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Differential peptidomics assessment of strain and age differences in mice in response to acute cocaine administration

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Abstract

Neurochemical differences in the hypothalamic-pituitary axis between individuals and between ages may contribute to differential susceptibility to cocaine abuse. This study measured peptide levels in the pituitary gland (Pit) and lateral hypothalamus (LH) in adolescent (age 30 days) and adult (age 65 days) mice from four standard inbred strains, FVB/NJ, DBA/2J, C57BL/6J, and BALB/cByJ, which have previously been characterized for acute locomotor responses to cocaine. Individual peptide profiles were analyzed using mass spectrometric profiling and principal component analysis. Sequences of assigned peptides were verified by tandem mass spectrometry. Principal component analysis classified all strains according to their distinct peptide profiles in Pit samples from adolescent mice, but not adults. Select pro-opiomelanocortin-derived peptides were significantly higher in adolescent BALB/cByJ and DBA/2J mice than in FVB/NJ or C57BL/6J mice. A subset of peptides in the LH, but not in the Pit, was altered by cocaine in adolescents. A 15 mg/kg dose of cocaine induced greater peptide alterations than a 30 mg/kg dose, particularly in FVB/NJ animals, with larger differences in adolescents than adults. Neuropeptides in the LH affected by acute cocaine administration included pro-opiomelanocortin-, myelin basic protein-, and glutamate transporter-derived peptides. The observed peptide differences could contribute to differential behavioral sensitivity to cocaine among strains and ages.

Keywords: adolescent, cocaine, label-free quantitation, matrix-assisted laser desorption ionization MS, peptidomics, principal component analysis.

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Recent studies of commonly abused drugs have focused on elucidating neurobiological differences between adolescent and adult responses to drug exposure. This is because drug use typically starts during adolescence (Chen and Kandel 1995; Nelson *et al.* 1995; Degenhardt *et al.* 2008) when the brain is still developing (Giedd *et al.* 1999), and adolescents respond differently to drugs as compared to adults in ways that appear to increase their risk for dependence (Laviola *et al.* 1999; Collins and Izenwasser 2002; Schramm-Sapyta *et al.* 2009; Zakharova *et al.* 2009). Cocaine use often begins in adolescence and can lead to long-lasting impacts on quality of life (Weiss *et al.* 2001; Lawrence *et al.* 2008; Koob and Volkow 2010). Cocaine also is one of the best-characterized drugs of abuse in terms of its neurological

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impact in animal models. Mouse models in particular have provided useful information due to the wide array of stable genotypes available for the study of the neurobiological features hypothesized to contribute to behavioral changes induced by drug exposure.

We recently documented significant differences in acute locomotor responses to cocaine in adolescent and adult mice across four common inbred mouse strains (Zombeck *et al.* 2010b). Acute locomotor responses are relevant as they provide a straightforward way to measure behavioral sensitivity to cocaine, and because these responses are correlated with other drug abuse-related behavioral measures such as conditioned place preference and self-administration (Allen *et al.* 2007; Mandt *et al.* 2008; Romanova *et al.* 2010). The underlying neurological differences between the strains and ages that explain the differences in acute locomotor responses to cocaine are not known. This study probes endogenous peptides in the pituitary gland (Pit) and lateral hypothalamus (LH) because peptides have been broadly implicated in mediating cocaine responses (Kreek 1996; Abul-Husn and Devi 2006; Borgland *et al.* 2006; Geng *et al.* 2006; Romanova *et al.* 2010; Haass-Koffler and Bartlett 2012; Rao *et al.* 2013). Using the same mice that were used in our earlier report of strain and age differences in acute locomotor responses to cocaine (Zombeck *et al.* 2010b), we explore whether peptide levels in the Pit and LH vary between genetically defined strains and animal age, as well as in response to acute cocaine exposure. These neuroendocrine regions were chosen for analysis as they each have a well-documented role in cocaine-responsive pathway modulation (Zhou *et al.* 2003, 2012; Aston-Jones *et al.* 2010), are accessible, and are morphologically distinguished from neighboring brain regions or tissues.

Label-free matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) was the method selected for executing this study. Due to its low sample volume requirements and excellent detection limits, MALDI MS is able to provide relative levels of peptides from rodent brain tissue samples that have not been pooled across multiple animals and/or brain regions (Li and Sweedler 2008; Miura *et al.* 2010; Uys *et al.* 2010; Romanova *et al.* 2013, 2014). Comparative MALDI MS-based screening of peptides, lipids, and metabolites is actively being explored as a potential method for a nearly universal high throughput, cost-effective, sensitive, and discriminative diagnostic tool for various cancers (Shin *et al.* 2007; Qiu *et al.* 2009; Shevchenko *et al.* 2010; Li *et al.* 2013) and other pathological conditions (Hachani *et al.* 2011; Ollero *et al.* 2011; Perez *et al.* 2014; Rico Santana *et al.* 2014), and even cognitive dysfunction (Zhang *et al.* 2012).

