Published in final edited form as:

Genes Brain Behav. 2013 November ; 12(8): 780–791. doi:10.1111/gbb.12081.

Behavioral metabolomics analysis identifies novel neurochemical signatures in methamphetamine sensitization

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Abstract

Behavioral sensitization has been widely studied in animal models and is theorized to reflect neural modifications associated with human psychostimulant addiction. While the mesolimbic dopaminergic pathway is known to play a role, the neurochemical mechanisms underlying behavioral sensitization remain incompletely understood. In the present study, we conducted the first metabolomics analysis to globally characterize neurochemical differences associated with behavioral sensitization. Methamphetamine-induced sensitization measures were generated by statistically modeling longitudinal activity data for eight inbred strains of mice. Subsequent to behavioral testing, nontargeted liquid and gas chromatography-mass spectrometry profiling was performed on 48 brain samples, yielding 301 metabolite levels per sample after quality control. Association testing between metabolite levels and three primary dimensions of behavioral sensitization (total distance, stereotypy and margin time) showed four robust, significant associations at a stringent metabolome-wide significance threshold (false discovery rate < 0.05). Results implicated homocarnosine, a dipeptide of GABA and histidine, in total distance sensitization, GABA metabolite 4-guanidinobutanoate and pantothenate in stereotypy sensitization, and myo-inositol in margin time sensitization. Secondary analyses indicated that these associations were independent of concurrent methamphetamine levels and, with the exception of the myo-inositol association, suggest a mechanism whereby strain-based genetic variation produces specific baseline neurochemical differences that substantially influence the magnitude of MA-induced sensitization. These findings demonstrate the utility of mouse metabolomics for identifying novel biomarkers, and developing more comprehensive neurochemical models, of psychostimulant sensitization.

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Keywords

addiction; sensitization; metabolomics; homocarnosine; myo-inositol; stimulant

INTRODUCTION

Repeated administration of psychostimulants, including methamphetamine (MA), results in a progressive enhancement of the drugs' behavioral activating effects through a process known as behavioral sensitization (BSn). Observed in both animal models and humans (Pierce & Kalivas, 1997, Strakowski & Sax, 1998), BSn is thought to reflect aspects of the neural adaptations underlying addictive behaviors and motivational states (Robinson & Berridge, 2008, Steketee & Kalivas, 2011, Wise & Bozarth, 1987). Research into the neurochemistry of MA-induced BSn has traditionally focused primarily on dopaminergic neurotransmission. This approach has shown that the psychostimulatory effects of MA are associated with elevated synaptic dopamine (DA) levels due to increased DA release from presynaptic terminals and inhibited reuptake, particularly in mesolimbic neurons projecting from the ventral tegmental area to the nucleus accumbens. Thus, activity-dependent synaptic plasticity and remodeling of the mesolimbic dopaminergic pathway are known to play an important role in psychostimulant sensitization and dependence (Nestler, 2001). However, the neurochemical mechanisms underlying BSn are complex and incompletely understood, extending beyond mesolimbic DA to include GABAergic and glutamatergic neurotransmission (Pierce & Kalivas, 1997, Steketee, 2003), as well as recently identified molecular mediators (e.g., shati and piccolo (Niwa et al., 2008)). While trending toward broader consideration beyond established pathways, no research to date has applied a nontargeted metabolomics approach to comprehensively investigate neurochemical variation associated with BSn.

Metabolomics is an emerging platform that seeks to interrogate the full complement of endogenous small molecules (typically <1500 Da) within a sample in a nontargeted fashion. Thus, instead of limiting focus to specific candidate metabolites or metabolic reaction sets, metabolomics approaches quantitatively assay the entire range of metabolites present in a sample to characterize its overall biochemical state. Although MA sensitization has not yet been investigated using metabolomics, the platform has successfully identified biochemical signatures for other CNS pathologies including schizophrenia, Parkinson's and motor neuron disease (Bogdanov *et al.*, 2008, Pears *et al.*, 2005, Prabakaran *et al.*, 2004). Thus, metabolomics offers the possibility of comprehensively mapping neurochemical profiles associated with MA-induced BSn, facilitating the identification of novel mechanisms, biomarkers and therapeutic targets.

To date, metabolomics studies of psychiatric disorders have generally focused on readily obtained biomaterials in humans (e.g., urine and serum) (Kaddurah-Daouk & Krishnan, 2009). While human metabolomics research has obvious translational clinical potential, it is also characterized by various limitations including expense, difficulty controlling confounders and inability to access relevant tissue. Mouse metabolomics circumvent many of these limitations, cost-effectively providing control of genetic background and environmental confounders, primary tissues access, and precise behavioral phenotype measurement (Dunn et al., 2011). These advantages, paired with extensive genomic and phenotypic database resources, make mouse metabolomics a valuable research strategy for disorders with robust experimental models (Peters et al., 2007). Following this rationale, we analyzed the relationship between longitudinal measures of MA-induced BSn and 301 metabolite levels quantified via nontargeted liquid and gas chromatography-mass spectrometry of 48 murine brain samples.

MATERIAL AND METHODS

Animals

Male mice aged 7–9 weeks, representing 8 inbred strains chosen to maximize genetic variability (129S1/SvImJ, A/J, C3H/HeJ, C57BL/6J, CAST/EiJ, DBA/2J, NOD/ShiLtJ, PWD/PhJ) (Yang et al., 2007), were obtained from Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate approximately 1week prior to testing. Mice were housed at a maximum of four per cage in an AAALAC-accredited animal facility with food (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories, Inc., Indianapolis, IN) and water available ad libitum under a 12-h/12-h light/dark cycle (lights on at 0700-h to 1900-h). Testing occurred during light phase. All procedures were carried out in accordance with the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal, 1996) and approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Apparatus

Behavioral testing was conducted using 8 commercially obtained, automated activity monitoring devices, each enclosed in sound- and light-attenuating chambers. Variables considered potentially relevant BSn indicators included: distance traveled (cm), horizontal activity (cm), number of movements, resting time (s), stereotypy count, stereotypy number, stereotypy time (s), margin distance (cm), margin time (s), center time (s) (Supplemental Methods for variable definitions and selection rationale). Variables were collected in 10-min bins during 1-h test sessions via computer-controlled circuitry (AccuScan Instruments, Columbus, OH). Each device’s interior was divided into separate 20×20×30 cm arenas permitting independent and simultaneous measurement of two mice. Sixteen photobeam sensors per axis, spaced 2.5 cm apart along the chambers’ walls, were used to detect movement.

