HHS Public Access

Author manuscript

J Neurochem. Author manuscript; available in PMC 2018 November 01.

Published in final edited form as:

J Neurochem. 2017 November ; 143(3): 268–281. doi:10.1111/jnc.14209.

ProSAAS-derived peptides are regulated by cocaine and are required for sensitization to the locomotor effects of cocaine

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Abstract

To identify neuropeptides that are regulated by cocaine, we used a quantitative peptidomic technique to examine the relative levels of neuropeptides in several regions of mouse brain following daily intraperitoneal administration of 10 mg/kg cocaine or saline for seven days. A total of 102 distinct peptides were identified in one or more of the following brain regions: nucleus accumbens, caudate putamen, frontal cortex, and ventral tegmental area. None of the peptides detected in the caudate putamen or frontal cortex were altered by cocaine administration. Three peptides in the nucleus accumbens and seven peptides in the ventral tegmental area were significantly decreased in cocaine-treated mice. Five of these ten peptides are derived from proSAAS, a secretory pathway protein and neuropeptide precursor. To investigate whether proSAAS peptides contribute to the physiological effects of psychostimulants, we examined acute responses to cocaine and amphetamine in the open field with wild-type (WT) and proSAAS knockout (KO) mice. Locomotion was stimulated more robustly in the WT compared to mutant mice for both psychostimulants. Behavioral sensitization to amphetamine was not maintained in

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CONFLICTS OF INTERESTS

The authors declare that they have no personal, commercial, or other conflicts of interests.

proSAAS KO mice and these mutants failed to sensitize to cocaine. To determine whether the rewarding effects of cocaine were altered, mice were tested in conditioned place preference (CPP). Both WT and proSAAS KO mice showed dose-dependent CPP to cocaine that was not distinguished by genotype. Taken together, these results suggest that proSAAS-derived peptides contribute differentially to the behavioral sensitization to psychostimulants, while the rewarding effects of cocaine appear intact in mice lacking proSAAS.

Keywords

Neuropeptide; peptidomics; proteomics; drug abuse

INTRODUCTION

Peptides play important roles in cell-cell signaling and are involved in many physiological processes, including feeding, body weight regulation, memory, pain, reproduction, anxiety, depression, and drug addiction (Strand 2003). Previous studies investigating the regulation of neuropeptides by drugs of abuse used radioimmunoassays for analyzing peptide levels. While this approach is useful for the detection of peptides for which highly specific antisera are available, these techniques are limited to known peptides and require a separate assay for each peptide. Peptidomic techniques have allowed the detection, quantification, and identification of hundreds of peptides in a single sample (Baggerman *et al.* 2004, Fricker *et al.* 2006, Schoofs & Baggerman 2003, Svensson *et al.* 2003, Hummon *et al.* 2006). These techniques permit the detection of a wide variety of peptides in the mouse brain, including known neuropeptides, novel forms of peptides from known neuropeptide precursors, and novel peptides from previously unreported precursors (Fricker 2010). An example of the latter category is the novel precursor named proSAAS (Fricker *et al.* 2000), which was found to be processed into a number of different peptides in mouse brain, including big and little SAAS, GAV, PEN, C-terminally truncated forms of PEN (such as PEN-20), as well as big and little LEN (note that these are names, not abbreviations).

ProSAAS was originally identified as an inhibitor of prohormone convertase 1 (also known as prohormone convertase 3) (Fricker et al. 2000). Because only proSAAS and some of its processing intermediates are able to inhibit prohormone convertase 1/3, and not any of the final products of proSAAS processing, it is likely that these peptides have alternative functions (Basak *et al.* 2001, Qian *et al.* 2000, Cameron *et al.* 2000). ProSAAS has been overexpressed in transgenic mice and the animals had elevated body weights, while mice lacking proSAAS-derived peptides showed decreased body weights (Wei *et al.* 2004, Morgan *et al.* 2010). Neither adult transgenic nor the proSAAS KO mice showed altered peptide processing, suggesting that the altered body weight was independent of the action of proSAAS on prohormone convertase 1/3. ProSAAS-derived peptides are secreted from cells (Fricker *et al.* 2000) and exert biological effects in brain (Hatcher *et al.* 2008) (Wardman *et al.* 2011). Recently, big LEN was found to activate the G protein-coupled receptor GPR171 and PEN was found to activate GPR83 (Gomes *et al.* 2013, Gomes *et al.* 2016). Thus, proSAAS-derived peptides are functional neuropeptides.

In the present study, we used a quantitative peptidomics approach to examine the effect of cocaine administration on peptide levels in various regions of the mouse brain. These brain regions include those known to be involved in reward pathways, such as the nucleus accumbens (NAc), ventral tegmental area (VTA), caudate putamen (CPU), and frontal cortex (FC). Cocaine has been shown to affect the expression of a number of neuropeptides or peptide precursors, including substance P (Adams *et al.* 2001, Arroyo *et al.* 2000, Hanson *et al.* 1989, Hurd & Herkenham 1992), dynorphin (Hurd & Herkenham 1992, Shippenberg *et al.* 2007, Werme *et al.* 2000), nociceptin/orphanin FQ (Romualdi *et al.* 2007, Lutfy *et al.* 2008), neurotensin/neuromedin N (Adams et al. 2001), thyrotropin-releasing hormone (Sevarino & Primus 1993, Eugene *et al.* 2002), secretogranin II (Kuzmin & Johansson 1999), neuropeptide Y (Wahlestedt *et al.* 1991, Westwood & Hanson 1999), vasopressin, oxytocin (Sarnyai *et al.* 1992), and corticotropin-releasing factor (Maj *et al.* 2003).

A previous peptidomic analysis examined the effects of cocaine treatment on brain peptide levels using $Cpe^{fat/fat}$ mice (Che *et al.* 2006) that lack carboxypeptidase E activity due to a naturally-occurring point mutation in this gene (Naggert *et al.* 1995). This peptidomics analysis found that several peptide-processing intermediates were altered by twice-daily injections of 10 mg/kg cocaine for 5 consecutive days (Che et al. 2006). However, because this study used $Cpe^{fat/fat}$ mice, it examined processing intermediates containing C-terminal basic residues rather than the fully mature peptides (Fricker & Leiter 1999). For this purpose, it was important to examine wild-type (WT) mice to assess the effect of cocaine treatment on the mature forms of these peptides in this study.

The present peptidomics analysis in WT mice revealed that cocaine decreased the levels of several proSAAS-derived peptides in the NAc and/or VTA. To evaluate a possible role for proSAAS-derived peptides in cocaine-mediated behaviors, we assessed psychostimulant induced locomotor activity, behavioral sensitization to psychostimulants in the open field, and cocaine-induced conditioned place preference (CPP) in WT and proSAAS KO mice. These analyses revealed that proSAAS KO mice show blunted acute responses to cocaine- and amphetamine (AMPH) in the open field, an absence of behavioral sensitization to cocaine, an inability to maintain sensitization to AMPH, and normal responses to the rewarding properties of cocaine in CPP.

MATERIALS AND METHODS

Peptidomics Study

Mice (C57BL/6J; RRID:IMSR_JAX:000664) were purchased from The Jackson Laboratory (Bar Harbor, ME) and group-housed in the Albert Einstein College of Medicine barrier facility on a 12:12 hr light:dark cycle. The animal studies conducted at Einstein were approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine. A total of 30 male mice, aged 9–11 weeks, were used in the experiment and were arbitrarily divided into two treatment groups: cocaine versus saline. Fifteen mice were administered daily injections of cocaine (10 mg/kg, i.p.) dissolved in 0.9% isotonic saline, while the other fifteen mice were given the same volume of saline alone. Following 7 consecutive days of injections, mice were euthanized by decapitation 1 hour after the last injection and the heads were immediately microwave-irradiated to raise the tissue

temperature to 80°C, which has been previously shown to stabilize peptides by inactivating tissue proteases (Che *et al.* 2005). The CPU, NAc, FC, and VTA were dissected and frozen on dry ice until further analysis. Tissue samples were pooled from 3–4 mice in each treatment group, resulting in 4 pools of cocaine-treated and 4 pools of saline-treated tissue, for each of the 4 brain regions, for a total of 32 tissue pools. The number of mice per pool (3–4) and the number of biological replicates (4 pools of tissue for each treatment and brain region) was based on previous studies using the peptidomics technique to detect statistically significant changes in mouse brain regions (Wardman *et al.* 2010, Zhang *et al.* 2010); it was not based on power analysis. Peptides were extracted from each of the 32 tissue pools as previously described (Che *et al.* 2007). Briefly, tissue was sonicated with 50 pulses at 1 pulse/sec in ice-cold water using an ultrasonic processor (W-380, Ultrasonic Inc., Farmingdale, NY). The homogenate was incubated in a 70°C water bath for 20 min, cooled in an ice bath, acidified with ice-cold HCl (final HCl concentration of 10 mM), and centrifuged at 13,000 × g for 30 min at 4°C. The supernatant (peptide extract) was removed and stored at −70°C until labeling.