Given the high complexity of the peptide complement in brain tissue extracts, the multivariate statistical methods that measure relationships between many variables are useful for comparisons of spectral features. By comparing ions in the MALDI MS spectra from individual mice via principal component analysis (PCA), the dimensionality of the MS data can be reduced to reveal unique, trait-dependent trends in the detected peak patterns. PCA allows the extraction of functionally relevant information from the MALDI MS spectra, especially when multiple peaks have similar trends. When used with one-step extraction (Romanova *et al.* 2008), MALDI MS-based analyses are rapid and require minimal sample handling steps, thereby reducing bias due to sample handling and treatment. Compared with other '–omics' approaches, MALDI MS measures the final players in the biochemical mediation of cocaine responses, and offers potential insights into previously observed phenotypic differences between strains and ages (Yin *et al.* 2011). We hypothesized that cocaine exposure would lead to differences in the peptide profiles of the Pit and LH as measured by MALDI MS, and that those differences would vary in mice of different strains and ages. We also predicted that different strains and ages would have variable cocaine dose-sensitivity in their peptide responses.

Methods

Chemicals

All chemicals were purchased from Sigma Aldrich, St. Louis, MO, USA.

Animals and husbandry

A total of 96 male mice were used from four different standard inbred stains: C57BL/6J (C57BL), FVB/NJ (FVB), BALB/cByJ (BALB), and DBA/2J (DBA) (Jackson Laboratory, Bar Harbor, ME, USA). All four strains are listed as Tier 1 priority in the Mouse Phenome Database (Grubb *et al.* 2014) and were chosen because they are highly genetically divergent, each representing different branches of the phylogenetic tree for inbred mouse strains, as previously described (Rhodes *et al.* 2007). All mice were housed on a 12 : 12 reverse light/dark cycle (lights off at 7 AM and on at 7 PM) with the room temperature maintained at $21 \pm 1°C$. Mice had *ad libitum* access to food and water at all times.

The mice arrived in two different age groups: postnatal day 21 and 56. Mice were initially housed in groups of 3–4 for 5 days before being individually housed for an additional 4 days. Hence, adolescent mice were tested at postnatal day 30 and adults at day 65. These are commonly accepted ages to study adolescent and adult rodents (Spear and Brake 1983; Spear 2000).

On day 4, starting at 8 AM (1 h after lights off), all mice received a single intraperitoneal (ip) injection of 0.9% saline, and then were immediately returned to their home cage to acclimate to the injection. Exactly 1 h after the saline injection, starting at 9 AM (2 h after lights off), mice were administered another injection of either saline or cocaine (15 or 30 mg/kg), and then immediately returned to their cages. Animals were killed exactly 1 h later (details below). Doses were chosen based on previous studies that have shown reliable behavioral differences between age groups in C57BL males (Zombeck *et al.* 2009, 2010a,b). The behavioral data from

these same animals has already been published (Zombeck *et al.* 2010b). All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to both the NIH and ARRIVE guidelines. The Beckman Institute Animal Facility where the mice were housed is accredited by AAALAC International.

Tissue sampling and peptide extraction

Animals were anesthetized with 150 mg/kg sodium pentobarbital (ip), then perfused transcardially with ice-cold physiological saline solution to remove the blood. Brains were immediately removed and snap frozen in cold isopentane. Fresh pituitaries were collected by scooping them out of the skull following brain removal, and transferring directly into the extraction solution. Frozen brains were sectioned in a cryostat with 1 mm steps, and tissue was collected using a 1 mm biopsy punch from the region corresponding to the LH. Tissue samples from each animal were immersed in a $\geq 10 \times$ volume of aqueous DHB solution (DHB: 2,5-dihydroxybenzoic acid, 20 mg/mL) promptly after dissection and incubated at +4°C for 48 h to facilitate peptide extraction, as described elsewhere (Romanova *et al.* 2008).

MALDI–TOF MS analysis

Sampling for peptide profiling was performed as previously described (Romanova *et al.* 2008, 2010, 2012). Briefly, 0.7 μL of the DHB-treated tissue extracts were co-spotted 1 : 1 (v/v) with fresh concentrated DHB matrix (50 mg/mL 50% acetone), applied in triplicate onto a target sample plate, and analyzed using a Bruker ultrafleXtreme mass spectrometer with Smartbeam technology (Bruker Daltonics, Billerica, MA, USA) operated at 500 Hz in the positive reflectron mode. Sample measurement was randomized, and spectra in the m/z 700–5500 range were collected automatically using the AutoXecute™ software (Bruker Daltonics) with real-time fuzzy control logic, optimized for peptides. During acquisition, quadratic calibration constants were adjusted every 15–25 spots on a square area. Laser intensity was optimized separately for LH and Pit samples and maintained at a constant level during analysis. The number of laser shots was set to 1600 for Pit, 4000 for LH, and accumulated in 200- or 100-shot steps, respectively; laser movement was set to random walk over the entire sample area. Peak evaluation was based on a minimum resolution of 5000 and medium signal intensity for LH, and 7000 resolution/high-signal intensity for Pit, with the intensity being defined as high or medium by the Bruker software as the ratio of base peak height to the number of fired laser shots; 20 failed judgments within the sample area were required before moving to the next sample. At the end of the automatic run, failed spots were retested individually using the same instrument parameters.