Locomotor Activity Procedure

The behavioral analysis sample included two experimental arms, a 3 mg/kg MA-test group and a control group receiving vehicle-only injections. MA-test and control groups each included 64 mice, 8 per strain per arm (128 total). For both arms, Test-day 1 consisted of two consecutive, 1-h behavioral activity test sessions. A vehicle injection was given immediately prior to Test-day 1 Session 1 for both groups and vehicle or 3 mg/kg MA injection, for control and test groups respectively, was given immediately prior to Test-day 1 Session 2. For Test-days 2–4 a single 1-h test session occurred, immediately preceded by vehicle or 3 mg/kg MA injection, for control and test groups respectively. Test-day 5 exactly replicated the procedure from Test-day 1. Measurements from the initial, vehicle-only sessions on Test-days 1 and 5 were excluded from analysis, resulting in 30 repeated assessments (5 days × 6 bins) per mouse in the behavioral analysis. Boxplots summarizing all activity data for the MA-test group by day, strain and measure, are presented in Supplemental Figures 1 and 2.

After the last activity test session, mice were immediately euthanized by microwave fixation (Muromachi Microwave Fixation System, TMW-4012C, Tokyo). Many neurochemicals undergo rapid post-mortem alterations due to ongoing metabolism, resulting in a distorted characterization of in vivo metabolism. These alterations are minimized by microwave irradiation, which instantaneously denatures all enzymes in the brain, preserving metabolites in the pre-sacrifice state (De Graaf *et al.*, 2009, Ikarashi *et al.*, 1985, Login & Dvorak, 1994). Brain tissue was then extracted by dissection, placed into 2 ml vials, snap-frozen in liquid nitrogen and kept frozen at −80°C (Ultima II, ULT2856-9-A40, Thermo Electron Corporation) prior to shipment to Metabolon. Inc. (Research Triangle Park, NC) for mass

spectrometry profiling. Forty-eight MA-test mice were selected for whole brain metabolomics assay (6 randomly selected per strain). Given the study's aim of characterizing neurochemicals associated with variation in MA-induced BSn, a phenomenon only observable with MA administration, vehicle treated controls were not considered in the metabolomics analysis, but these data have been presented elsewhere (Mcclay *et al.*, 2013).

Drugs

(±)-Methamphetamine (National Institute on Drug Abuse, Rockville, MD) was prepared in 0.9% saline stock solutions sterilized by filtration through 0.2 μm filtration disks. Working MA solutions were injected intraperitoneally at 10 ml/kg body weight. The 3 mg/kg MA test dose has been shown to induce sustained behavioral response, persisting longer than test session duration, in multiple mouse strains (Peachey *et al.*, 1976, Snider *et al.*, 2012, Zhang *et al.*, 2006).

Metabolomics Sample Preparation

Sample preparation was carried out using the automated MicroLab STAR® system (Hamilton Robotics, Reno NV) (Evans *et al.*, 2009, Ohta *et al.*, 2009). Recovery standards were added prior to the first extraction process step for QC purposes. A proprietary (Metabolon) series of organic and aqueous extractions were used to remove the protein fraction while maximizing recovery of small molecules. The resulting extract was divided into liquid chromatography (LC/MS) and gas chromatography (GS/MS) mass spectrometry fractions. Samples were placed briefly on a TuboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for either LC/MS or GC/MS. A small aliquot of each experimental sample for a specific matrix was extracted and pooled together as a "client matrix". Aliquots of client matrix samples were injected throughout the platform day run and served as technical replicates. As such, the variability in the quantitation of all consistently detected biochemicals in the experimental samples was monitored to provide a process variability metric for run-day normalization and quality control.

Liquid chromatography/Mass Spectrometry (LC/MS, LC/MS^2)

The LC/MS portion of the platform was based on a Waters Acquity UPLC and a Thermo-Finnigan LTQ mass spectrometer, consisting 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 ≥11 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 basic extracts, also using water/methanol, contain 6.5mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion.

The LC/MS accurate mass portion of the platform was based on a Surveyor HPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which has a linear ion-trap (LIT) frontend and a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer backend. For ions with counts >2 million, an accurate mass measurement can be performed on both parent ions, as well as fragments. The typical mass error was <5 ppm. Ions with <2 million counts require greater effort to characterize. Fragmentation spectra (MS/MS) were typically generated in data dependent manner, but if necessary targeted MS/MS were employed (i.e., for lower concentration signals). Further details of LC/MS methods can be found elsewhere (Evans et al., 2009).

Gas chromatography /Mass Spectroscopy (GC/MS)

The samples intended for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). The GC column is 5% phenyl and the temperature ramp was from 40° to 300° C in a 16 minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis. The information output from the raw data files were automatically extracted as discussed below. Further details regarding GC/MS methods can be found elsewhere (Ohta et al., 2009).

Data Extraction and Compound Identification

Raw MS data files were loaded into a relational database and peaks identified using Metabolon proprietary peak integration software. Biochemicals (and MA) were identified by comparison to library entries of purified standards or recurrent unknown entities, including approximately 1500 commercially available purified standard biochemicals for LC and GC platforms. The combination of chromatographic properties and mass spectra gave an indication of a match to the specific compound or isobaric entity. As previously described, a variety of curation and QC procedures were implemented to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, misassignments or background noise, and to confirm the consistency of peak identification among the various samples (Evans et al., 2009). Biochemicals with <3-fold greater abundance than background were excluded. A data normalization step was 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 dividing all samples by that compound's median (Evans et al., 2009). Thus, while extraction efficiencies, ionization efficiencies and abundance in murine brain are variable across metabolites, they are comparable within metabolites, allowing unbiased comparisons of relative abundance between samples.

Statistical analysis

Linear mixed models were used to model MA-induced BSn effects and generate mouse-specific sensitization measures for MA-test mice (Willett et al., 1998). It was necessary to simultaneously model control and MA-test animal data to accurately estimate sensitization effects by partialling out the effect of protocol habituation (i.e., response to handling and experimental procedures), which was present in both control and MA-test arms, from BSn, which was present only in MA arm. For each phenotype, behavioral activity (Y) for each mouse (i) during each test period (j) was modeled as a function of: (b_0) baseline activity level (i.e., intercept); (b_1) habituation, modeled as a linear function of study day; (b_2) baseline effect of MA administration (i.e., dichotomous indicator of experimental arm); (b_3) sensitization to MA exposure, modeled as an interaction between arm and study day; and mouse-level deviations (random effects) from the mean: (u_{0i}) intercept, (u_{1i}) habituation effect, and (u_{3i}) sensitization effect; and a residual (e_{ij}). This model is formalized as:

$$Y_{ij} = b_0 + b_1 day_{ij} + b_2 arm_i + b_3 arm_i \times day_{ij} + u_{0i} + u_{1i} + u_{3i} + e_{ij}$$

with random effects specified as orthogonal (Willett et al., 1998).