Peptides were isotopically labeled with the N-hydroxysuccinimide (NHS) ester of D0 or D9-trimethylammonium butyric acid (TMAB) following the scheme shown in Figure S1. Note that for runs 1 and 3, the peptides extracted from cocaine-treated mice were labeled with D0-TMAB and the peptides from saline-treated mice were labeled with D9-TMAB, while the cocaine- and saline-treated samples for runs 2 and 4 were labeled in the converse (Figure S1). This approach provided a control for potential variations in the reactivity of the isotopic labels. The isotopic labeling was conducted as described (Morano *et al.* 2008). In brief, the extracts were neutralized to pH 9.5 with 75 mM phosphate buffer and the addition of 1 M NaOH. D0- or D9-TMAB-NHS dissolved in DMSO was added separately to the peptide extracts. After 10 min, an appropriate volume of 1.0 M NaOH was added to adjust the pH back to 9.5 and the tubes were incubated for 10 min at room temperature. The addition of TMAB and NaOH was repeated seven times. After the last addition, the mixtures were incubated at room temperature for 1 hr after which 2.5 M glycine was added to quench the remaining TMAB reagents. D0- and D9-labeled samples were pooled as indicated in Figure S1, resulting in a total of 16 samples (four for each of four brain regions). After pooling, peptides were purified by microfiltration through a 10 kDa membrane (Microcon® YM-10 unit; EMD Millipore, Billerica, MA). To remove any labels from Tyr residues in the peptides, the pH of filtrate was adjusted to 9.0 and 2.0 M hydroxylamine (in DMSO) was added in three aliquots, with an interval of 10 min between each addition. Following the reaction, peptides were desalted using a PepCleanTM C-18 spin column (ThermoFisher Scientific). Peptides were eluted from the column with 70% acetonitrile in 0.1% trifluoroacetic acid in water, frozen, concentrated in a vacuum centrifuge, and stored at −70°C until further analysis.

The 16 pools of labeled and purified peptides were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described (Morano et al. 2008), where solvent A was 2% acetonitrile in 0.1% formic acid and solvent B was 80% acetonitrile in 0.1% formic acid. In brief, the peptide mixture was trapped and washed on a PepMapTM C18 trapping column (5 μm, 100Å, 300 μm i.d. × 5 mm; LC Packing, Marlton, NJ) using a Eldex MicroProTM Syringe Pumping System with 5% solvent B for 20 min at a flow rate of 4 μL/min. Peptides

were separated on a Grace Vydac MS C18 capillary column (3 μm, 100Å, 75 μm i.d. × 150 mm, Hesperia, CA) at 4 μL/min with a gradient elution of 5% solvent B for 45 min, which then increased to 35% solvent B in 40 min, and to 95% solvent B in the remaining 20 min. Flow from the column was directed to an API Q-Star Pulsar-iTM quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The nano-electrospray ion source was operated in the positive mode with a spray voltage of 2 kV. The information-dependent acquisition mode was used with a 1-sec survey scan and 2-sec MS/MS scan on the most intense ions in the MS survey scans. The collision energy for MS/MS (20–45 eV) was dynamically selected based on the m/z value and charge state of the ion selected.

Quantification of peptides was performed by measurement of peak intensity and expressed as a ratio of the peak for the cocaine-treated sample versus the peak for the saline-treated sample. Peptide identification was based on tandem mass spectrometry (MS/MS). The Mascot program (http://www.matrix-science/com) was used to search the NCBInr database (*mus musculus*) with variable modifications of the N-termini and Lys residues with GIST-Quat and GIST-Quat:2(H)9 (Mascot names for D0- and D9-TMAB labels). Additional variable modifications allowed in the searches included C-terminal amidation, N-terminal acetylation, methionine oxidation, and Ser/Thr phosphorylation. To reduce the number of false-positives, all search results were inspected manually using previously described criteria (Morano et al. 2008). Briefly: (i) the observed parent mass was within 50 ppm of the theoretical mass; (ii) the observed number of TMAB tags on the peptide matched with the predicted number of free amines available (i.e., Lys residue and N-terminus); (iii) the observed charge state of the peptide matched the expected number of positive charges; (iv) 80% or more of the major fragments observed in MS/MS matched predicted fragments (minimum of five matches). Furthermore, the observed isotopic tag(s) incorporated into the peptide (i.e., D0- vs D9) had to match the one identified by Mascot. Finally, we also considered whether the identified peptide was derived from a protein known to be expressed in mouse brain, based on the previous literature as well as bioinformatic analyses of databases (Unigene, Allen Brain Atlas, and NCBI). No animals were excluded from the peptidomics analysis.

Animals in the Behavioral Tests

The proSAAS mice were backcrossed onto the C57BL/6J stain for more than 10 generations. Adult male and female WT and proSAAS KO mice were generated by mating heterozygous females with WT or KO males for these experiments. All mice were housed 3–5/cage in a temperature- and humidity-controlled room on a 14:10 hr (lights on at 06:00 hr) light:dark cycle with food and water provided *ad libitum*. All behavioral experiments were conducted between 10:00 and 14:00 hr with approved protocols from the Penn State University College of Medicine and the Duke University Institutional Animal Care and Use Committees. The number of mice used in each experiment are reported in the figure legends. For the behavioral experiments, sample sizes of 8–12 mice per group was determined using Shapiro-Wilk or Kolmogorov-Smirnov tests in the SPSS program. Both male and female mice were used in the behavioral studies and this variable was collapsed because no significant sex differences emerged. Since behaviors were assessed by computer programs

(described below), there was no need to blind the investigators as to the genotype or treatment paradigm. For the studies in which mice were treated with either saline or a drug, the mice were arbitrarily divided between treatment groups. No animals were excluded from the behavioral analysis.

Cocaine or Amphetamine Stimulated Hyperlocomotion

Psychostimulant-induced locomotion following cocaine or amphetamine (AMPH) administration was tested in the open field arena (Omnitech, Columbus, OH) illuminated at 340 lux as described (Deng *et al.* 2010). Mice were placed individually into the open field and baseline locomotion was monitored over 60 min. Animals were removed, injected (i.p.) with vehicle (sterile water, 4 ml/kg; Hospira Inc., Lake Forest IL), or 10, 20 or 30 mg/kg cocaine (Sigma-Aldrich, St. Louis, MO), or 2 or 3 mg/kg AMPH (Sigma-Aldrich), and returned immediately to the open field for 120 min.

For behavioral sensitization, mice were administered 20 mg/kg cocaine or 3 mg/kg AMPH for 5 consecutive days using the same procedure as described above. On day 11, mice were challenged with the same doses of cocaine or AMPH. All results are presented as cumulative distance traveled in 5-min blocks or as cumulative locomotion (pre- and post-injection).

Cocaine CPP

CPP was assessed using three-chambered CPP apparatuses (Med Associates, St. Albans, VT) as described (Deng et al. 2010). The locations of the mice and the durations of time spent in each chamber were monitored by infrared diodes using MedPCIV software (Med Associates).

WT and proSAAS KO mice were arbitrarily assigned to the treatment groups: 10, 20, or 30 mg/kg cocaine (Sigma-Aldrich; NIDA Drug Supply, Bethesda, MD). Habituation to the entire apparatus was performed for 30 min on 3 consecutive days to ensure that none of the mice displayed a preference for one chamber over the other. Conditioning consisted of three 2-day conditioning cycles of alternating vehicle and cocaine injections. One-half of the mice received their assigned dose of cocaine, while the other half were given vehicle. Additionally, one-half of each of these groups had the vehicle or cocaine paired with the white chamber, whereas the other half had the vehicle or cocaine paired with the black chamber. Following injection, mice were placed immediately into their assigned chamber for 30 min. The next day, mice were given an injection of the other agent and placed into the other chamber. This cycle of conditioning was repeated over 6 days. Forty-eight hours later, mice were tested for CPP with free access to both chambers of the apparatus for 30 min. CPP was expressed as a preference score (time in the cocaine chamber – total time in vehicle chamber / total time in both chambers) for the vehicle- and cocaine-conditioned chambers at baseline (habituation) and on the challenge day. Scores approaching “0” indicated no preference for either chamber, positive scores signified a preference for the cocaine-conditioned chamber, and negative scores denoted a preference for the vehicle-conditioned chamber. For calculating baseline preference, the chamber where cocaine would be administered on the animal’s first conditioning day was designated as the cocaine-conditioned chamber.

