

Global Neuropeptidome Profiling in Response to Predator Stress in Rat: Implications for Post-traumatic Stress Disorder

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Abstract

Traumatic stress triggers or exacerbates multiple psychiatric illnesses, including post-traumatic stress disorder (PTSD). Nevertheless, the neurophysiological mechanisms underlying stress-induced pathology remain unclear, in part due to the limited understanding of neuronal signaling molecules such as neuropeptides in this process. Here, we developed mass spectrometry (MS) based qualitative and quantitative analytical strategies to profile neuropeptides in rats exposed to predator odor (an ethologically relevant analog of trauma-like stress) versus control subjects (no odor) to determine peptidomic alterations induced by trauma. In total, 628 unique neuropeptides were identified across 5 fear circuitry-related brain regions. Brain region-specific changes of several neuropeptide families including granin, ProSAAS, opioids, cholecystokinin, and tachykinin were also observed in the stressed group. Neuropeptides from the same protein precursor were found to vary across different brain regions, indicating site-specific effects of predator stress. This study reveals for the first time the interaction between neuropeptides and traumatic stress, providing insights into the molecular mechanisms of stress-induced psychopathology and suggesting putative novel therapeutic strategies for disorders such as PTSD.

Introduction

Post-traumatic stress disorder (PTSD) is a psychiatric disorder resulting from severe stress due to traumatic events such as exposure to life-threatening experiences or extreme violence¹. This debilitating disease occurs in 5–10% of the population and is characterized by the distressing recollection of traumatic memories long after the period of trauma exposure². Symptoms of PTSD include hypervigilance exaggerated stress responses, insomnia, and depression; PTSD patients also are at high risk for suicide^{3,4}. In mammals, the stress response involves a complex physiological reaction to respond to the immediate threat and then subsequently restore the body's homeostasis through activation of the sympathetic-adrenomedullary (SAM) system, the hypothalamic-pituitary-adrenocortical (HPA) systems, and the brain limbic system⁵⁻⁷. The pathophysiology of PTSD is thought to involve dysfunction within these stress-modulatory systems, including changes in brain region volume, and alterations in key neuromodulators⁴. Brain regions that have been implicated in PTSD include the basolateral amygdala (BLA), prefrontal cortex (PFC), hippocampus (HPC), paraventricular hypothalamus (PVN), and nucleus accumbens (NAcc)⁸. However, the mechanisms involved in the translation of traumatic stress to the long lasting and debilitating symptoms of PTSD remain poorly understood. While functional neuroimaging and preclinical animal studies have delineated certain brain circuits as putative candidates for the etiology of PTSD, a clear elucidation of coordinated brain-wide changes of neuromodulators following traumatic stress remains largely unexplored⁹.

One kind of neuromodulator, neuropeptides (NPs), are widely present in all animal species¹⁰. NPs are cleaved from prohormones by endogenous peptidases and act through both autocrine and paracrine signaling¹¹. They are involved in diverse fundamental biological activities, including reproduction¹², food intake¹³, and sleep/circadian rhythm¹³⁻¹⁶. In addition to physiological processes, NPs regulate psychological processes closely related to mood, emotions, cognition, and memory and have been implicated in several psychiatric illnesses including PTSD¹⁷. Perhaps the most widely studied NP for stress-related disorders such as PTSD is corticotropin-releasing factor (CRF). CRF elicits behavioral and endocrine responses to

stress through multiple brain regions and is altered in PTSD and depression¹⁸. In contrast, Neuropeptide Y (NPY) acts in opposition to CRF, and has been suggested as a potential NP-based treatment for PTSD¹⁹. Substance P, a member of the tachykinin neuropeptide family, responds to stress by mediating pain perception²⁰ and anxiety regulation²¹, and substance P antagonist is used to prevent emesis induced by chemotherapy treatments²². These diverse roles in stress and behavioral regulation thus implicate NP as potential biomarkers and therapeutic targets. It is crucial to identify and quantify stress-related neuropeptides with high confidence and to understand their biological functions and mechanisms associated with the nervous system.

Studies of neuropeptides are hampered by their innate characteristics including low *in vivo* abundance, high structural complexity and variability, and their tendency towards rapid degradation. Most of the prior studies investigated NP regulation under stress and only studied one or very few NPs at a time and relied on techniques such as radioimmunoassay (RIA)²³, gene modification²⁴, and mRNA expression²⁵. The techniques are limited due to their low-throughput, cost-inefficiency and are time-consuming. The emergence of liquid chromatography coupled with electrospray ionization (LC-ESI) with tandem mass spectrometry (MS/MS) makes large-scale identification of endogenous peptides possible and cost-effective^{10, 26}. With LC-MS/MS, only minimal sample (as low as picomolar or even femtomolar) is necessary for accurate profiling. Additionally, specific information for peptide sequences with various post-translational modifications can be obtained. LC-MS/MS can also be used to quantify neuropeptides through multiple approaches, such as label-free quantitation²⁷, isobaric labeling quantitation²⁸, and isotopic labeling quantitation²⁹.