The term of primary interest was the sensitization random effect—u_{3i}. For each of the 10 behavioral phenotypes this term, quantifying the magnitude of each test mouse's MA-induced sensitization, was output from the linear mixed model as best linear unbiased predictors (BLUPs) (Robinson, 1991). Sensitivity analyses modeling the effects of habituation and sensitization quadratically yielded equivalent (r>0.95) mouse-level

sensitization estimates. Measures of longitudinal change estimated in this fashion reliably increase phenotype signal-to-noise ratio, and thus, statistical power, compared to conventional BSn measurement approaches, such as last minus first MA exposure, or post-sensitization MA challenge in test and control animals (Willett, 1989, Willett *et al.*, 1998). Next, to avoid redundant analysis of multiple variables representing the same underlying sensitization phenomena, we conducted factor analyses to assess dimensionality in the 10 sensitization measure using the standard principle factor method. Several orthogonal rotations were implemented (e.g., varimax, quartimax) and yielded comparable three factor solutions. Additionally, one way ANOVA were used to calculate the proportion of variance explained by strain (i.e., heritability) for all sensitization and metabolite measures.

Primary metabolomics analyses were conducted as a series of bivariate linear regressions, with a separate model estimated for each metabolite-sensitization combination, resulting in 903 total associations (301 metabolites × 3 outcomes). As mice are nested within strains, they are nonindependent, which violates regression assumptions and generally downwardly biases standard errors, increasing Type I errors (Williams, 2000). To account for nonindependence of mice within strains, the Huber/White/Sandwich clustered variance estimator was used to calculate standard errors (Rogers, 1994, Williams, 2000). This method explicitly adjusts for within-cluster (i.e., strain) correlation, allowing unbiased significance testing of clustered data and efficient use of all available information. For each sensitization outcome, false discovery rate (FDR) methods used to adjust significance criterion for multiple testing. As argued previously (Van Den Oord & Sullivan, 2003), we prefer an FDR-based approach to declaring significance because it: (a) balances competing goals of finding true effects versus avoiding false discoveries, (b) provides comparable standards across studies, as it is less affected by number of tests and importantly, (c) is relatively robust to correlated tests (Fernando *et al.*, 2004, Storey, 2003), as were present here. We used an FDR<0.05 threshold for declaring metabolome-wide significance, specifying that, on average, 5% of associations declared significant are expected to be false discoveries. Operationally, FDR levels were controlled using *q*-values (Storey, 2003).

Additionally, we conducted several sensitivity analyses to test the robustness of the metabolome-wide significant associations. First, to eliminate the possibility that the observed metabolite differences reflect acute MA response, we adjust for levels of locomotor activity observed in the first test session again using cluster-robust standard errors. Second, to eliminate the possibility that metabolome-wide significant associations are driven by outlying mice, we calculate Spearman rank correlations with cluster-robust standard errors. Third, analyses were repeated systematically excluding one strain at a time, to ensure that statistical significance was not driven by a single outlying strain. Only associations satisfying these three sensitivity criteria ($p<0.05$) were considered robust and included in the final sets of analyses.

Next, multiple regression was used to consider whether the robustly significant metabolite-outcome associations were mediated by concurrent MA level in brain and/or genetic differences between strains. To this end, we compared sensitization variance explained by metabolite level in a bivariate regression model (R^2) to marginal sensitization variances explained by metabolite level controlling for MA level (R^2_M) and strain (R^2_S), respectively. R^2_M and R^2_S were calculated as a partial R^2 (Cohen et al., 2003). Substantively, a partial R^2 may be interpreted as the additional variance explained by the metabolite, over and above that explained by the putative mediator. Heritability (H^2) was calculated and is defined as the proportion of the sensitization-metabolite covariance explained by strain. Lastly, we collapsed metabolite levels into strain means and examined the correlations of these metabolite strain means between the MA-test mice analyzed here and mice sacrificed at baseline (i.e., no MA exposure). This was done to investigate the degree to which associated

metabolites reflected baseline strain differences. All formulae are detailed in Supplemental Methods.

RESULTS

As expected, results indicated that for all 10 phenotypes the mean behavioral activating effects of MA significantly sensitized with repeated administration ($p<0.05$; Supplemental Figure 3). The degree of sensitization significantly varied across individual mice ($p<.05$; Supplemental Table 1). For each phenotype, measures quantifying mouse-specific MA-induced BSn were extracted from linear mixed models as BLUPs (Robinson, 1991). All BSn measures were approximately normally distributed with no significant outliers/skewness (Supplemental Figure 4). Factor analysis of sensitization measures indicated three substantial factors underlying the measures (explaining 91% cumulative variance). Factor 1 (65% variance) was well-represented by sensitization in total distance (TD) ($\lambda=0.97$), Factor 2 (15% variance) by stereotypy number (SN) ($\lambda=0.78$), and Factor 3 (11% variance) by margin time (MT) ($\lambda=0.74$) (Supplemental Table 2; Supplemental Figure 5). These three BSn measures were used as metabolomics outcomes and raw data used to generate them is presented, by strain, in longitudinal scatterplots with superimposed strain mean trajectories in Figure 1. ANOVA results indicated that these three sensitization measures varied in heritability, with approximately half of sensitization variance due to strain differences for TD ($p=6.60E-06$, $R^2=0.46$) and SN ($p=9.63E-06$, $R^2=0.45$), while a nonsignificant proportion of sensitization variance was attributable to strain for MT ($p=0.19$, $R^2=0.15$). See Supplemental Table 3 for ANOVA results for strain effects on all 10 behavioral indicators.