Statistical analysis of peptide profiles

Comparisons of the peptide profiles were performed on the MS data in their original format using PCA and individual peak statistics functions (Student *t*-test or one-way ANOVA for normally distributed peak signal intensities determined by the Anderson-Darling normality test) in ClinProTools 2.2 (Bruker Daltonics). The Benjamini-Hochberg p-value adjustment procedure was automatically applied to correct for the multiple testing hypothesis problem commonly associated with the MS data. A total of 24 parameters (24 = 4

strains $\times$ 2 ages $\times$ 3 treatments) were paired in a number of different ways for numerous PCA iterations. Only distinct results are reported here. Upon loading files into ClinProTools and prior to PCA calculations, spectra were normalized to total ion count with a built-in algorithm. The automatic data pretreatment was completed using the following settings in ClinProTools: convex hull baseline correction with 0.5 flatness, mass filter m/z 800–4500, Savitsky-Golay smoothing over m/z 1.0 with four cycles, a data reduction factor of 2, null spectra exclusion, and spectra grouping from technical replicates into an average sample spectrum. Peak picking for PCA calculation (unlimited for LH and restricted to the 100 most intense peaks for Pit) was done on the group average spectrum with a signal-to-noise threshold of 6 and relative intensity of 1%. The number of peaks was restricted in the Pit samples due to overall higher signal intensity and the greater number of peaks detected from the Pit samples compared to the LH samples. Additionally, unsupervised hierarchical clustering was performed on PCA-transformed data (95% variance explained) using Euclidean distance and average linkage methods.

Peptide identification by liquid chromatography (LC)–MS

The workflow for LC-MS was the same as previously described (Romanova *et al.* 2012). After all tissue samples were processed individually for peptide extraction, and a small portion of each sample was used for high throughput semi-quantitative screening of peptides by MALDI-TOF MS, the larger leftover portions were combined according to strain and processed for peptide structural characterization using LC-tandem MS (MS/MS). The one-step extraction protocol (Romanova *et al.* 2008) used here is not directly compatible with LC due to excess DHB. Therefore, we initially removed the bulk of the DHB by solid phase extraction, as described previously (Romanova *et al.* 2010), and then used multistage high performance LC separation guided by off-line MALDI-TOF MS peptide scanning to choose peptide-containing fractions for further analysis.

We used two different LC-MS systems for our identification efforts. The first was a Dionex U3000 RSLC system with a capillary flow selector (Thermo Scientific, Sunnyvale, CA, USA) and an Acclaim PepMap RSLC column (Thermo Scientific, 300 μm × 150 mm, 2 μm, 100 Å) interfaced to a Bruker HCT Ultra PTM Discovery mass spectrometer via an electrospray ionization source (Bruker Daltonics). MS and MS/MS measurements were performed in the data-dependent acquisition mode with dynamic exclusion (top 4 ions, 2 spectra each, released after 1 min), a full scan mass range of m/z 300–2000, and a collision-induced dissociation scan at 35% energy. The second LC-MS/MS system was a Dionex U3000 split flow nanoLC (Thermo Scientific) with a sample pre-concentration set-up that included an Acclaim PepMap100 Nano-Trap column (200 μm × 2 cm, 5 μm, 300 Å) and an Acclaim PepMap RSLC column (75 μm × 150 mm, 2 μm, 100 Å) interfaced to a maXis 4G ultra high-resolution-QTOF mass spectrometer (Bruker Daltonics) via a Bruker on-line nanoESI source.

Fragmentation spectra obtained on both LC-MS platforms were charge-deconvoluted and exported as Mascot Generic Files (mgf) for identification via the International Protein Index (mouse 3.21) database search using Peaks Studio 5.3 (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Search parameters included a precursor mass tolerance of 0.5 Da for ion trap data and 0.1 Da

for QTOF data; a fragment ion mass tolerance of 0.5 Da and 0.1 Da, respectively; a maximum of three PTMs per peptide; and variable PTMs included amidation, oxidation, acetylation, pyroGlu formation from E or Q, and disulfide bonds. The database was downloaded from ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/ (Kersey *et al.* 2004). Peptides identified in the peptidomics experiments were back-referenced to the peptide peaks detected in the MALDI-TOF MS measurements based on their monoisotopic mass and accuracy of detection.

Results

Mouse strain differences under baseline (saline) conditions

Initially, we investigated peptide profiles from the extracts of the individual LH and Pit samples from control animals in each age and strain group to determine whether inbred strains have common detectable peptide complements at different life stages. A variety of known and putative signaling peptides were detected. In Pit samples, masses matching peptides encoded by pro-opiomelanocortin (POMC) and arginine vasopressin precursors were detected in all samples (Fig. 1). Detection of multiple known peptides resulting from proteolytic processing of the POMC prohormone adds confidence to our assignments, similar to the peptide fingerprinting of the trypsin-digested protein used for protein identification (Cottrell 1994). Peptide assignments were confirmed by a follow-up peptidomics approach on pooled samples, separately for the Pit and LH (Tables S1–S4).