Statistical Analyses

Statistical analyses were performed with the SPSS-21 statistical programs (IBM SPSS Inc., Chicago, IL, USA) and results were presented as means and standard errors of the mean. Omnibus repeated-measures analysis of variance (RMANOVA) within- and between-subject tests evaluated open field locomotion during baseline (0–60 min) and following acute vehicle or psychostimulant administration (61–180 min). To examine within genotype effects in more detail, separate RMANOVAs were run for WT and proSAAS KO mice. Finally, cumulative post-injection locomotor activities were analyzed by two-way ANOVA. For behavioral sensitization to cocaine and AMPH, separate omnibus RMANOVAs analyzed baseline (0–60 min) and psychostimulated locomotion (61–180 min). Cumulative baseline and stimulated locomotor activities were analyzed by RMANOVA. For CPP, the within subjects effects of the RMANOVA included the baseline and challenge days for preference scores. The between subjects effects included genotype and cocaine dose (10, 20 and 30 mg/kg). In all cases, Bonferroni corrected pair-wise comparisons were used as the *aposteriori* tests and a $p<0.05$ was considered significant.

RESULTS

The peptidomics analyses compared 4 distinct pools of cocaine- and saline-treated mice (each pool representing 3–4 different mice), and this was performed for 4 different brain regions. The dose of cocaine (10 mg/kg once per day) and length of treatment (7 days) were based on previous studies (Adams et al. 2001, Arroyo et al. 2000, Hanson et al. 1989, Hurd & Herkenham 1992, Shippenberg et al. 2007, Werme et al. 2000, Romualdi et al. 2007, Lutfy et al. 2008, Sevarino & Primus 1993, Eugene et al. 2002, Kuzmin & Johansson 1999, Wahlestedt et al. 1991, Westwood & Hanson 1999, Sarnyai et al. 1992, Maj et al. 2003). Brain regions were labeled with isotopic tags as shown (Supplemental Figure S1); two of the replicates (runs 1 and 3) had the labels reversed relative to the other two replicates (runs 2 and 4) to control for possible differences due to the isotopic tags. Relative peptide levels in the cocaine-treated versus the saline-treated mice were determined from the peak intensities. For example, a peptide with monoisotopic m/z values of 815.5 (D0-TMAB) and 818.5 (D9-TMAB), which was subsequently identified from MS/MS analysis as PEN, showed a consistent decrease in the VTA (Figure 1) with cocaine treatment. This peptide was also decreased in the cocaine-treated samples from the NAc (Table S1).

Peptides were identified by MS/MS sequence analysis using a combination of database searches with the Mascot program followed by manual verification of the data to eliminate false positives. Altogether, 102 distinct peptides were identified that were derived from secretory-pathway proteins. In addition to these 102 peptides, many other fragments of non-secretory cytosolic or mitochondrial peptides were identified (Fricker 2010). The non-secretory pathway peptides were not reproducibly affected by cocaine treatments and were not considered in subsequent analyses.

All data for the secretory pathway peptides are shown in supplemental Table S1. Each row in this table represents a peak set for a particular peptide in one of the LC/MS runs. Altogether, there are 926 rows in this table, each with quantitative data on the relative level of peptide in the cocaine- versus the saline-treated groups. Many of the same peptides were found in

multiple brain regions. These 926 entries represent 102 distinct peptides derived from 29 secretory pathway proteins (Table S1). In some brain regions, several peptides were decreased consistently in each of the cocaine-treated groups relative to the saline-treated controls. The isotopic label-based quantification method provides a ratio of the amount of peptide in the cocaine-treated group relative to the amount of the same peptide in age- and sex-matched saline-treated group. Because this quantification technique generates a ratio between the two groups, it is not possible to perform statistical testing of the differences between groups. Therefore, the peptides were compared to a database of peptide ratios from untreated WT animals that were previously analyzed using the same peptidomics technique (Fricker 2010). This control database provides an estimate of the animal-to-animal variation for each peptide and represents data from some of the same brain regions used in the present study (frontal cortex and striatum). When compared to the control database, 10 peptides were altered significantly by cocaine treatment (Figure 2, Table S2). Several of these changes represent proSAAS-derived peptides: decreased PEN and little SAAS in the NAc and VTA, and reduced PEN-20 in the VTA (Figure 2, Table S2). In addition, nociceptin was decreased in the NAc. Four other peptides were reduced in the VTA: the C-terminal region of provasopressin/neurophysin (residues 154 to end), the phosphorylated form of corticotropin-like intermediate lobe peptide (CLIP), cerebellin 4, and chromogranin B residues 588–597 (Figure 2, Table S2). While some other peptides showed a trend towards decreasing, especially proopiomelanocortin-derived peptides, the differences were not statistically significant (Table S2). Because several of the peptides that were affected significantly by cocaine treatment were derived from proSAAS, we examined cocaine-mediated behaviors in proSAAS KO mice.

Cocaine-Stimulated Hyperlocomotion in the Open Field

Mice were placed into the open field for 60 min to assess baseline activities, injected with different doses of cocaine, and returned to the open field for 120 min (Figure 3A–B). An omnibus RMANOVA within subjects test for baseline activity (0–60 min) observed a significant time effect [$F_{(11,748)}$=77.798, p<0.001] and time by genotype [$F_{(11,748)}$=2.894, p=0.010] and time by treatment [$F_{(33,748)}$=1.922, p=0.020] interactions; the between subjects tests and none of the Bonferroni *post-hoc* tests were significant. Baseline locomotor activities declined over the first 60 min for both WT and proSAAS KO mice, demonstrating habituation to the open field. However, no genotype effects were observed for baseline activities. Following vehicle or cocaine injection (61–180 min), an omnibus RMANOVA within subjects test discerned a time effect [$F_{(23,1564)}$=56.620, p<0.001], and time by genotype [$F_{(23,1564)}$=15.138, p<0.001], time by treatment [$F_{(69,1564)}$=9.163, p<0.001], and time by genotype by treatment interactions [$F_{(69,1564)}$=4.702, p<0.001]; the between subjects test found significant genotype [$F_{(1,68)}$=47.524, p<0.001] and treatment effects [$F_{(3,68)}$=19.243, p<0.001], and a genotype by treatment interaction [$F_{(3,68)}$=13.698, p<0.001] (Figure 3A–B). Bonferroni corrected pair-wise comparisons determined there were no genotype differences between the vehicle controls. By contrast, locomotor activities for WT mice given 20 or 30 mg/kg cocaine were higher between 75–145 min than those for the mutant mice (p-values≤0.051).

To examine the cocaine effects within genotype, a RMANOVA within subjects test for locomotion (61–180 min) detected a significant time effect [$F_{(23,783)}$=35.320, p<0.001] and a time by treatment interaction [$F_{(69,783)}$=7.669, p<0.001]; the between subjects effect of treatment was also significant [$F_{(3,34)}$=18.538, p<0.001]. *Post-hoc* tests showed that WT locomotion was augmented by 20 mg/kg cocaine at 90 min (p=0.035) and by 30 mg/kg at 65–150 and 160 min (p-values≤0.026) relative to vehicle. Additionally, the activities of WT mice following 30 mg/kg cocaine was increased over that for the 10 mg/kg dose at 70–150 and 160 min (p-values≤0.047) and for the 20 mg/kg dose at 65 and 75–150 min (p-values≤0.053). For proSAAS KO mice a RMANOVA for locomotion (61–180 min) demonstrated a significant time effect [$F_{(23,782)}$=38.437, p<0.001] and a time by treatment interaction [$F_{(69,782)}$=3.165, p<0.001]; the between subjects effects were not significant. Locomotion in proSAAS KO animals was enhanced only at 75–80 min with 20 mg/kg cocaine (p-values≤0.001) and at 70–80 min for 30 mg/kg cocaine (p-values≤0.043). Locomotor activity in mutants following injection of 30 mg/kg cocaine was higher than with 10 mg/kg dose at 80 min (p=0.053).