Metabolomics association analyses were conducted as a series of 903 bivariate linear regressions using cluster-robust standard errors, one for each metabolite-outcome combination. Q-Q plots show that associations were generally stronger than expected by chance, with nine associations achieving metabolome-wide significance ($q<0.05$), 2 for TD, 5 for SN and 2 for MT (Figure 2). Specifically, TD was significantly associated with ribulose and homocarnosine. SN was significantly associated with isovalerylcarnitine, pantothenate, glutarylcarnitine, N-acetylglutamate and 4-guanidinobutanoate. MT was significantly associated with betaine and myo-inositol (Table 1). All results were robust to adjusting for the acute locomotor effects of MA. However, Spearman rank sensitivity analyses indicated that the ribulose-TD, glutarylcarnitine-SN, N-acetylglutamate-SN and betaine-MT associations were driven by outlying mice. Similarly, sensitivity analyses examining the influence of individual strains indicated outlying metabolite levels among 129S1/SvImJ drove the isovalerylcarnitine-SN association. Consequently, these five non-robust associations are excluded from further analysis (Supplemental Figure 6). The four robust associations are visualized in Figure 3. As shown by standardized regression coefficients (β column, Table 1), for each of the robust associations a 1 SD change in metabolite level was associated with 0.35–0.5 SD change in the corresponding BSn measure. Thus, metabolite levels explained 12–25% of sensitization outcome variance in bivariate models (R^2 column, Table 1). Among the robustly significant metabolites, ANOVA results indicated high heritability for homocarnosine ($p=5.12E-17$, $R^2=0.89$), pantothenate ($p=1.40E-07$, $R^2=0.66$) and 4-guanidinobutanoate ($p=1.62E-13$, $R^2=0.83$), and nonsignficant heritability for myo-inositol ($p=0.17$, $R^2=0.21$). Strain ANOVA results for all 301 assayed metabolites, summarized in Supplemental Figure 7, demonstrate significant heritability ($p<0.05$) for a majority (68.8%) of metabolites, and high heritability ($h^2>0.70$) for substantial minority (13.0%) of metabolites.

Next, to determine whether the observed robustly significant associations were a direct result of concurrent MA level, we adjusted the sensitization-metabolite associations for MA level in brain homogenate at sacrifice. Results indicated that MA level did not significantly

mediate associations, with marginal metabolite-sensitization associations remaining highly significant ($p<.001$) (Figure 4; Supplemental Table 4). Next, we adjusted the metabolite-sensitization associations for strain differences to determine to what extent significant associations were due to genetic differences. Results indicated that associations were generally largely determined by genetic variation between strains, with marginal metabolite effects attenuated to nonsignificant levels when adjusting for strain for homocarnosine-TD, pantothenate-SN and 4-guanidinobutanoate-SN ($p>.05$; Figure 4). The myo-inositol-MT association was an exception—it was not substantially influenced by strain differences, with its marginal metabolite-sensitization association remaining large and significant ($p<.001$; Figure 4). These results were consistent with one way ANOVA results showing significant heritability for all implicated metabolites and sensitization outcomes, with the exceptions of myo-inositol and MT.

Finally, we collapsed metabolite levels into strain means and examined the correlations of the metabolite strain means between the MA-test mice analyzed here and mice sacrificed at baseline (i.e., no MA exposure). The rationale for this analysis is that if, (a) the strain differences explain a large majority of metabolite-sensitization associations, and (b) the correlations of strain metabolite means pre- and post- MA sensitization are high, it can be transitively inferred that, (c) significant results reflect associations between baseline strain neurochemical differences and sensitization. This proved to be the case for metabolites in associations substantially accounted for by strains differences, with homocarnosine ($p=0.0001$, $R^2=0.94$), pantothenate ($p=0.0001$, $R^2=0.94$) and 4-guanidinobutanoate ($p=0.0003$, $R^2=0.90$) all showing a very high degree of correspondence between strain means pre- and post- MA sensitization. A lower, though still substantial, correlation was observed for myo-inositol ($p=0.013$, $R^2=0.67$), further suggesting a unique mechanism for the myo-inositol-MT association relative to the other robust associations. The full results of pre- and post-sensitization strain mean correlations for all 301 assayed metabolites are summarized in Supplemental Figure 8 and indicate variable correlation strength, but with far more significant associations than expected by chance (61.8% at $p<0.05$).

DISCUSSION

Here we report the first nontargeted metabolomics analysis to globally characterize neurochemical differences associated with MA-induced BSn. Association testing between sensitization measures and 301 mass spectrometry-derived metabolite levels, measured across eight inbred mouse strains, identified four novel, robust BSn-metabolite associations. Previous research indicates plausible biological mechanisms for several of these associations.

In addition to the primary sensitization metabolomics association analysis, we also analyzed the longitudinal activity data and metabolite battery separately. Results from our activity phenotype modeling yielded several notable findings. First, mean MA-induced BSn was significant across all activity measures and the magnitude of sensitization varied markedly across strains (Supplemental Figure 3; Figure 1). While previous research on strain differences in psychostimulant BSn has yielded mixed findings due to modest statistical power, the strain differences observed here were consistent with the most commonly reported patterns, showing increased BSn in DBA/2J and C57BL/6J, particularly relative to A/J and 129/J strains (Chen *et al.*, 2007, Eisener-Dorman *et al.*, 2011, Mead *et al.*, 2002, Thomsen & Caine, 2011). Next, consistent with findings from BXD recombinant inbred strains (Philip *et al.*, 2010), we found that strain accounted for a significant proportion of sensitization variance ($h^2=33–50\%$, $p<0.001$) for all sensitization phenotypes except margin time ($h^2=15\%$, $p=0.19$) (Supplemental Table 3). Independent analyses of the metabolite battery indicated that heritability ranged widely for the 301 metabolites considered, with

most metabolites significantly heritable, many metabolites, including homocarnosine (h^2=89%), highly heritable, but 31.2% of metabolites, including myo-inositol, showing nonsignificant heritability (p<0.05) (Supplementary Figure 7).