The Pit profiles of adolescent mice were distinguishable by strain (Fig. 2a), with >60% of the variation accounted for by PC1 and >20% by PC2. As shown in Fig. 2c, among 54 peaks detected by MALDI-TOF MS, a subset of peaks matched to peptides sequenced in pooled samples fitted statistically in the 90% confidence interval, with two peptides having $p \leq 0.05$, thus demonstrating differential levels among mouse strains. The majority of differentially detected peptides were POMC-derived peptides. One interesting finding was the differently cleaved corticotropin-like intermediate peptide residues [141–162] flanked by basic residues on both termini. Traditionally, corticotropin-like intermediate peptide is thought to be cleaved at residues [142–162], and we did detect and confirm the sequence of this form in the Pit. However, only the alternate [141–162] form contributed to strain classification. Spectra classification by unsupervised hierarchical clustering was 100% correct for the BALB, DBA, and FVB strains (Fig. 2b). The data indicated that even a small population of adolescent mice (*n* = 16) can be classified by overall peptide profiles in the Pit using the multivariate statistical approach. Pit extracts from adult mice had more variability and hence could not be classified correctly using PCA or univariate sorting approaches. Recognition of adolescent and adult mice according to strain by peptide profiles in the LH was not achieved.
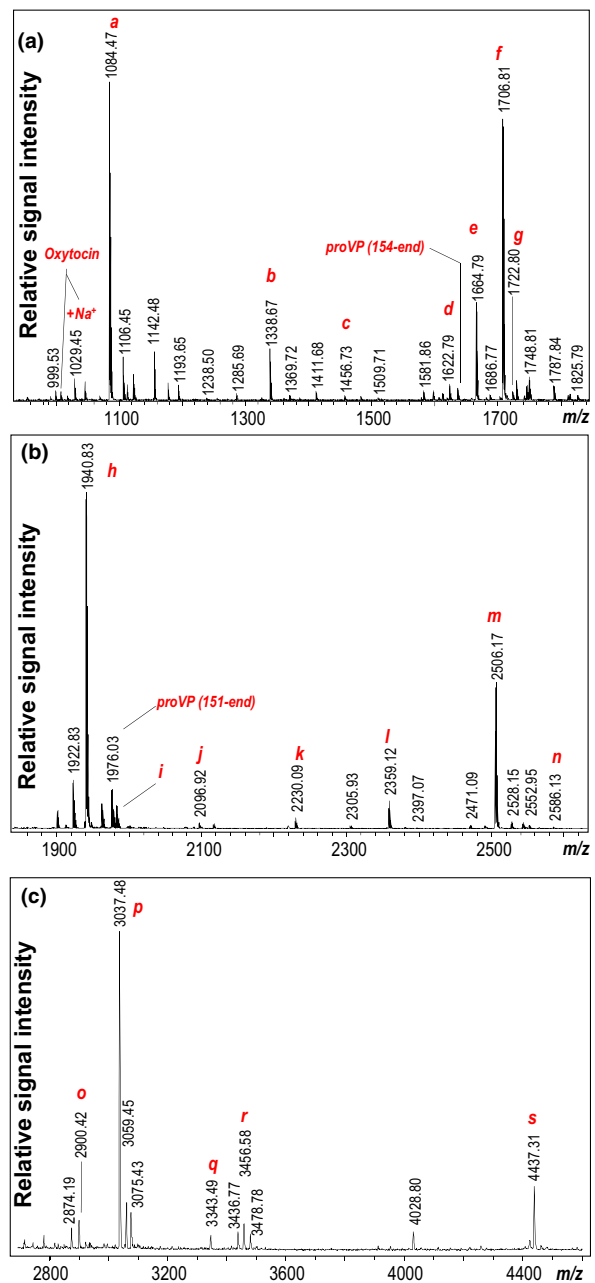

Fig. 1 Pituitary peptide profiles obtained using direct matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF) mass spectrometry (MS). Panels (a–c) show peptides detected in different mass regions. Known peptides are labeled based on mass match to the LC-MS sequencing data: a–(AVP); b–γ-MSH; c–di-Ac-α-MSH (3–13); d–α-MSH; e–Ac-α-MSH; f–di-Ac-α-MSH; h–J-peptide; i–Ac-J-peptide; j–J-peptide + N-terminal Arg; k–corticotropin-like intermediate peptide (CLIP) (1–20); l–CLIP (1–21); m–CLIP, n–phosphorylated CLIP; o–Ac-β-endorphin (1–26); p–Ac-β-endorphin (1–27), q–ACTH (1–28); r–γ-LPH (10–38); s–γ-LPH. Abbreviations: proVP, provasopressin-neurophysin 2 copeptin; AVP, arginine vasopressin; ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone; Ac, acetylated; CLIP, corticotropin-like intermediate peptide; and LPH, lipotropin. Labeled *m/z* correspond to monoisotopic masses of protonated molecular ions.
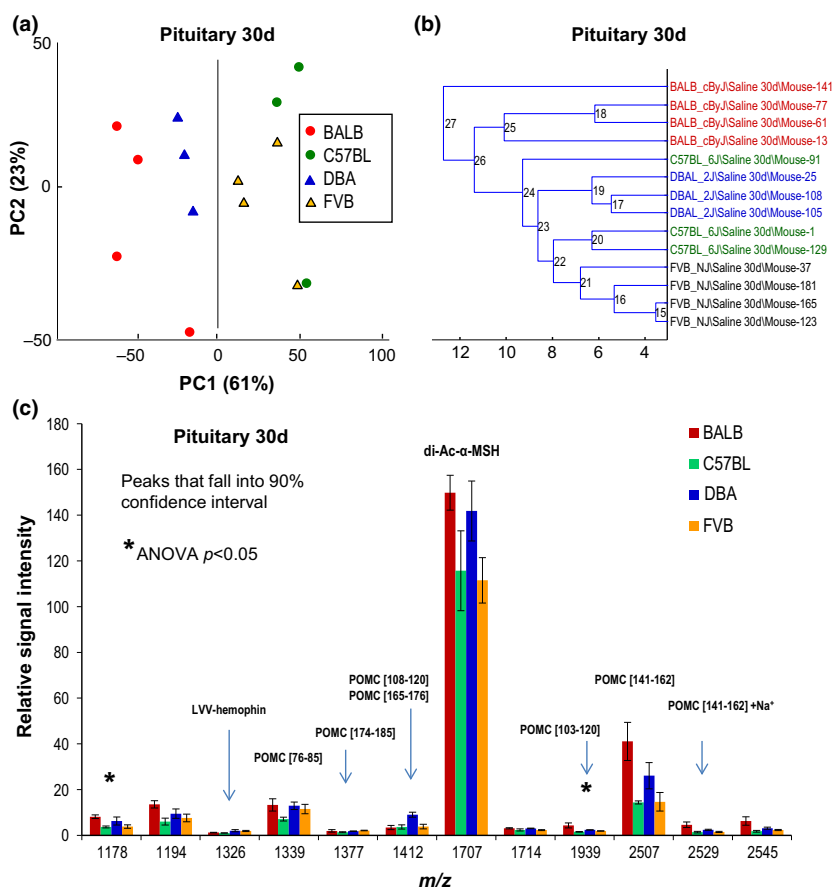
Fig. 2 Genotypes of adolescent mice can be distinguished according to their overall peptide profiles in pituitaries. (a) Principal component analysis (PCA) score plot for the first two principal components (PCs) with the variance described by each PC; (b) unsupervised hierarchical clustering of matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF) mass spectrometry (MS) spectra; (c) relative intensities of select peaks in Pit profiles that fall into the 90% confidence interval; known peptides are labeled. Mouse strains are shape and color coded; each data point represents one animal; DBA #153 and C57BL #49 mice were excluded due to sampling; *m/z* corresponds to maximum height at half width of the deisotoped centroid peak from the group mean spectrum.