To analyze the cocaine effects more readily, the data were expressed as cumulative locomotor activities (Fig. 3A–B, *insets*). An ANOVA for cumulative baseline activity failed to detect any significant effects. Following vehicle or cocaine injection, ANOVA reported main effects of genotype [$F_{(1,68)}$=52.909, p<0.001] and treatment [$F_{(3,68)}$=26.477, p<0.001], and a significant genotype by treatment interaction [$F_{(3,68)}$=15.878, p<0.001]. The Bonferroni tests demonstrated that WT locomotion was higher with 20 and 30 mg/kg cocaine compared to proSAAS KO mice given the same doses (p-values≤0.003). Analyses of responses within genotypes demonstrated that motor activities in WT mice were dose-dependently increased with 20 and 30 mg/kg cocaine (p-values≤0.001), whereas this relationship was not found with the proSAAS KO mice. Thus, acute locomotor responses to cocaine were transient and severely blunted in the proSAAS KO mice, whereas activity was robustly stimulated in a dose-dependent manner in WT animals.

Amphetamine-Stimulated Hyperlocomotion in the Open Field

Acute responses to AMPH were examined next (Figure 3C–D). An omnibus RMANOVA within subjects test for baseline activity (0–60 min) observed a significant time effect [$F_{(11,462)}$=49.885, p<0.001] and a time by treatment interaction [$F_{(22,462)}$=2.859, p=0.020]; the between subjects tests were not significant. Bonferroni tests failed to detect any genotype effects for baseline activities. Following vehicle or AMPH injection (61–180 min), an omnibus RMANOVA within subjects test discerned a time effect [$F_{(23,966)}$=39.727, p<0.001], and time by genotype [$F_{(23,966)}$=7.431, p<0.001], time by treatment [$F_{(46,966)}$=8.946, p<0.001], and time by genotype by treatment interactions [$F_{(46,966)}$=2.900, p<0.001]; the between subjects test found the main effects of genotype [$F_{(1,42)}$=9.517, p=0.004] and treatment [$F_{(2,42)}$=15.138, p<0.001] to be significant. Bonferroni corrections noted that WT responses to 2 and 3 mg/kg AMPH were significantly enhanced at 65–115 and 125 min and at 75–115 min, respectively, over that of the proSAAS KO mice (p-values≤0.039).

When effects were analyzed within genotype, a RMANOVA within subjects test for locomotion (61–180 min) in WT mice revealed significant time effects [$F_{(23,483)}$=30.382, p<0.001] and a time by treatment interaction [$F_{(46,483)}$=7.574, p<0.001]; the between subjects test of treatment was also significant [$F_{(2,21)}$=11.757, p<0.001] (Figure 3C–D). Bonferroni *post-hoc* tests observed that WT locomotor activity was stimulated with 2 mg/kg AMPH at 65–115, 125, and 135 min (p-values≤0.050) and with the 3 mg/kg dose at 70–115, and 125–130 min (p-values≤0.054) relative to the vehicle control. For proSAAS KO mice, the RMANOVA within subjects test demonstrated significant time effects [$F_{(23,483)}$=10.417, p<0.001] and a time by treatment interaction [$F_{(46,483)}$=2.729, p<0.001]; the between subjects treatment effect was significant [$F_{(2,21)}$=3.782, p=0.040]. The *post-hoc* tests for mutants found locomotion to be induced with 2 mg/kg AMPH at 65–75 min (p-values≤0.021) and with the 3 mg/kg dose at 65–100 min (p≤0.040).

Responses to AMPH were studied also according to cumulative activities (Figure 3C–D, *inset*). An ANOVA confirmed there were no significant genotype differences at baseline. However, for post-injection activities an ANOVA observed significant main effects of genotype [$F_{(1,428)}$=16,422, p<0.001] and treatment [$F_{(2,42)}$=22.697, p<0.001], and a genotype by treatment interaction [$F_{(2.42)}$=5.087, p=0.011]. Here, locomotion in WT mice was potentiated with 2 and 3 mg/kg AMPH over that of proSAAS KO mice administered the same doses (p-values≤0.001). When activity was examined within WT mice, both doses of AMPH stimulated locomotion to similar extents above that of the vehicle control (p-values<0.001). By comparison, only the 3 mg/kg dose was successful in this regard in the proSAAS KO mice (p=0.028). Together, these results show that in proSAAS KO mice the acute locomotor responses to AMPH are more transient and much less robust than in the WT animals.

Behavioral Sensitization to Cocaine

To further evaluate the responses of the proSAAS mice to cocaine, locomotor sensitization was examined. Mice were treated with vehicle or 20 mg/kg cocaine for five consecutive days and then challenged with the same cocaine dose on day 11 (Figure 4A–F). An omnibus RMANOVA within subjects test for baseline activity (0–60 min) across all 6 days noted a time effect [$F_{(11,924)}$=78.938, p<0.001] and a significant time by day interaction [$F_{(55,924)}$=4.494, p<0.001]; the between subjects tests for genotype was significant [$F_{(1,84)}$=11.161, p<0.001]. The Bonferroni *post-hoc* tests for the interaction at baseline were not significant. Following injection of vehicle or cocaine (61–180 min) an omnibus RMANOVA within subjects test for locomotion revealed a time effect [$F_{(23,1932)}$=157.473, p<0.001], and time by day [$F_{(115,1932)}$=2.929, p<0.001] and time by genotype interactions [$F_{(23,1932)}$=15.753, p<0.001]; the between subjects test observed significant day [$F_{(5,84)}$=2.583, p=0.032] and genotype effects [$F_{(1,84)}$=69.060, p<0.001]. Since many different significant effects were found across times, among days, and between genotypes and because these effects are difficult to follow in Figure 4A–F, the results were presented as cumulative distance traveled for each day.

RMANOVA were run where pre-/post-injection was nested within day comparing pre- and post-injection locomotion across each of the 5 days of repeated dosing and at challenge (day

11) (Figure 4G–H). The RMANOVA within subjects test demonstrated significant day [$F_{(1,17)}$=30.590, p<0.001] and pre-/post-injection effects [$F_{(1,14)}$=97.319, p<0.001], and day by genotype [$F_{(1,14)}$=7.555, p=0.016], pre-/post-injection by genotype [$F_{(1,14)}$=20.529, p<0.001], day by pre-/post-injection [$F_{(1,14)}$=47.349, p<0.001], and day by pre-/post-injection by genotype interactions [$F_{(1,14)}$=11.644, p=0.004]; the between subjects test of genotype was significant [$F_{(1,14)}$=18.342, p=0.001]. Baseline activities in proSAAS KO mice were lower than those for WT animals on day 1 (p=0.013) (Figure 4G). Despite this fact, cumulative baseline activities prior to cocaine administration on all other days were statistically indistinguishable between the genotypes. By contrast, cumulative locomotor responses in WT mice were significantly augmented by cocaine over those of proSAAS KO animals on all five sensitization days as well as on challenge day 11 (p-values≤0.034) (Figure 4H). Notably, post-injection activities on all 6 days were augmented over baseline for WT mice (p-values<0.001), whereas for mutants they were only elevated on sensitization days 1, 3, and 5 and at challenge (p≤0.046). Within each genotype, locomotion at baseline was not significantly different across the 6 days. Moreover, WT responses on days 5 and 11 (challenge) following cocaine injection were increased over those of days 1 and 2 (p-values≤0.048), clearly demonstrating behavioral sensitization to cocaine. In striking contrast, cocaine-enhanced locomotion was not increased across days for the proSAAS KO mice, indicating that they did not show behavioral sensitization to 20 mg/kg cocaine. These results were replicated in separate cohorts of WT and proSAAS KO mice using 20 mg/kg cocaine at the Penn State University College of Medicine (data not shown). Together, our results demonstrate that WT mice develop behavioral sensitization to cocaine, whereas proSAAS KO mice do not develop this response.

Behavioral Sensitization to Amphetamine

Behavioral sensitization to AMPH was also evaluated. Mice were treated with vehicle or 3 mg/kg AMPH for five consecutive days and then challenged with the same AMPH dose on day 11 (Figure 5A–F). An omnibus RMANOVA within subjects test for baseline activity (0–60 min) across all 6 test days reported only a time effect [$F_{(11,924)}$=46.046, p<0.001]; the between subjects tests were not significant. Hence, no genotype effects were seen at baseline on any day. Following injection of vehicle or cocaine (61–180 min) an omnibus RMANOVA within subjects test for locomotion demonstrated a time effect [$F_{(23,1909)}$=145.924, p<0.001], and time by genotype [$F_{(23,1909)}$=7.383, p<0.001], time by day [$F_{(115,1909)}$=2.496, p<0.001], and time by genotype by day interactions [$F_{(115,1909)}$=1.261, p=0.036]; the between subjects test showed significant genotype [$F_{(1,83)}$=15.875, p<0.001] and day effects [$F_{(5,83)}$=6.907, p<0.001]. As there were numerous significant effects for times, days, and genotypes, the data were presented and analyzed as cumulative distance traveled to see these effects more clearly.

