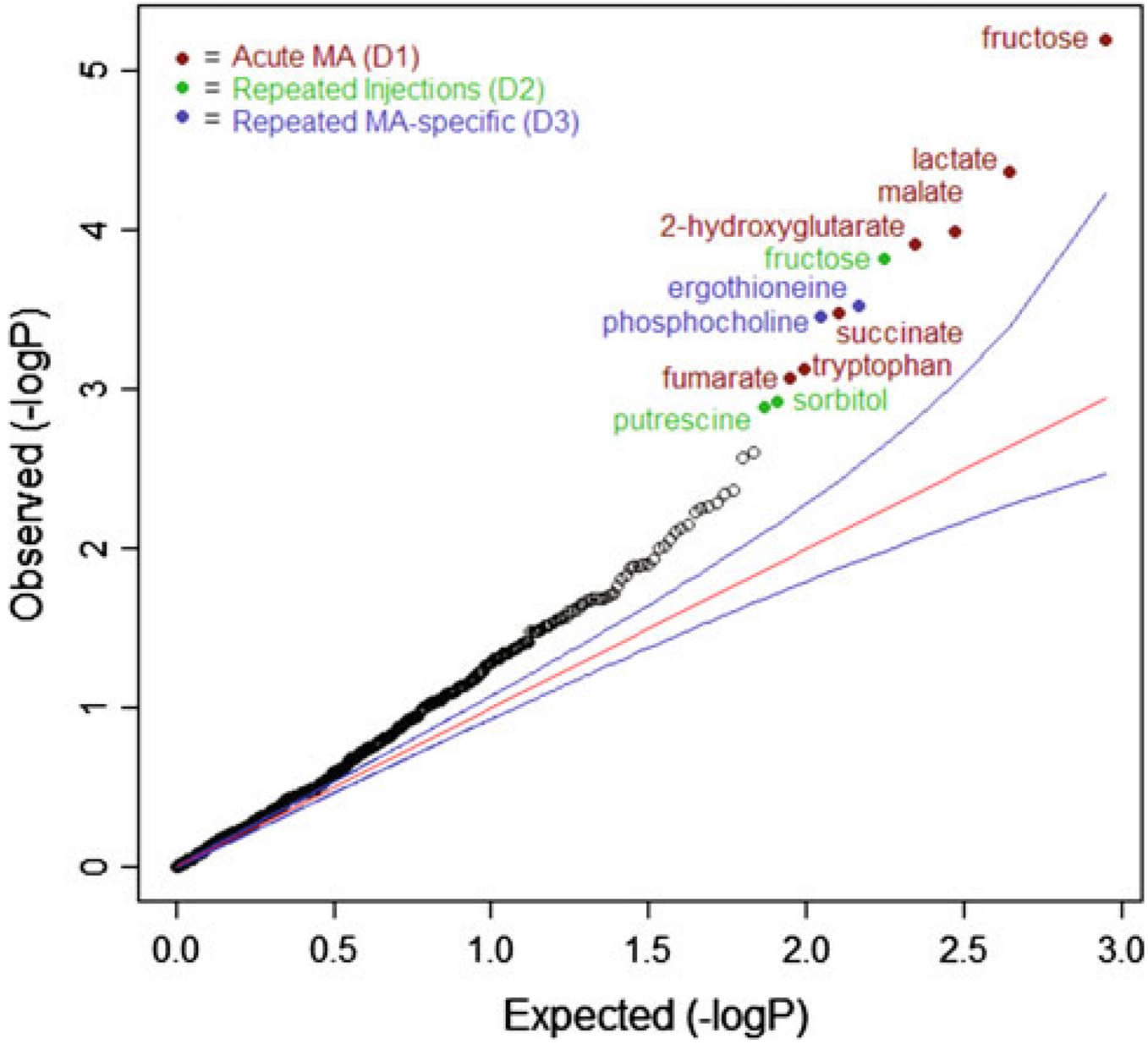

Fig. 1.

Quantile-quantile (QQ) plot of all *p* values in primary analysis. The plot compares observed *p* values to expected *p* values under the null distribution. Each point represents a neurochemical association *p* value and points falling above the null expectation, represented by the *red line*, are higher than expected by chance. The *blue line* represents the 95 % confidence interval. A total of 301 metabolites were tested for association with each of the three experimental conditions. The fact that many *p* values fall above the *red* (and *upper blue*) *line* indicates many small effects. Twelve findings were significant after false discovery rate control at the 10 % level ($q < 0.1$) and these are identified in the figure (Color figure online)

Table 1

Outline of study design, showing drug doses, locomotor activity test sessions and time points of tissue fixation by microwave irradiation

Days	Acute vehicle (AV)	Acute 3 mg/kg MA (AM)	Repeated vehicle (RV)	Repeated 3 mg/kg MA (RM)
1	1-h baseline 1-h test (vehicle) + Microwave fixation	1-h baseline 1-h test (3 mg/kg) + Microwave fixation	1-h baseline 1-h test (vehicle)	1-h baseline 1-h test 3 mg/kg
2,3,4			1-h test (vehicle)	1-h test 3 mg/kg
5			1-h baseline 1-h test (3 mg/kg) + Microwave fixation	1-h baseline 1-h test (3 mg/kg) + Microwave fixation
Number of animals per strain Number of animals total	6 48	3* 27*	3* 27*	6 48