Further, as shown in Fig. 3, using PCA pairwise comparisons of strains across ages revealed that DBA mice can be distinguished from FVB mice, regardless of age (Fig. 3a), due to statistically higher levels of select POMC peptides in the Pit (Fig. 3c). The peptide peak that matches masses of both the amidated POMC [108–120] and acetylated POMC [165–176] (Table S2) is higher in DBA adolescents (2.3 fold) and adults (2.8 fold) than in FVB mice, and POMC peptide [165–182] is higher at 1.5 and 1.8 fold, respectively. Likewise, FVB mice contrast from BALB mice, regardless of age (Fig. 3b), due to significantly lower levels of the unknown peptides *m/z* 1178, 1194, and 2545, and higher levels of *m/z* 2924, 2940, and 2986. The LH peptide profiles were not significantly distinct in either age group or when compared across age (data not shown).

Effect of acute cocaine on peptide dynamics in LH

Exposing mice to acute cocaine treatment resulted in measurable alterations of peptide levels in LH extracts, but not Pit. Speculating that the neurochemical response to cocaine is mediated by a fundamental mechanism that may be strong enough to override baseline phenotypic differences, we combined the LH spectra from all saline-treated adolescent mice and compared it against the combined spectra from cocaine-treated mice, in a dose-dependent manner, to see if these overall spectra show specific peptides that differentiate between control and treated adolescents. By PCA, from 226 detected peaks, a number of peptides contributed to the distinction (Fig. 4a) between the saline- and cocaine-treated groups, with the peptides contributing to these differences being peptides from myelin basic protein (MBP), claudin-11, excitatory amino acid transporter, and pyruvate dehydrogenase (Fig. 4b).

Effects of age and acute cocaine administration on peptide profiles in FVB mice

Of the four tested strains, male FVB mice showed exceptionally low locomotor responses to acute cocaine treatment, and no age differences or dose dependence in our behavioral study (Zombeck *et al.* 2010b). However, PCA analysis of peptide detection levels in the Pit and LH indicates dramatic dose-dependent changes among adolescent FVB mice that clearly differentiate cocaine-treated mice from those that received control injections (Fig. 5). The LH spectra, in particular, are well separated between the control and 15 mg/kg cocaine treatment groups by PC1 and PC2 (Fig. 5a), with the greatest difference being due to an increase in the level of peptides from MBP (1.8 fold), excitatory amino acid
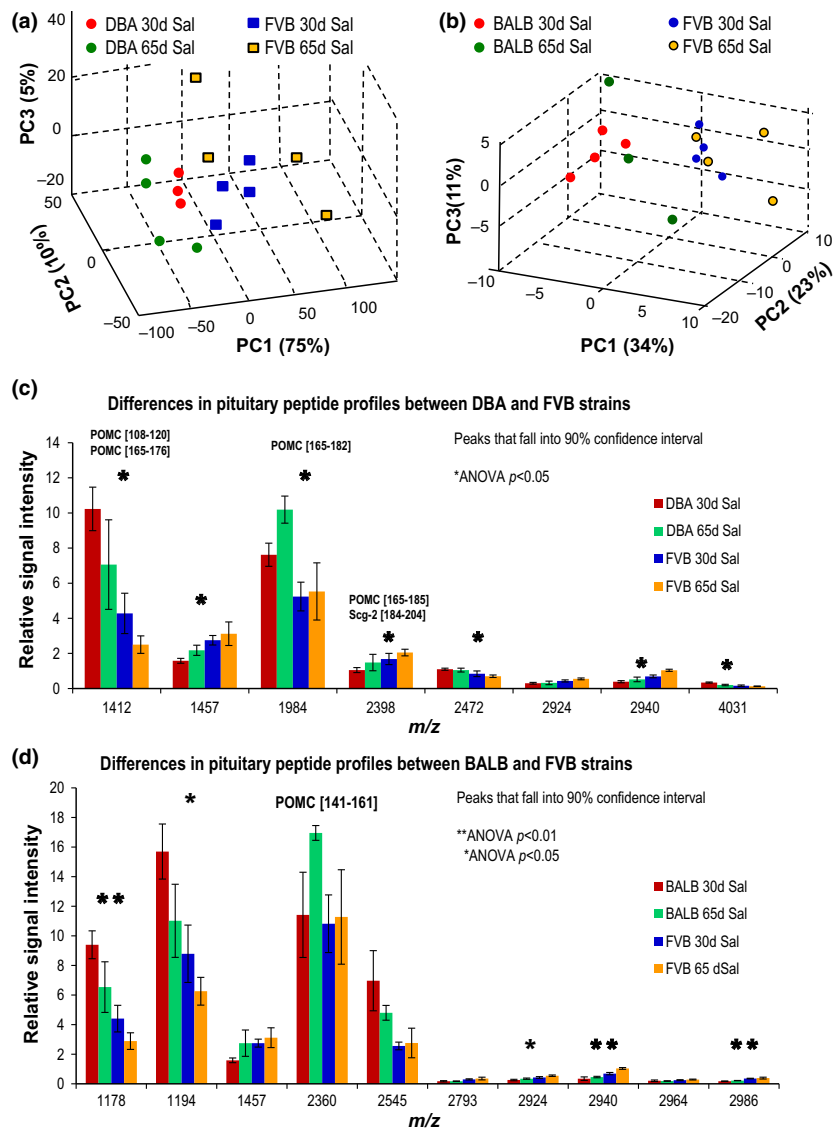
Fig. 3 The DBA and BALB strains can be distinguished from the FVB strain by their overall peptide profiles in Pit, independent of age. (a and b) Principal component analysis (PCA) plots for the first three principal components (PCs) are shown, along with the % of variance described by each PC; DBA mouse #153 was excluded due to sampling. (c and d) Intensities of peptides detected in Pit at significantly different levels between strains; *p*-value is for the Student's *t*-test; *m/z* corresponds to the maximum height at half width of the deisotoped centroid peak from the group mean spectrum as calculated by ClinProTools.