To examine behavioral sensitization to AMPH in more detail, RMANOVA were run comparing pre- and post-injection locomotion across each of the 5 days of repeated dosing and at challenge (day 11) (Figure 5G–H). The RMANOVA within subjects test demonstrated significant day [$F_{(1,14)}$=6.394, p=0.024] and pre-/post-injection effects [$F_{(1,14)}$=277.402, p<0.001], and day by genotype [$F_{(1,14)}$=8.889, p=0.010], pre-/post-injection by genotype [$F_{(1,14)}$=7.998, p=0.013], day by pre-/post-injection [$F_{(1,14)}$=6.296, p=0.025], and day by

pre-/post-injection by genotype interactions [$F_{(1,14)}$=7.969, p=0.014]; the between subjects test of genotype was significant [$F_{(1,14)}$=11.110, p=0.005]. Baseline locomotion activities across all test days were statistically indistinguishable between and within genotypes (Figure 5G). Following AMPH administration, WT cumulative locomotion was significantly higher than in the proSAAS KO mice on days 1, 2, 5, and on the challenge day 11 (p-values≤0.040) (Figure 5H). Additionally, WT mice demonstrated behavioral sensitization since their locomotor response to the challenge injection (day 11) was significantly higher than it was on day 1 (p=0.050). By comparison, AMPH-stimulated activities in proSAAS KO animals were elevated on days 2, 4, and 5 over that of day 1 (p-values≤0.021). The increased locomotion across days indicated that the mutants were becoming sensitized to AMPH. However, since their locomotor responses on day 1 and day 11 (challenge injection) were not statistically significant, these data indicate that they were unable to maintain AMPH sensitization over time.

Cocaine Conditioned Place Preference

To examine the rewarding effects of cocaine, mice were tested in CPP. Following habituation to the apparatus, mice were subjected to three 2-day conditioning cycles of alternating vehicle and cocaine injections and tested for CPP 48 hr after the last pairing. A RMANOVA within subjects test found a significant day (baseline and CPP) effect [$F_{(1,53)}$=126.122, p<0.001] and a significant day by dose interaction [$F_{(2,53)}$=11.319, p<0.001]; the between subjects test for dose was also significant [$F_{(2,53)}$=11.447, p<0.001]. During habituation neither genotype displayed any preference for one chamber over the other regardless of subsequent dose assignment for cocaine (Figure 6). Additionally, no significant differences in cocaine CPP were observed between genotypes. Cocaine CPP in both WT and proSAAS KO mice was lower at the 10 mg/kg than at the 20 and 30 mg/kg doses (p-values≤0.001), which were not statistically different from each other. The results showing identical CPP for 20 mg/kg cocaine between WT and proSAAS KO mice were replicated at the Penn State University College of Medicine (data not shown).

DISCUSSION

Like most other drugs of abuse, cocaine administration induces increased dopamine efflux in the nucleus accumbens. Neuropeptides also appear to play central roles in reward mechanisms that are either upstream or downstream of dopamine. For example, mice lacking the mu opioid receptor show reduced physiological responses to a number of drugs of abuse including nicotine, delta-9-tetrahydrocannabinol, ethanol, and cocaine (Hall *et al.* 2004, Mathon *et al.* 2006, Mathon *et al.* 2005, Becker *et al.* 2002, Hummel *et al.* 2004, Berrendero *et al.* 2002, Ghozland *et al.* 2002). Mice lacking the receptor for substance P (neurokinin 1 receptors) show reduced rewarding effects to opioids, but not to cocaine (Gadd *et al.* 2003, Murtra *et al.* 2000, Ripley *et al.* 2002). Thus, endogenous peptides can play a critical role in the signaling pathways involved in the response to drugs of abuse.

The results of the present study are consistent with the emerging view that proSAAS-derived peptides are functional neuropeptides. Specifically, the finding that several proSAAS peptides were significantly decreased by cocaine treatment and that proSAAS KO mice

showed a reduced acute dose-response to the locomotor-stimulating effects of cocaine support the hypothesis that proSAAS-derived peptides contribute to these responses. The observed behavioral effects were not specific to cocaine since acute exposure to AMPH exerted similar results. In the sensitization studies, locomotor responses in proSAAS KO mice to cocaine did not increase across days or in the day 1 to challenge day comparison, indicating that the mutants did not sensitize to this psychostimulant. By comparison, the locomotor-stimulating effects to AMPH were increased across days in both the WT and the proSAAS KO mice. In the day 1 to challenge day (day 11) comparison, WT mice clearly demonstrated behavioral sensitization to AMPH. However, while sensitization did occur across days in the proSAAS KO mice, it was not maintained because locomotion on day 1 and 11 were not statistically different from each other. Since the mechanisms of action to cocaine and AMPH are different (Harris & Baldessarini 1973, Taylor & Ho 1978, Sulzer *et al.* 1993), these results suggest that proSAAS peptides may play distinct roles in the behavioral sensitization to different psychostimulants.

In contrast to the failure of proSAAS KO mice to display or maintain behavioral sensitization to psychostimulants, CPP was observed at each dose of cocaine for both WT and proSAAS KO animals, and no significant differences were observed between genotypes. Hence, proSAAS peptides do not appear to be involved in the neural circuitry associated with the rewarding effects of cocaine. In contrast, the inability of the mutants to develop behavioral sensitization suggests that loss of proSAAS peptides specifically perturbs the neural networks involved in the neuroadaptations associated with sensitization.

Based on the distribution of proSAAS mRNA and peptides (Wardman & Fricker 2014, Wardman et al. 2011, Feng *et al.* 2001, Fricker et al. 2000), the proSAAS derived peptides may either directly or indirectly influence dopaminergic transmission. Because proSAAS mRNA is expressed in brain regions such as the VTA and substantia nigra that contain high levels of mRNAs for enzymes required for dopamine biosynthesis (i.e., tyrosine hydroxylase and *L*-aromatic amino acid decarboxylase), proSAAS peptides may directly influence dopaminergic neurotransmission. Alternatively, proSAAS mRNA is highly enriched in the bed nuclei of the stria terminalis, paraventricular nucleus of the thalamus, medial preoptic area, nucleus of the solitary tract, locus coeruleus, and arcuate nucleus; peptides in these regions may modulate other neural systems to affect responses to psychostimulants.

In addition to the cocaine-induced decreases in proSAAS-derived peptides, peptides derived from provasopressin, proopiomelanocortin, cerebellin 4, and chromogranin B were also decreased in the VTA of cocaine-treated mice. It is not known if any of these observed peptides are biologically active, although each of the precursors is known to produce peptides with biological activity (Solomon 1999, Baribeau & Anagnostou 2015, Zhao *et al.* 2009, Matsuda & Yuzaki 2011). In addition to changes in levels of these other peptides, nociceptin was decreased in the NAc of cocaine-treated mice. Nociceptin is the endogenous ligand for the nociceptin receptor (also known as ORL-1), a G protein-coupled receptor related to the opioid receptors (Civelli 2008). Administration of cocaine was previously shown to decrease nociceptin levels in some brain regions, as measured by radioimmunoassay (Lutfy *et al.* 2008), and the magnitude of the decrease was comparable to that measured in the present study. Administration of exogenous nociceptin has been shown

to prevent sensitization for cocaine in rodent models (Lutfy *et al.* 2002, Bebawy *et al.* 2010). Our finding that levels of nociceptin are altered upon cocaine treatment is consistent with a role for this peptide in the response to this drug.

Two previous studies from another laboratory used a peptidomics approach to examine peptides in rat brain in response to acute cocaine administration. However, the vast majority of peptides detected in both studies were derived from intracellular proteins, and either no neuropeptides (Romanova *et al.* 2010) or a very small number of neuropeptides (Romanova *et al.* 2015) were detected. Neither of these two studies from Romanova and colleagues used heat-stabilization of the brain tissue prior to peptide extraction, and without this treatment it is difficult to detect neuropeptides over the high background level of intracellular peptides (Svensson *et al.* 2003, Che *et al.* 2005, Scholz *et al.* 2011, Skold *et al.* 2007). In our present analysis, we also detected peptides derived from cytosolic and mitochondrial proteins and analyzed relative levels in response to cocaine administration. Although some of these peptides showed large changes between the cocaine and saline groups in some of the biological replicates, none of the peptides showed consistent changes in all four of the replicates (data not shown).