Each test group comprised either 3 or 6 animals from each of the 8 strains, with 18 animals tested per strain, * except for C57BL/6 J with 24 (6 animals in all groups), to give 150 subjects in total. A 1 h baseline locomotor activity session, preceded by vehicle injection (i.p. saline), was followed by a 1 h test session where the mouse received either vehicle or 3 mg/kg MA. Locomotor activity was assessed using automated infrared recording equipment in 10 min periods. “Microwave fixation” indicates brain tissue fixation, which occurred immediately after the final test session. Note that the AM and RV groups both received a single 3 mg/kg dose of MA 1 h prior to sacrifice. However, the RV group allows us to estimate the control effect of repeated vehicle injections (see text)

Table 2

Metabolome-wide significant neurochemical associations with the different MA treatment conditions

Compound	ChEBI ID	Beta	StdErr	*p* value	*q* value	Variable
Fructose	28,757	−0.282	0.063	6.30E-06	0.003	D1 (acute MA)
Lactate	24,996	0.305	0.075	4.36E-05	0.013	D1 (acute MA)
Malate	15,595	0.223	0.057	0.0001	0.022	D1 (acute MA)
2-hydroxyglutarate	11,596	0.129	0.034	0.0001	0.022	D1 (acute MA)
Succinate	30,031	0.191	0.053	0.0003	0.035	D1 (acute MA)
Tryptophan	27,897	0.156	0.046	0.0007	0.066	D1 (acute MA)
Fumarate	29,806	0.118	0.035	0.0008	0.068	D1 (acute MA)
Fructose	28,757	0.273	0.072	0.0002	0.022	D2 (repeated injections)
Sorbitol	30,911	0.289	0.089	0.0012	0.087	D2 (repeated injections)
Putrescine	17,148	0.313	0.097	0.0013	0.087	D2 (repeated injections)
Ergothioneine	4,828	0.191	0.053	0.0003	0.035	D3 (repeated MA-specific)
Phosphocholine	18,132	0.118	0.033	0.0004	0.035	D3 (repeated MA-specific)

Samples were collected at 1 h post-treatment on the final test day (see Table 1) and subjected to large scale metabolomics analysis (301 compounds assayed following QC). Compounds are identified by their entry in the chemical entities of biological interest (ChEBI) database (http://www.ebi.ac.uk/chebi/). A multi-level mixed modeling approach was used to analyze the data to account for the fact that observations are nested within inbred strains. The parameterization of the model tested for the acute effects of 3 mg/kg MA (D1), then the marginal effects of receiving repeated injections (D2), then the marginal effects of being subjected to repeated MA (D3), over and above all other effects. The sign of the regression coefficient (Beta) indicates the effect direction, with negative indicating a decrease following MA administration and vice versa

Table 3

Metabolite associations $p < 0.05$ with acute MA-induced change in locomotor activity

Metabolite	ChEBI ID	Beta	Std Err	*p* value
Succinate	30,031	1,606.11	270.44	3.85E-07
Malate	15,595	907.07	370.19	0.0182
Tyrosine	18,186	1,101.14	475.81	0.0253
Serotonin	28,790	−665.32	298.97	0.0311
3-dehydrocarnitine	57,885	362.82	166.22	0.0343

Results of a mixed model regression analysis, whereby metabolite levels were tested for association with MA-induced change in total distance traveled, for animals receiving a single 3 mg/kg MA injection. The most proximal 10 min period of locomotor activity to sacrifice and collection of brain material was used as the outcome variable, minus the mean baseline activity for that animal. The sign of the regression coefficient (Beta) indicates the effect direction, with positive value indicating a positive correlation between metabolite levels and locomotor activity and vice versa

Table 4

Pathways showing significant enrichment for metabolites affected by MA

	Pathway	Total	Hits	Expected	*p* value
Acute MA	Alpha linolenic acid and linoleic acid metabolism	9	4	0.316	0.00014
	Citric acid cycle	23	4	0.808	0.007
	Mitochondrial electron transport chain	15	3	0.527	0.013
Repeated MA	Oxidation of branched chain fatty acids	14	2	0.255	0.025

For each analysis, those metabolites showing association in the main analysis with *p* values <0.05 were examined using metabolite set enrichment analysis (Xia and Wishart 2010), except that fructose, putrescine and sorbitol (associated with repeated injections—see Table 2) were dropped. "Unknown" metabolites, or those without matching library entries, were also not included since these cannot be assigned to specific pathways. Complete lists of the compounds entered into each analysis are provided in the Supplementary Material

Total total number of metabolite entries for reference pathway, *Hits* number of metabolites in data matching to pathway, *Expected* number of hits by chance