transporter (1.6 fold), and unknown peptide *m/z* 1478 (1.8 fold). It is interesting that the low dose cocaine effect was not found among adult FVB mice, where classification of low and high cocaine dose samples was 50% for either group, and 75% for the control saline samples (data not shown). Contrary to behavioral observations, these findings suggest that age differences in brain neuropeptide dynamics coincide with acute cocaine treatment in FVB mice. Cocaine-induced changes in the Pit were less prominent than in the LH, but levels of di-acetylated α-melanocyte-stimulating hormone from POMC increased 1.3 fold, and unidentified peptide *m/z* 1123 decreased significantly (Fig. 5b).

Diversity of peptides detected in the Pit and LH

The mouse Pit and LH have been well investigated using peptidomics approaches, and lists of peptides from known prohormones have been published (Che *et al.* 2006;

Romanova *et al.* 2008; Perroud *et al.* 2009; Sasaki *et al.* 2010; Nilsson *et al.* 2012). We performed our own sequencing to aid in the interpretation of the MALDI and PCA data because many of the detected masses were either not previously reported or could not be reliably assigned to a protein by the peptide mass fingerprinting approach alone. We also wanted to provide consistent results across the strains used here, as several of them have been more extensively studied in the past than others. The peptides we identified in the mouse Pit and LH are presented in the Supporting Information (Tables S1–S4).

Discussion

Phenotypic assessment of inbred mouse strains has recently become of interest in light of variable drug-induced behaviors among numerous strains used in
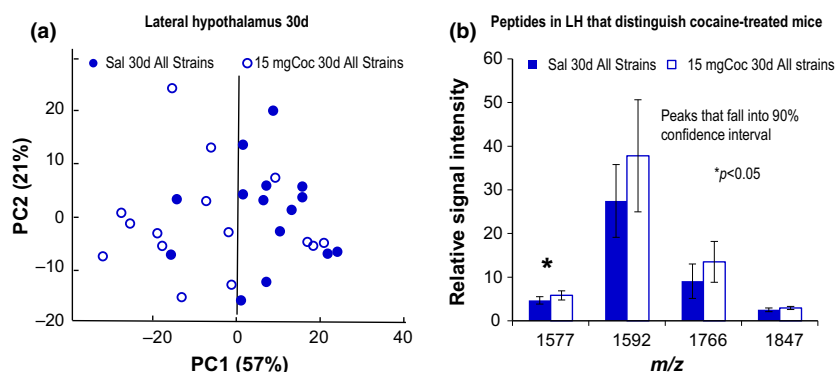
Fig. 4 (a) Principal component analysis (PCA) plot showing that the effect of acute cocaine exposure on peptide profiles in the LH is similar in adolescent mice from all four strains. Data points are color coded according to treatment. (b) Intensities of peptides that allow distinction of peptide profiles between control and cocaine-treated (15 mg/kg) adolescent mice; *m/z* 1577, excitatory amino acid transporter 1; *m/z* 1592, myelin basic protein (MBP) (*m/z* corresponds to the maximum height at half width of the deisotoped centroid peak from the group mean spectrum as calculated by ClinProTools).
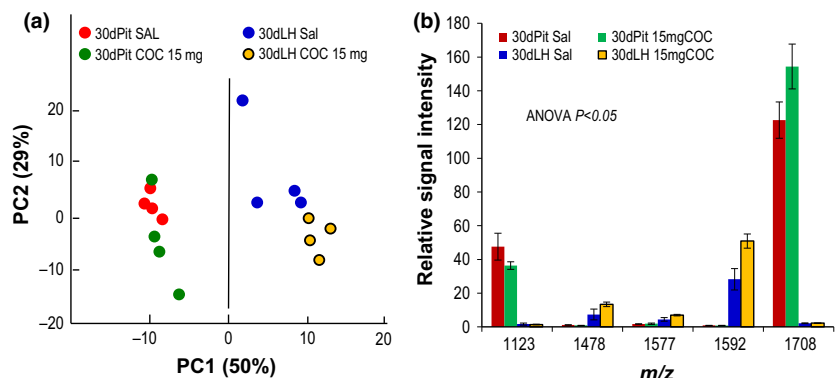