Results from the primary association analyses of MA-induced activity sensitization (TD, SN and MT) and the 301 brain metabolites indicated four metabolite-sensitization associations were metabolome-wide significant (q<0.05) and satisfied all sensitivity criteria: homocarnosine-TD (β=−0.50, p=2.25E-05), pantothenate-SN (β=0.47, p=1.29E-04), 4-guanidinobutanoate-SN (β=−0.35, p=4.59E-04) and myo-inositol-MT (β=−0.47, p=4.86E-04) (Figure 3). Among these implicated metabolites, the association of homocarnosine to TD provides clear biological rationale, suggesting that strain-based differences in homocarnosine-mediated cortical excitability moderate the magnitude of MA-induced psychomotor sensitization. This interpretation is supported by research showing that homocarnosine, a CNS dipeptide of GABA and histidine, is intimately related to GABAergic function and serves as a potent inhibitory neuromodulator (Petroff *et al.*, 1998b). Brain homocarnosine levels dose-dependently increase in response to antiepileptic drugs including vigabatrin and topiramate, with the degree of homocarnosine increase strongly correlating with seizure control (Petroff *et al.*, 1998a, Petroff *et al.*, 1999). Moreover, homocarnosine has been indirectly implicated in MA-induced sensitization by studies showing that vigabatrin is effective in treating psychostimulant dependence (Brodie *et al.*, 2005, Gerasimov *et al.*, 1999). Vigabatrin is thought to influence psychostimulant addiction through tonically inhibiting both endogenous and psychostimulant-induced extracellular striatal DA release (Dewey *et al.*, 1992, Gerasimov *et al.*, 1999). Additional clinical research has shown that homocarnosine's cortical inhibitory effects are relevant to several related outcomes, including alcohol dependence and panic disorder (Behar *et al.*, 1999, Goddard *et al.*, 2001).

Homocarnosine's neural function remains incompletely understood. Although it has been established that hydrolysis of homocarnosine can rapidly liberate GABA in brain, the balance of evidence suggests that homocarnosine most likely exerts inhibitory effects in of itself, without conversion to GABA (Jackson *et al.*, 1994, Petroff, 2002). This is supported by recent studies of homocarnosine function in rat hippocampus indicating that while both homocarnosine and GABA exerted inhibitory effects, homocarnosine showed no effect on membrane potential; its effects were blocked by pretreatment with $GABA_A$ inhibitors; and its presence reduced the frequency but not the burst characteristics of induced seizure-like activity (Petroff & Williamson, 2009). Thus, consistent with our findings, it appears that homocarnosine's inhibitory action does not function through increasing GABA levels, but rather through shifting the balance from intracellular to synaptic locations of neurotransmitter.

The 4-guanidinobutanoate-SN association suggests a related GABAergic mechanism. 4-guanidinobutanoate is a relatively poorly studied metabolite formed by the transfer of the guanidino group from arginine to GABA via transamination (Jansen *et al.*, 2006, Schulze *et al.*, 1998). While research on the neural function of this metabolite is sparse, there is some indication that it exerts GABAergic effects in rodent CNS (Bowery & Brown, 1974). Intriguingly, as with homocarnosine, 4-guanidinobutanoate levels are elevated by vigabtrin treatment (Schulze *et al.*, 1998). These facts, combined with the substantial correlation observed with homocarnosine in the current data (r=0.58; p<0.001), suggest that 4-guanidinobutanoate is plausible biomarker of GABAergic inhibition and perhaps a causal factor in attenuating MA-induced BSn.

The myo-inositol-MT association is also consistent with previous research. Margin time, or thigmotaxis, is a well-established index of anxiety in mice, which has been validated using

several anxiogenic and anxiolytic drugs (Simon *et al.*, 1994). Consistent with our results, there is a large body of research in both rodents and humans indicating a negative correlation between CNS myo-inositol levels and anxiety disorders /behaviors (Benjamin *et al.*, 1995, Einat & Belmaker, 2001). This research has demonstrated that chronic myo-inositol administration, but not acute, increases open field activity in rodents and that chronic exposure increases rodent myo-inositol levels in various brain regions, including cortex and hippocampus (Cohen *et al.*, 1997, Einat & Belmaker, 2001). Human postmortem research has shown that reduced myo-inositol levels in frontal cortex are associated with bipolar disorder and suicide, suggesting that endogenous levels are relevant to anxiety-related phenotypes (Shimon *et al.*, 1997). While the precise mechanism by which myo-inositol exerts its anxiolytic effects remains obscure, the metabolite is a key precursor of the phosphoinositide secondary messenger cycle, which is activated following ligand binding with G-protein coupled receptors across several neurotransmitter systems including serotonergic (5-HT_{1C} and 5-HT_2) and dopaminergic (D_1) receptor types (Kim *et al.*, 2005). Finally, the pantothenate-SN finding is entirely novel, with no notable support from previous research.

Synthesizing all results yields a consistent causal interpretation, with the notable exception of the myo-inositol-MT finding. Thus, the homocarnosine-TD, 4-guanidinobutanoate-SN and pantothenate-SN associations all share several notable features—all of these metabolites and sensitization dimensions are highly heritable in this mouse panel; strain means of the metabolites are virtually perfectly correlated between untreated and MA sensitized mice; and the covariances of metabolites and sensitization measures are highly heritable. Furthermore, analyses controlling for acute MA activity effects and MA brain levels at sacrifice rule out the possibilities that these findings reflect differences in MA metabolism rates or the acute activity-inducing effects of MA. Additionally, none of the significant associations identified here overlap findings from our previous metabolomics analysis of the direct neurochemical effects of MA exposure (Mcclay *et al.*, 2013). Cumulatively, these findings strongly suggest that baseline strain differences in the implicated neurochemicals substantially influence the magnitude MA-induced TD and SN sensitization.

The myo-inositol-MT finding suggests a different, and less certain, causal interpretation. Unlike the other significant associations, myo-inositol and MT were not significantly heritable in this sample, nor was their covariance. Furthermore, the correlation of strain means in untreated and MA sensitized mice indicated substantially less stability than was observed for the other significant metabolites. Thus, this finding is not attributable to strain differences and it is possible that the association may be due to myo-inositol levels differentially shifting as a consequence of repeated MA exposure, rather than baseline neurochemical differences. Given the causal ambiguity of this finding, it may be reasonable to regard it with a heightened degree of skepticism. However, the result is entirely consistent with a large body of previous research showing negative correlations between CNS myo-inositol levels and anxiety traits, including open field measures similar to MT (Einat & Belmaker, 2001, Shimon *et al.*, 1997), supporting its validity as a true discovery.