With caveats, such as the need to heat-stabilize brain tissue prior to peptide extraction, the quantitative peptidomics technique used in the present study is relatively unbiased and permits the detection of a large number of neuropeptides and other peptides in brain tissue (Fricker 2015). The proSAAS-derived peptides are the most commonly detected peptides in peptidomic studies, consistent with the high abundance of these peptides in brain (Fricker 2012). The finding that levels of several proSAAS-derived peptides are altered by cocaine suggests that these peptides may play an important role in cocaine abuse. However, this role appears to be complex as the sensitization responses to cocaine and AMPH are different in the proSAAS KO mice, while the CPP responses are normal. ProSAAS is processed to a variety of peptides that include big and little forms of SAAS, GAV, PEN, and LEN (Fricker 2010, Fricker et al. 2000) and these peptides may play synergizing or opposing roles in response to psychostimulants.. Future experiments with mice lacking receptors of PEN (GPR83) and big LEN (GPR171) may add unique insights into the roles of specific proSAAS peptides in the responses to psychostimulants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by National Institutes of Health grants R01 DA-004494 (L.D.F.), R21 DA-036385 (D.J.M.), K01 DA-037355 (D.J.M.), and DA-008622 (to J.P.). Work was also funded, in part, under a grant from the Pennsylvania Department of Health using Tobacco CURE Funds. Special thanks to Drs. Ruth Angeletti and Ilona Vathy (formerly professors at Albert Einstein College of Medicine) for helpful discussions, assistance with mass spectrometry, and providing reagents for the peptidomic studies performed at Einstein, and to Dr. Juan Sironi for assistance with peptidomic data analysis.

Abbreviations

CLIP	corticotropin-like intermediate lobe peptide

CPP	conditioned place preference
KO	knockout
LS/MS	liquid chromatography/mass spectrometry
MS/MS	tandem mass spectrometry
CPU	caudate putamen
NAc	nucleus accumbens
FC	frontal cortex
VTA	ventral tegmental area
TMAB	trimethylammonium butyric acid. Note that SAAS, PEN, and LEN are names, not abbreviations

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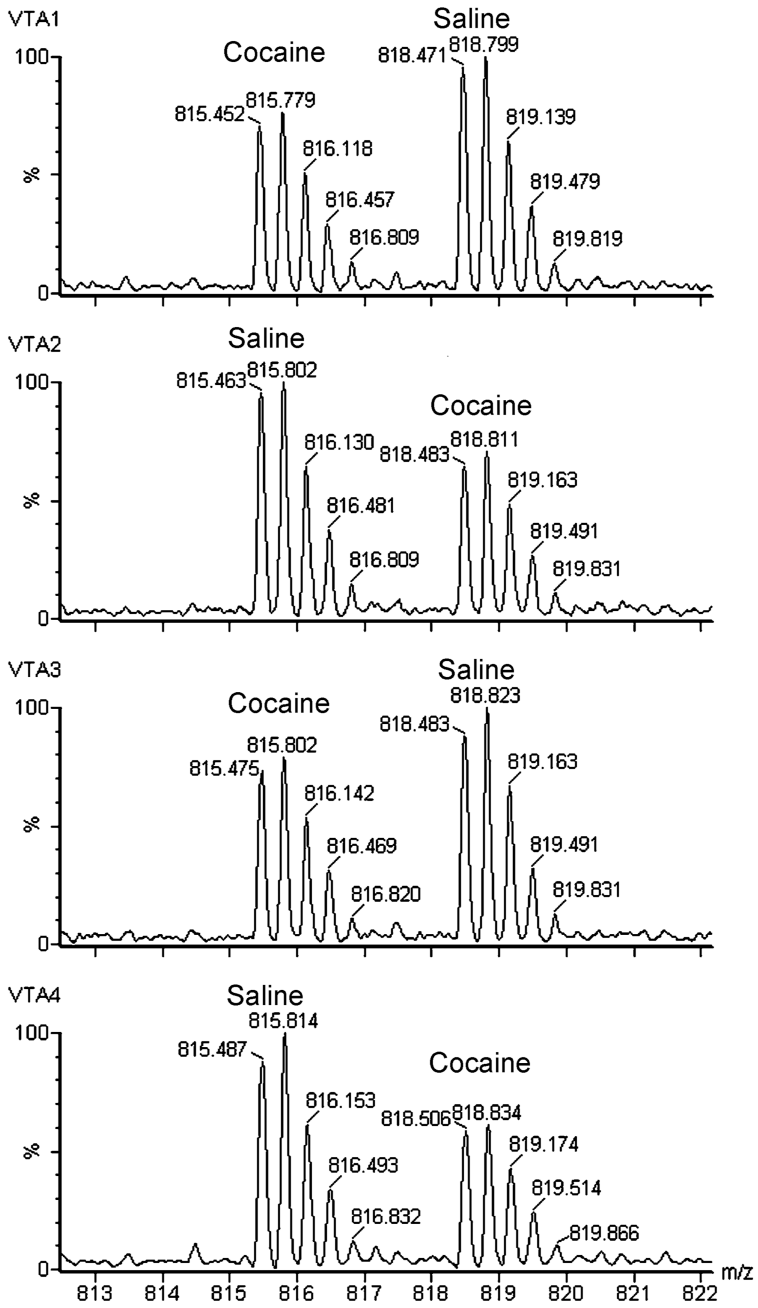

Figure 1. Representative MS data

Peptide extracts from distinct groups of mice treated for 7 consecutive days with 10 mg/kg cocaine in saline or saline alone were labeled with D0-TMAB and D9-TMAB, as shown in Figure S1 and analyzed by LC/MS and MS/MS. The spectra show monoisotopic ions with m/z of 815.5 and 818.5 for each of the 4 LC/MS runs performed on ventral tegmental area (VTA) extracts; these ions were identified from MS/MS analysis as the D0-TMAB and D9-TMAB-labeled triply-charged form of PEN. Note that the peak intensities for the cocaine-treated groups are lower than the peak intensities for the saline-treated groups.

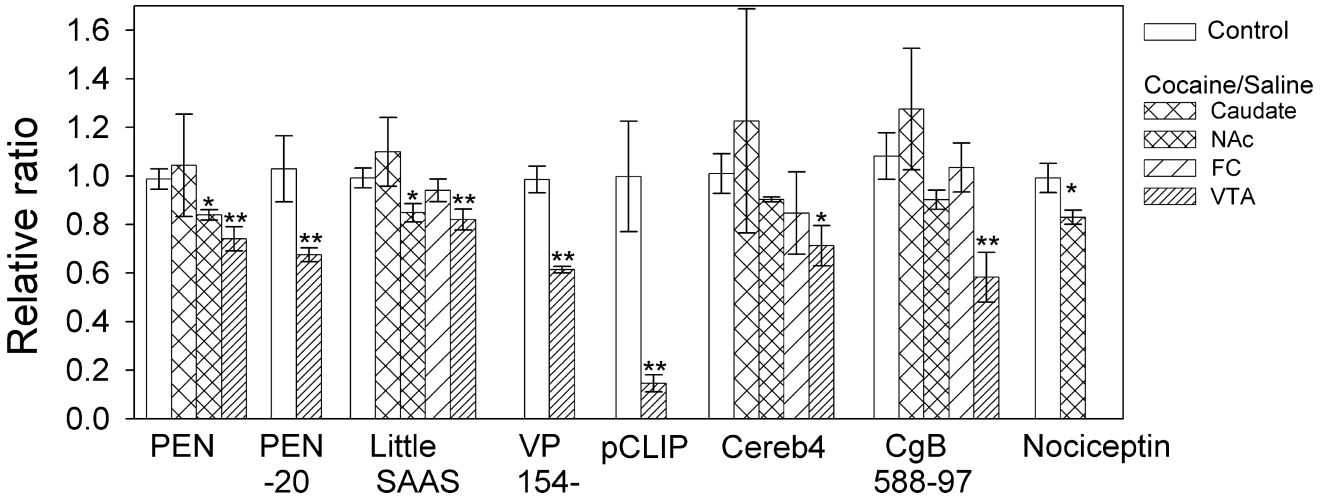

Figure 2. Peptides affected by cocaine administration

Twenty peptides showing the largest changes between cocaine- and saline-treated mice were compared to a dataset of these peptides in untreated mice (open bars). Bars: wide cross-hatch, caudate putamen (CPU); narrow cross-hatch, nucleus accumbens (NAc); wide vertical lines, frontal cortex (FC); narrow vertical lines, ventral tegmental area (VTA). For most data, n=4 (see Table S2). Error bars represent standard error of the mean. * $p<0.05$; ** $p<0.01$ using Student's two-tailed t-test, compared to the control dataset.