Fig. 5 Cocaine induces peptide changes in the Pit and LH of adolescent FVB mice. (a) Principal component analysis (PCA) plot for the first two principal components (PCs) showing segregation of spectra by sample type and treatment according to peptide profile features. (b) Relative intensities of peptides detected at significantly different levels as determined by ANOVA; known peptides are: *m/z* 1577, excitatory amino acid transporter 1; *m/z* 1592, myelin basic protein (MBP); *m/z* 1708, di-Ac-α- melanocyte-stimulating hormone (*m/z* corresponds to maximum height at half width of the deisotoped centroid peak from the group mean spectrum as calculated by ClinProTools); remaining *m/z* are not identified.

addiction studies (Zombeck *et al.* 2010b; Eisener-Dorman *et al.* 2011; Thomsen and Caine 2011; Wiltshire *et al.* 2015). With the exception of a few biochemical investigations, (Brodkin *et al.* 1998; McCarthy *et al.* 2004; Adkins *et al.* 2013), the focus had been placed on genetic and behavioral differences between strains. We sought to determine biochemical differences in the hypothalamic-pituitary axis relevant to drug responsiveness in the strains we studied. We show that just as in rats (Romanova *et al.* 2010), acute cocaine induces detectable changes in the peptide profiles of mice, and that some changes are common to all strains, whereas others are unique to particular strains. Neuropeptides and hormones play important roles in modulating neural circuits that control behavior. Therefore, an understanding of how genetic differences between strains affect peptide concentrations in

tissues could provide an important missing link between the genetics and the complex behavioral phenotypes. The genotypes of standard inbred strains are reproducible, hence, genetic (e.g., DNA sequence, polymorphisms) and phenotypic data are cumulative. In addition to many gross morphological, physiological, and behavioral traits, data are also available for gene expression profiles in particular tissues in response to various treatments (Grubb *et al.* 2014). This information can be used to identify causal connections between structural DNA variants (i.e., alleles) and phenotypic traits (Grupe *et al.* 2001). Notably missing from the data, however, is peptide information from various tissues in response to treatments that connect the gene expression differences with behavioral patterns. This work offers a robust way to perform peptidomic analysis for comparisons between strains.

Differences in peptide profiles are more prominent in adolescent mice than adults

A surprising finding was that the peptide profiles of adolescents were easier to classify based on strain and cocaine dose than adults. Prominent peptide differences among genotypes found in adolescent mice allowed strain classification according to overall baseline peptide profiles in the Pit, as well as according to peptide profile alterations in the LH upon cocaine treatment. Moreover, in one of the strains, FVB, multiple peptides from POMC in the LH of adolescent mice provided the basis for dose-dependent differentiation between control and cocaine-treated mice. It proved more challenging, however, to discriminate between the genotypes in adult mice, where only the DBA and FVB strains, compared against each other, could be distinguished by overall peptide profiles in the Pit and LH. One possible explanation is that adolescent mice of different strains develop at different rates and thus, peptide profiles in their brains and hypothalamic-pituitary axis are distinct at a certain age during development, but converge to the same endpoint as adults. An alternative possibility is that as the mice age, they develop more idiosyncratic differences in peptide biochemistry related to the accrual of subtle individual-specific experiences. With time, these subtle differences may accumulate, causing individuals within a genotype to diverge, and effectively increasing the individual within-strain variation for the trait.

Mouse strain differences in Pit peptide profiles

Previous work using radioimmunoassay analysis showed that the contents of β-endorphin and adrenocorticotropic hormone in the Pit vary among inbred strains of mice. For example, DBA and BALB mice β-endorphin values are 2.5-fold lower, and adrenocorticotropic hormone values are 1.5-fold lower, than in C57BL mice (Crabbe et al. 1981). Based on this information, one might expect other peptides encoded by POMC prohormone to be differentially expressed among strains. Our data do show significant differences in levels for specific POMC-related peptides, although differences among adolescent mice are more prominent than adult mice under baseline conditions. One possible explanation is that the circadian dynamics of the hypothalamic-pituitary axis show individual, age, and strain differences, but are hard to account for during tissue sampling when working with multiple genetically diverse strains. Another possible explanation is that our small sample size represented in each group/condition ($n = 4$) was not sufficient to overcome individual variation in peptide signal intensity among mice. The data demonstrate that a straight-forward relationship between a specific peptide level and a genotype may not be obvious, and that a multivariate analytical approach such as PCA is better suited for discriminating complex traits because it is simultaneously controlling for the effects of each of the predictors (peptides) on the entire profile. In the future, it would be interesting to see if linear discriminant analysis would be helpful in overcoming the variability within each compared group and hence, document the peaks that allow for classification of mouse strains by neuroendocrine peptide profiles. Although differences in peptide profiles were observed in the Pit, strain-specific differences from the LH were not observed. This may be because the LH has lower levels of peptides and so it is easier to observe subtle changes in peptide levels in the Pit due to genetic diversity between strains.