These results suggest promising directions for future research. Given that the predominant pattern in the significant findings suggests common genetic variation producing baseline neurochemical differences that then substantially predict the magnitude of MA-induced sensitization, future research using a heterogeneous, genetically diverse sample of mice, such as latter generations of the Collaborative Cross (Iraqi *et al.*, 2012), would be advantageous for several reasons. First, the increased genetic diversity would offer greater statistical power and reduce the risk of spurious association (Flint & Eskin, 2012). Second, genomewide genotypes are freely available for the founder strains and hundreds of crosses of the Collaborative Cross panel (Iraqi *et al.*, 2012), enabling inexpensive genetic mapping

of both metabolite levels and sensitization variables. Notwithstanding these merits, an awareness of the limits of generalizability between mouse models and humans is warranted given known species differences in potentially relevant neurochemical pathways, such as neuroactive steroid modulation of GABAergic signaling (Nguyen *et al.*, 1995).

Another topic relevant to future research is the use of whole brain homogenate in the current study, the selection of which was influenced by volume requirements for the nontargeted metabolomic assay (Evans *et al.*, 2009, Ohta *et al.*, 2009). While, in principle, volume requirements could have been met through pooling specific regions across mice within strains, implicit in such pooling is the strong assumption of no within-strain variance, which would have precluded investigating heritability and missed the myo-inositol-MT finding, which was driven by within-strain variance. Another, more general limitation of the metabolomics platform is its exclusive focus on changes to the overall metabolite pool. Thus, 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. Despite these limitations, the multiple robust signals detected here demonstrate the method's value as a screening tool. Clearly, this is only the first stage in understanding the role of these metabolites in psychostimulant abuse. Accordingly, we encourage future research into the roles of the implicated metabolites in targeted assays of candidate brain regions.

In conclusion, using nontargeted metabolomic profiling, we identified multiple neurochemical signatures in MA-induced sensitization. Specific results include the finding that relative abundance of two specific GABAergic metabolites, homocarnosine and 4-guanidinobutanoate, attenuate the magnitude of MA psychomotor sensitization. Further, consistent with previous research, we found myo-inositol negatively associated with sensitization of MA's anxiogenic effects. While the estimated risk of false discoveries was quite low, independent replication and targeted follow-up will be necessary to confirm and elaborate these findings. To facilitate this, we have made *p*-values from the primary analysis available for download (http://www.people.vcu.edu/~ejvandenoord/) as a resource supporting future replication, follow-up or data integration efforts. It is hoped that this research will encourage further metabolomics applications to identify novel neurochemical mechanisms and potential therapeutic targets in neuropsychiatric disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by National Institute of Health grants R21DA021411 to Dr. van den Oord and K01MH093731 to Dr. Adkins.

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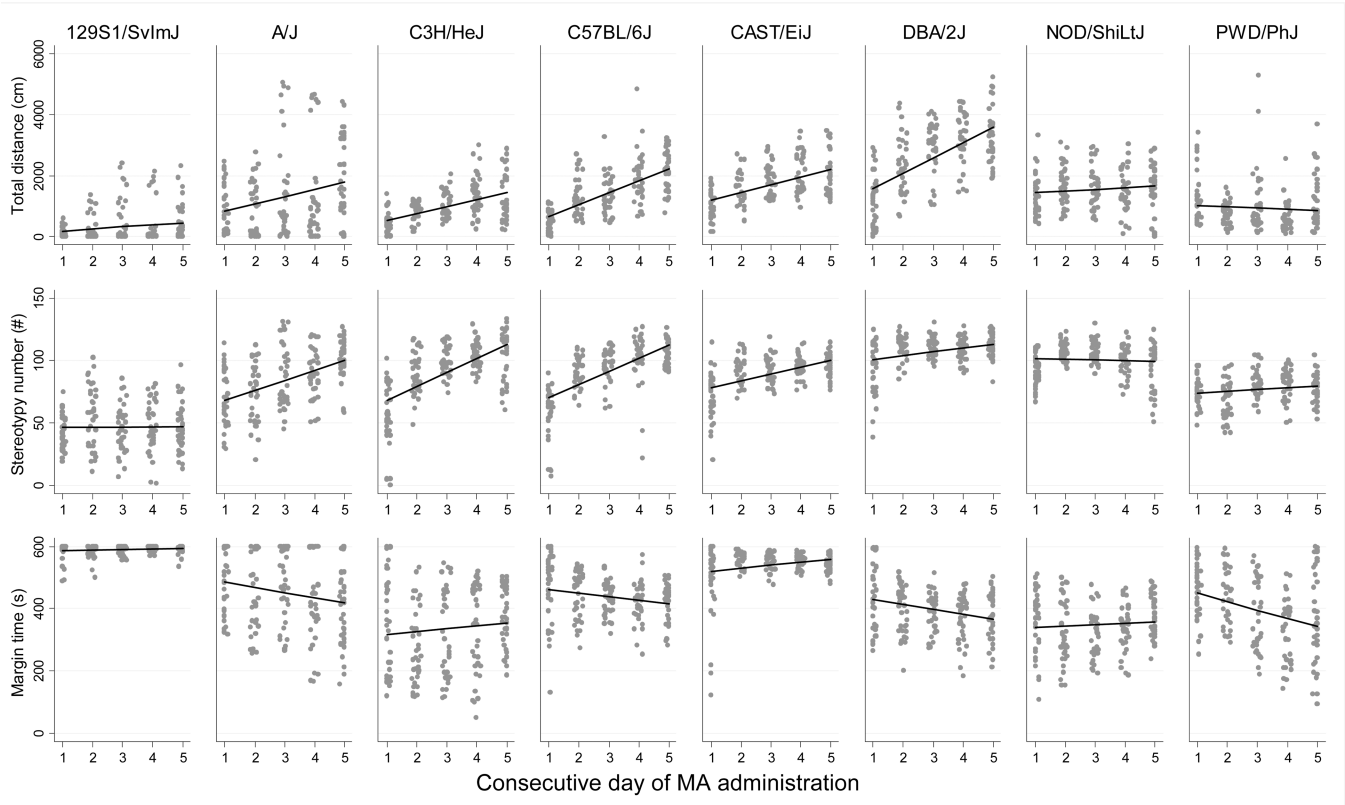

Figure 1. Longitudinal scatterplots, by strain, of all MA-test mice repeated assessments for behavioral outcomes used in metabolomics analyses

Mean strain sensitization trajectories are superimposed over scatterplots. A stochastic jitter was used to prevent overlay of points occurring on the same day.