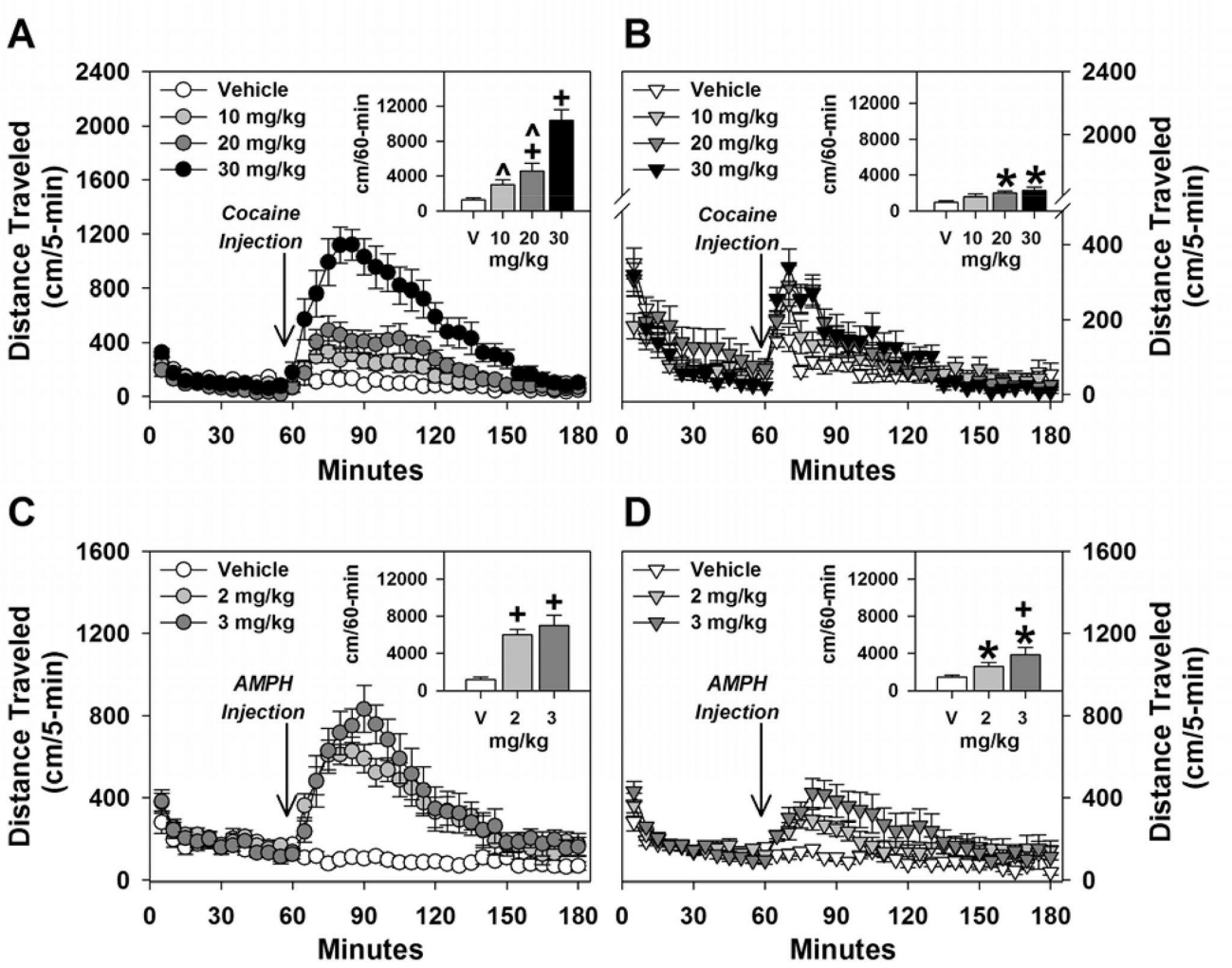

Figure 3. Locomotor activity in the open field for WT and proSAAS KO mice given an acute injection of the vehicle or different doses of cocaine or amphetamine

(A–B) Locomotor activity as distance traveled for WT (panel A) and proSAAS KO mice (panel B) in 5-min blocks across 180 min of testing; vehicle or cocaine were administered at 60 min (arrow). *Inset* shows the cumulative distance traveled over the 60 min following vehicle (V) or cocaine injection. **(C–D)** Locomotor activity as distance traveled for WT (panel C) and proSAAS KO mice (panel D) in 5-min blocks across 180 min of testing; vehicle or amphetamine (AMPH) were administered at 60 min (arrow). *Inset* shows the cumulative distance traveled over the 60 min following vehicle (V) or AMPH injection. N = 8–10 mice/genotype/treatment; *p<0.05, from WT controls; +p<0.05, from the vehicle within genotype; ^p<0.05, from 30 mg/kg cocaine within genotype.

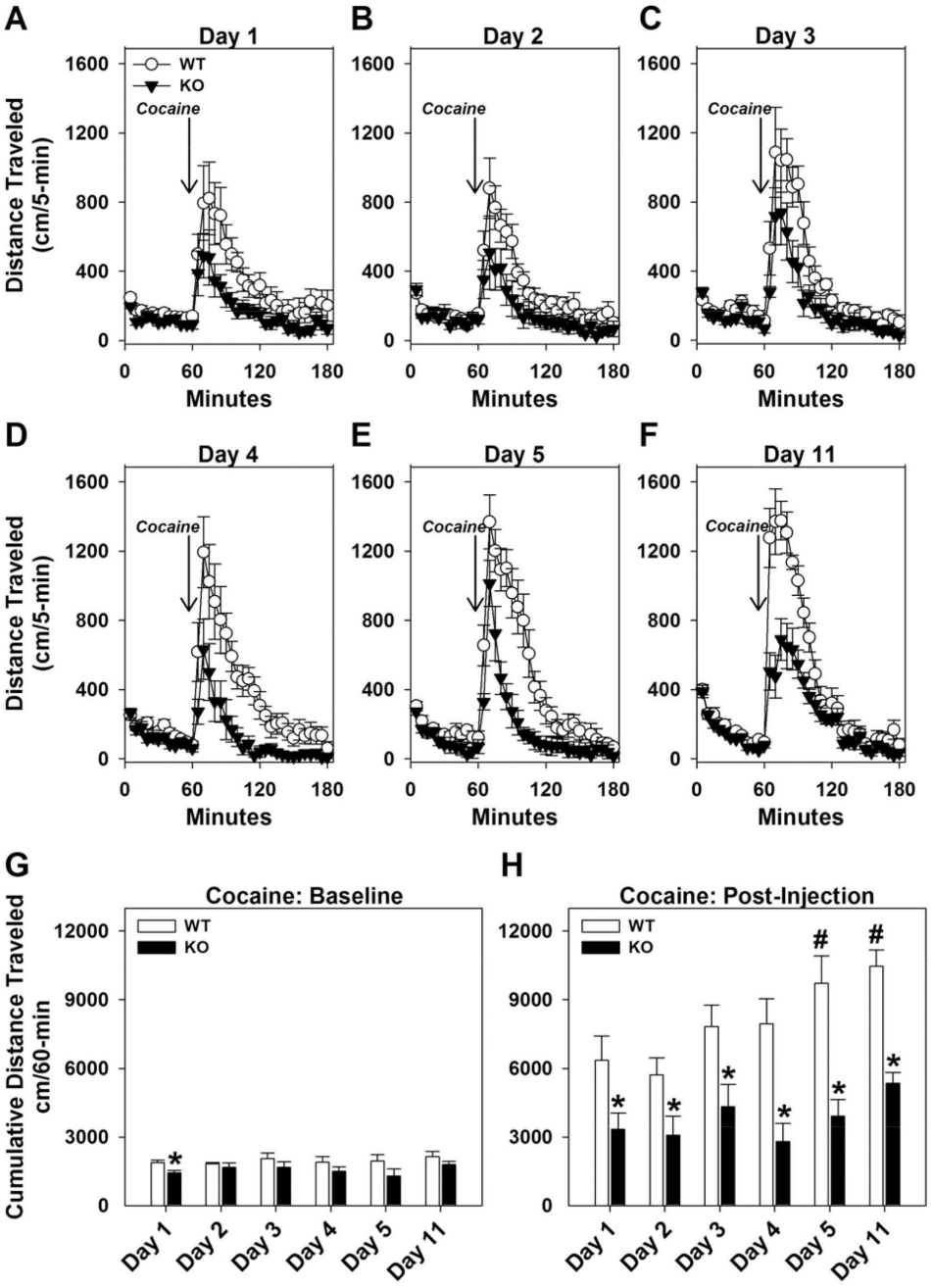

Figure 4. Behavioral sensitization of locomotor activity for WT and proSAAS KO mice treated with vehicle or 20 mg/kg cocaine

(A–F) Locomotor activity as distance traveled for WT and proSAAS KO mice in 5-min blocks across 180 min of testing over 5 consecutive days followed by a hiatus and a cocaine challenge at day 11; the vehicle or cocaine were administered at 60 min (arrow). **(G–H)** Cumulative locomotor activities in WT and proSAAS KO mice for baseline (G) and cocaine-stimulated activities (H). N = 8 mice/genotype/treatment group; *p<0.05 from the WT controls; #p<0.05, from day 1 within genotype.