Consistent peptide responses to acute cocaine in the LH of adolescents across 4 genotypes

Acute cocaine dosage appears to disrupt multiple signaling pathways in the LH of adolescent mice in a similar fashion, irrespective of strain. In our study, animals were killed exactly 1 h following an injection of saline or 15 or 30 mg/kg cocaine. The peptide response to cocaine is almost certainly a dynamic process, with certain peptides increasing in concentration and then falling in a tissue-specific manner at different time courses following exposure to the drug. Therefore, the peptide response to cocaine characterized in our study must be considered a snapshot that captured only changes that occurred within the first hour following acute cocaine exposure.

The 1 h period was chosen because this matches, at least approximately, the duration of the psychoactive effects of cocaine as measured, for example, by changes in locomotor activity. One hour is enough time for some genes to become expressed and translated, but perhaps not others. Peptides that manifest changes are derived from glutamate transporter 1, MBP, claudin-11, and pyruvate dehydrogenase. Previously, we identified via MALDI that MBP isoform 9 changed in cocaine-treated rats as compared to controls (Romanova et al. 2010, 2012; Uys et al. 2010). Alterations in myelin regulation were identified in postmortem samples of the human nucleus accumbens in cocaine users, when compared to controls, using Affymetrix microarray technology to measure gene expression on a global scale (Albertson et al. 2004). Endorphin, enkephalin, and corticotropin (derivatives of POMC) appear to play important roles in an animal's response to cocaine exposure, as well as in reward and aversion behaviors associated with cocaine. Previous studies have identified changes in POMC derivatives in response to acute cocaine administration (DuMars et al. 1988; Forman and Estilow 1988; Sarnyai et al. 1992, 1993; Zhou et al. 1996, 2002, 2003; Olive et al. 2001; Crespo et al. 2003; Roth-Deri et al. 2003, 2004; Marquez et al. 2008).

Changes in the abundance of peptides in our study may reflect altered gene expression, translation from already expressed genes, degradation of peptides within cells, or release of peptides into extracellular spaces followed by rapid degradation. Although this work only represents a snapshot of the peptides in several defined brain locations, given the

high throughput capability of the peptidomic analysis using MALDI MS, it is feasible to characterize a time-course for peptide responses in specific brain regions of interest using a series of animals in the future.

Correlations with locomotor behavioral sensitivity to cocaine

The animals in this study were also measured for acute locomotor responses to cocaine during the 1 h period after injection, and before euthanasia for tissue extractions. In our prior report of the behavior of these animals (Zombeck *et al.* 2010b), the main results were greater locomotor responses to cocaine in C57BL, followed by DBA, BALB, then FVB, and much greater locomotor responses to the 30 mg/kg dose than the 15 mg/kg dose across strains. BALB and C57BL adolescents were less sensitive than adults to the acute locomotor activating effects of cocaine. In DBA and FVB, adolescents and adults displayed similar sensitivity to cocaine. When combined with the results from the present study, these data suggest that locomotor responses to acute cocaine are inversely related to changes in peptide profiles in the LH. This conclusion comes from the following three pieces of evidence. First, adolescents displayed a stronger peptide signal from cocaine than adults, but a weaker locomotor response. Second, the low dose of cocaine elicited a greater peptide response than the high dose, but a weaker locomotor response. Third, the FVB displayed the least sensitivity to the acute locomotor activating effects of cocaine of all four strains, yet displayed the most robust peptide response in the LH to cocaine. One interpretation of the inverse relationship is that changes in peptide profiles in the LH in response to cocaine represent plasticity or tolerance-related mechanisms that enable the animal to compensate for the chemical perturbation and maintain homeostasis, despite the dramatic change in neurochemistry caused by the acute cocaine treatment. This interpretation posits that a larger peptide change represents greater plasticity, resulting in increased tolerance and a less overt behavioral response.

Conclusions

Proteins and peptides are widely considered critical molecules that affect most aspects of brain function and behavior (Strand 1999; Kandel *et al.* 2012). Genomics approaches are often used as proxies for measuring proteins and peptides, and it is assumed that protein levels and gene expression levels are correlated when often, in practice, they are not (Vogel and Marcotte 2012). Peptidomics is more complex than genomics because one gene transcript can produce many peptides or proteins, and each product can be heavily altered by post-translational modifications. Our study demonstrates the feasibility of using MALDI MS to reliably measure peptidome dynamics in a specific brain region and an

endocrine gland of genetically defined, standard inbred mouse strains at different ages and in response to cocaine. With larger studies, both in terms of number of animals and strains, it may be possible to combine peptide data with gene expression data and other phenotypic information that has been accumulated for inbred strains. Together, this information may be able to explain more of the variation in acute locomotor responses to cocaine (or any other phenotype of interest that has been measured in the same panel of inbred strains). We anticipate that future studies will be able to leverage the methods described herein to further advance our understanding of the neurochemical changes associated with individual behavioral variation.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Identification of arginine-vasopressin and oxytocin neuropeptides in the mouse pituitary.

Table S1. The proteins identified in the mouse Pit.

Table S2. Peptides identified in the mouse Pit.

Table S3. Proteins identified in the mouse LH.

Table S4. Peptides identified in the mouse LH.

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