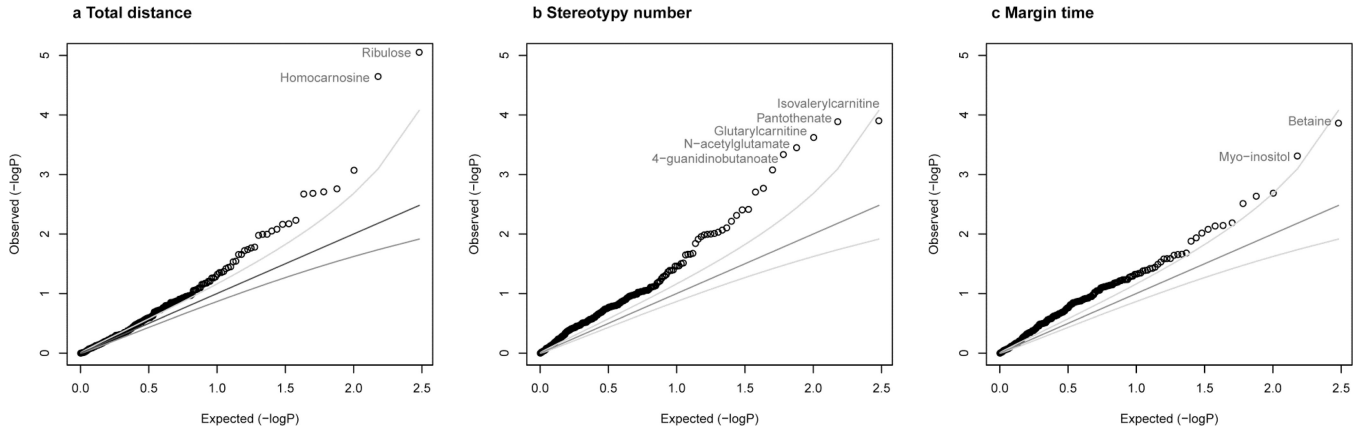

Figure 2. Nine neurochemical levels were significantly associated with MA-induced BSn ($q<0.05$)

Q-Q plots compare observed −log(10) *p*-values to expected –log(10) *p*-values under the null distribution for bivariate associations of BSn measures (**a** Total distance, **b** Stereotypy number, **c** Margin time) to 301 neurochemical levels. Each point represents a neurochemical-BSn association *p*-value and points falling above the null expectation, represented by the dark gray line, are higher than expected by chance. Light gray lines indicate 95% confidence intervals for each *p*-value rank. All metabolome-wide significant *p*-values are labeled.

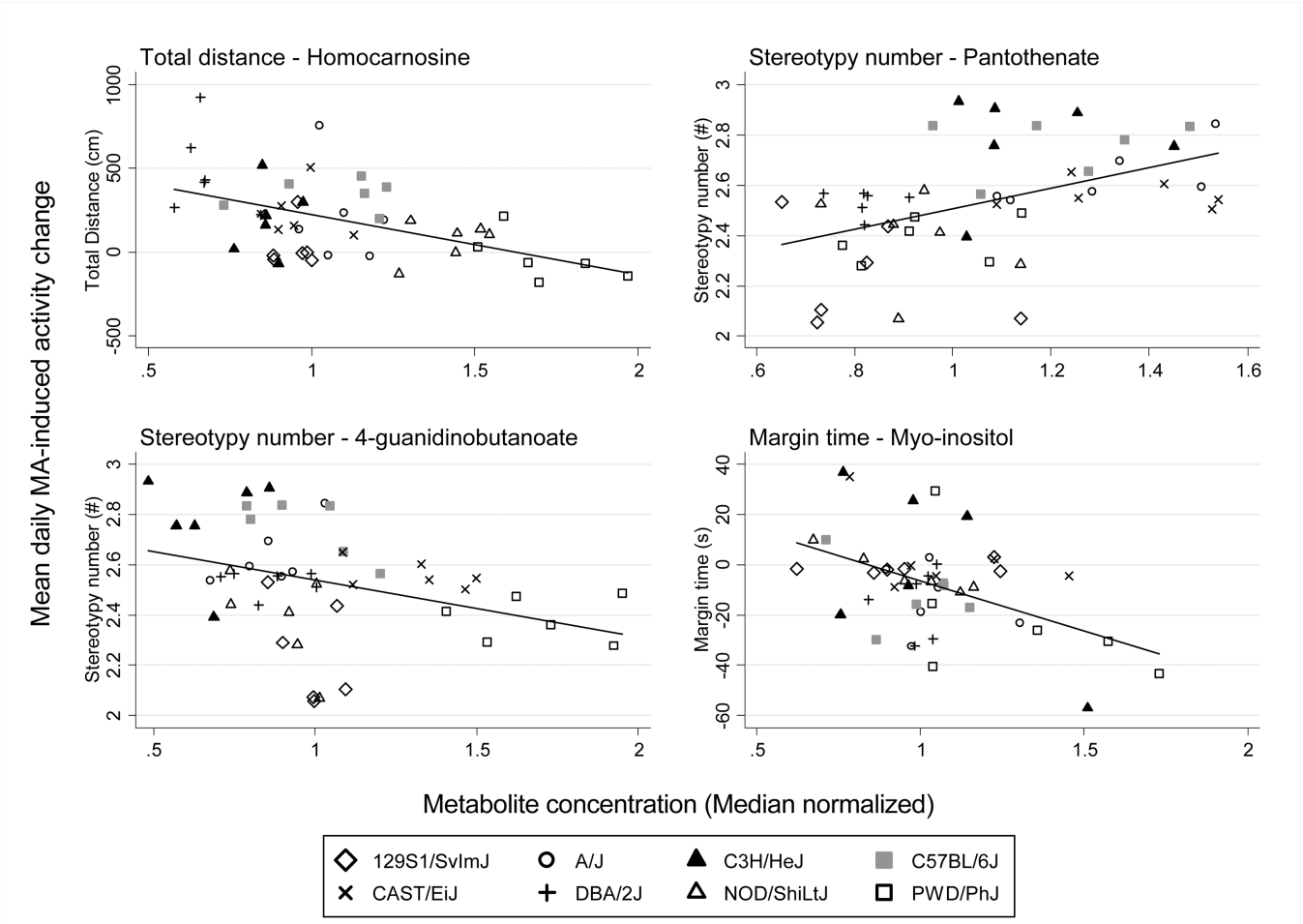

Figure 3. Associations of homocarnosine to TD, pantothenate and 4-guanidinobutanoate to SN, and myo-inositol to MT were robust across the examined inbred strain panel

Sensitivity analyses indicated that the statistical significance of these associations was robust to outlying mice and the exclusion of any single strain.