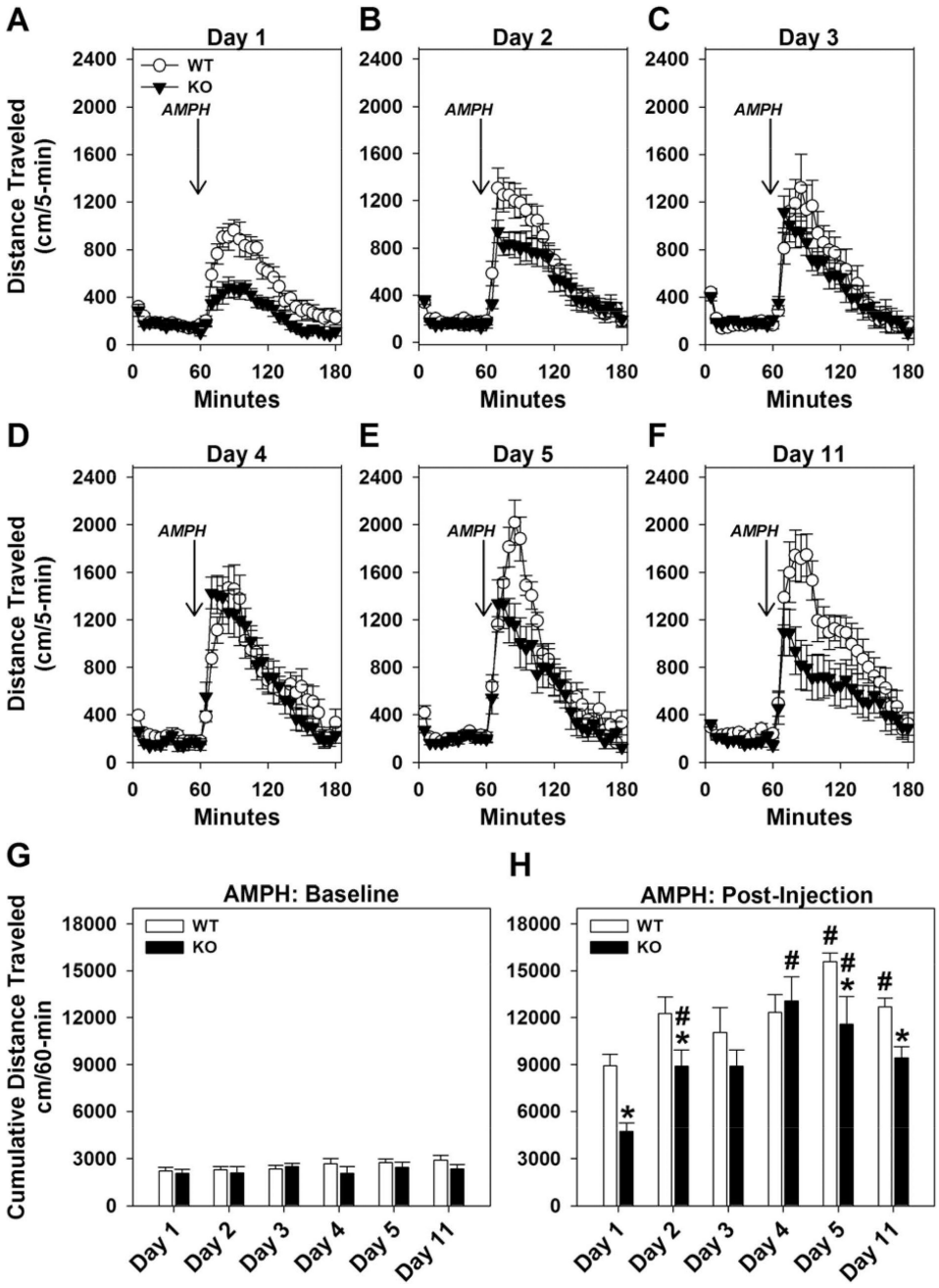

Figure 5. Behavioral sensitization of locomotor activity for WT and proSAAS KO mice treated with vehicle or 3 mg/kg amphetamine

(A–F) Locomotor activity as distance traveled for WT and proSAAS KO mice in 5-min blocks across 180 min of testing over 5 consecutive days followed by a hiatus and an amphetamine (AMPH) challenge at day 11; the vehicle or AMPH were administered at 60 min (arrow). **(G–H)** Cumulative locomotor activities in WT and proSAAS KO mice for baseline (**G**) and AMPH-stimulated activities (H). N = 8 mice/genotype/treatment group; *p<0.05 from the WT controls; #p<0.05, from day 1 within genotype.

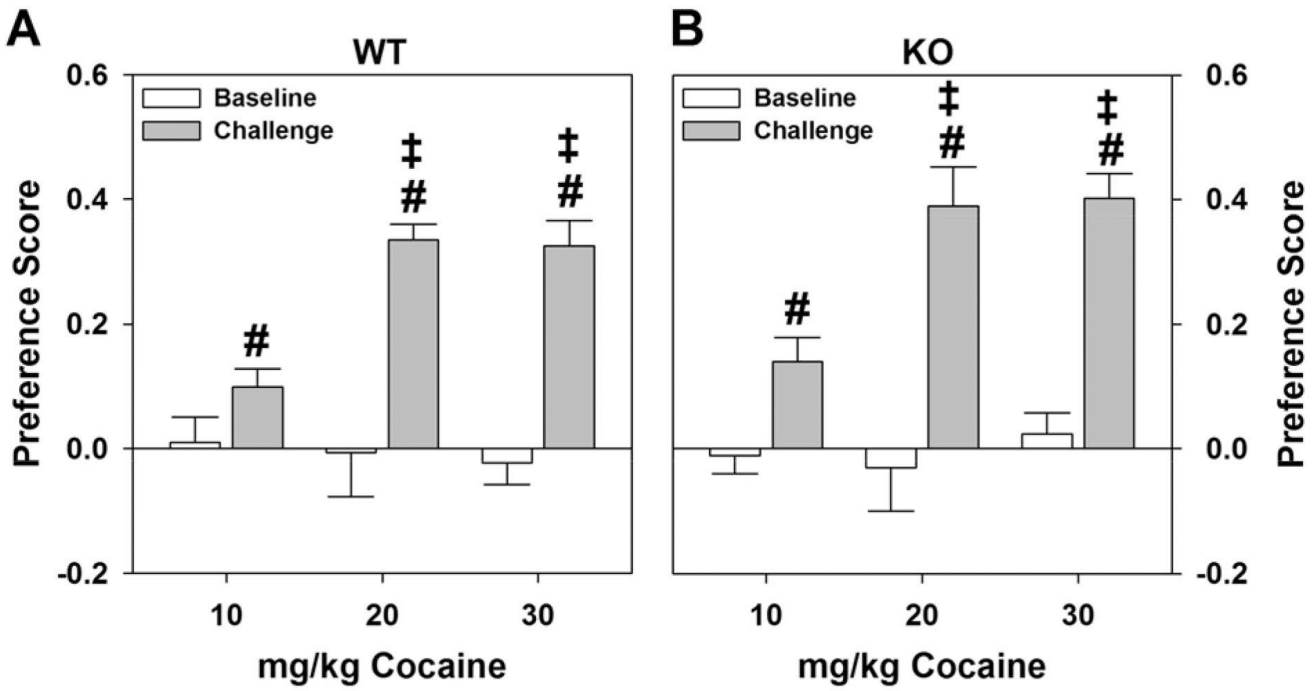

Figure 6. ProSAAS KO mice display normal conditioned place preference (CPP) for cocaine

(A–B) CPP for WT (**A**) and proSAAS KO mice (**B**); CPP was defined as an increase in the time (sec) spent in the cocaine-paired chamber on day 1 compared to that at challenge. N = 8–12 mice/genotype/treatment group; #$p<0.05$, from baseline within genotype; ‡$p<0.05$, from 10 mg/kg cocaine within genotype.