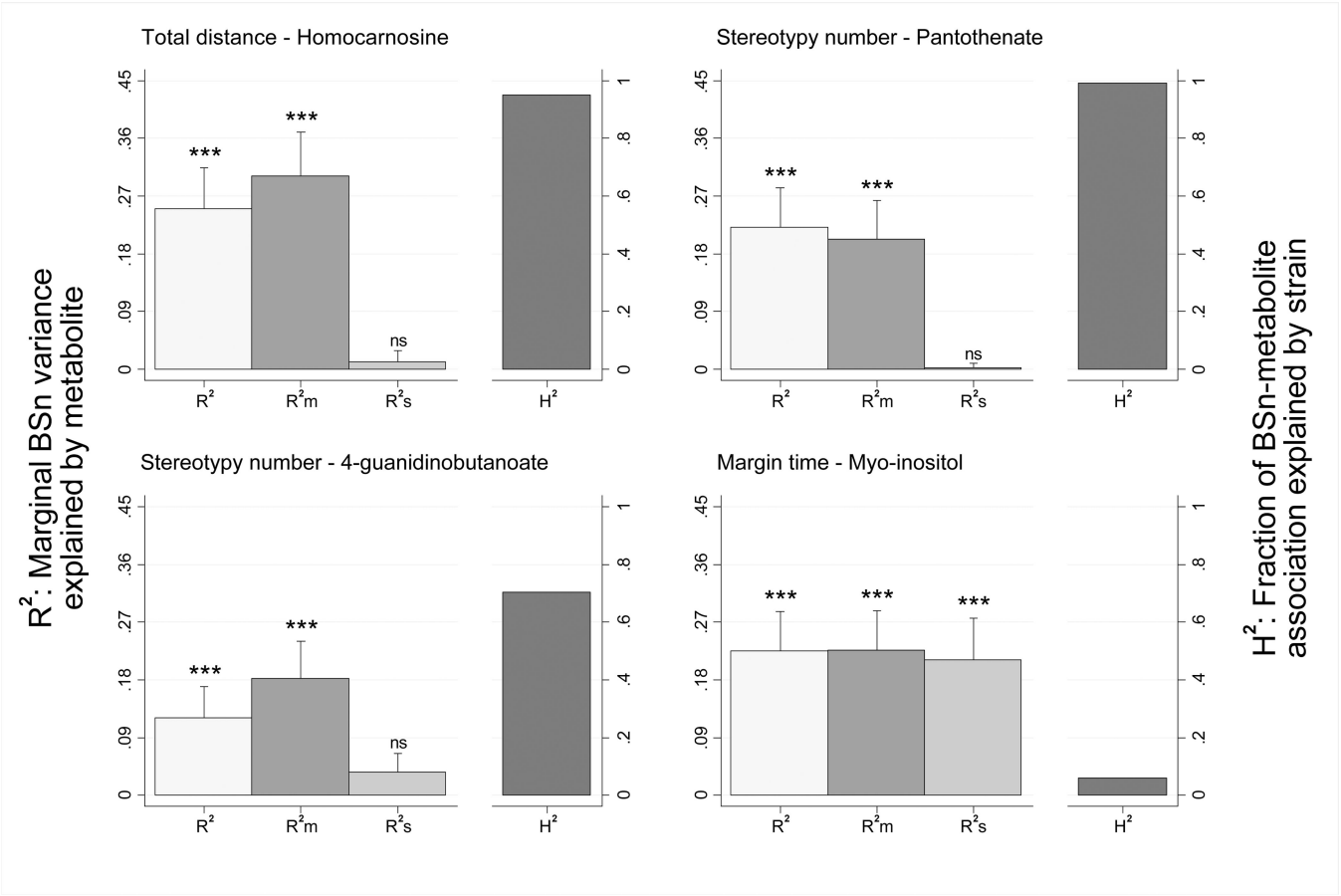

Figure 4. Among robust associations, neurochemical concentrations explained 12–25% of BSn variance and, with the exception of myo-inositol, were largely mediated by genetic differences

The first columns (light gray) describe unconditional BSn variance explained by the metabolite, with R^2 ranging from 0.251 (homocarnosine) to 0.121 (4-guanidinobutanoate) (p<.001; bars represent s.e.m.). The second columns (dark gray) describe marginal BSn variance explained by metabolite adjusting for concurrent MA brain levels (partial R^2), with R^2_m remaining highly significant (p<.001). The third columns (medium gray) represents the metabolite partial R^2 adjusting for strain, which was nonsignificant for homocarnosine, pantothenate and 4-guanidinobutanoate (p>.05), but significant for myo-inositol (p<.001). Thus, estimates of the proportion of metabolite-BSn associations explained by strain were high (H^2=0.70–0.99) for all associations except myo-inositol (H^2=0.06), as shown in the final column (black) of each panel.

Table 1

Parameter estimates for metabolome-wide significant (q<0.05) sensitization-neurochemical associations

		Primary analysis				Sensitivity analysis			
						Adj acute		Spearman rank	
Sensitization outcome	**Neurochemical**	**q**	**p**	**beta**	**R^2**	**t**	**p**	**t**	**p**
Total distance	Ribulose	0.01	8.81E-06	0.55	0.30	5.72	1.26E-06	1.20	0.274
Total distance	Homocarnosine	0.01	2.25E-05	−0.50	0.25	−4.97	1.00E-05	−3.28	0.013
Margin time	Betaine	0.02	1.37E-04	0.45	0.20	4.62	4.09E-05	1.58	0.165
Stereotypy number	Isovalerylcarnitine	0.02	1.25E-04	−0.50	0.25	−6.24	1.39E-07	−2.53	0.040
Stereotypy number	Pantothenate	0.02	1.29E-04	0.47	0.22	3.83	3.95E-04	3.68	0.008
Stereotypy number	Glutarylcarnitine	0.04	2.39E-04	0.46	0.22	3.70	5.93E-04	2.33	0.053
Stereotypy number	N-acetylglutamate	0.05	3.53E-04	−0.25	0.06	−3.98	2.48E-04	−1.03	0.338
Stereotypy number	4-guanidinobutanoate	0.05	4.59E-04	−0.35	0.12	−3.90	3.19E-04	−3.15	0.016
Margin time	Myo-inositol	0.05	4.86E-04	−0.47	0.22	−2.78	7.93E-03	−2.76	0.028