In the present study, we utilized a recently established rat model of trauma-like stress³⁰⁻³² (predator exposure) to mimic the type of stimuli that lead to PTSD³¹, and investigated the neuropeptidome change via mass spectrometric-based peptidomics analysis with label-free quantitation. This approach enabled for the first time a comprehensive investigation of the dynamic relationship of candidate markers across multiple brain regions in an ethologically relevant model of trauma. We identified 628 unique neuropeptide sequences from all tissues collected. Our method successfully detected more mature neuropeptides and

fewer peptide fragments. Different expression patterns of the neuropeptides were identified, including ProSAAS, cholecystokinin (CCK), vasoactive intestinal peptides (VIP), and opioid peptides, in the Stressed group. Notably, peptides from the same family were altered differently in different brain regions.

Experimental section

Materials and reagents

Optima-grade formic acid was purchased from Sigma Aldrich (St. Louis, MO). Ultracel® 30kDA molecular weight cutoff filters were purchased from Millipore Sigma (Burlington, MA). C18 OMIX tips were purchased from Agilent (Santa Clara, CA). Protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). All chemicals and solvents, unless noted otherwise, were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade solvents were used for sample preparation and Optima-grade solvents were used for instrumental analysis.

Animals and stress exposure

All animal procedures were in accordance with NIH guidelines and approved by University of Wisconsin-Madison IACUC. Male Sprague-Dawley rats (weighing 300-400g) were purchased from Envigo Labs. Rats arrived approximately two weeks prior to the experiment, and were housed in pairs in a temperature- and humidity-controlled vivarium and maintained on a 12h light schedule with lights on from 7am – 7pm. Three biological replications were conducted for both Ctrl and Stressed groups of rats. Food and water were available *ad libitum*, and rats were handled gently by the experimenter daily to reduce nonspecific stress at the time of experiments.

Rats were approximately 90 days old at the time of the experiments. On the test day, rats were placed individually in clear Plexiglas[®] cages with wire grid floors. Rats in the Stressed group had fox urine pellets mixed into their cages' bedding; Ctrl rats had no pellets in their bedding (no-stress group). Animals' behavioral responses during the exposure period were videorecorded. One hour later, rats were removed from cages and euthanized for brain tissue collection, described below.

Sample preparation

Rats were anesthetized with isoflurane and perfused with chilled physiological saline solution before being decapitated. Brains were immediately dissected after decapitation. The right side of the brain was then denatured using The Stabilizer[™] T1 (Denator AB, Uppsala, SE) with its fresh frozen program. The BLA, PFC, HPC, PVN and NAcc of the hypothalamus were dissected from the denatured right-side brain, flash frozen with dry ice and stored at -80°C until further use. For tissue extraction, 2 types of extraction solvent were used: 8M urea with protease inhibitor cocktail tablets, and acidified methanol (90/9/1 methanol/water/glacial acetic acid, v/v/v). For each mg of the tissue, 15 µL of 8 M urea, and 20 µL of acidified methanol were used. Tissues were first extracted with 8 M urea with a hand-held ultrasonic homogenizer with 50 % amplitude, 3 seconds on, 8 seconds off for 30 seconds. The homogenized samples were centrifuged at 20,000 rcf for 20 min at 4 °C. The supernatant was then filtered with Ultracel[®] 30 kDa molecular weight cutoff (MWCO) filters. The filter was preconditioned with 200 µL of 0.1 N NaOH, and 8 M urea by centrifugation at 14,000 rcf for 4 min and 8 min respectively. The supernatant was centrifuged at 14,000 rcf for 2 hours. The flowthrough was then kept at -80 °C until further use. The dead volume in the MWCO filter and the insoluble pelleted sample from the centrifugation were then extracted again with acidified methanol using the hand-held

ultrasonic homogenizer with 50% amplitude, 15 seconds on, 15 seconds off for 60 seconds. The homogenized samples were centrifuged at 20,000 rcf for 20 min at 4 °C. The supernatant was dried down using speedvac on medium heat and kept in -80 °C until further use. The urea-extracted portion and acidified methanol-extracted portion were combined and desalted with C18 Omix tips (Agilent, CA) following the packaged instructions. The eluent was then dried down with speedvac on medium heat and stored at -80 °C until further use.

LC-MS/MS data acquisition

Samples were reconstituted in water with 0.1% formic acid (FA). Sample concentrations were tested using a NanoDrop™ 2000 (Thermo Fisher Scientific, CA) at a wavelength of 205 nm and equal amount of peptide sample were loaded onto a 15 cm length, 75 µm i.d. in-house-packed Bridged Ethylene Hybrid C18 (1.7 µm, 130 Å) column and were analyzed on an Orbitrap Q Exactive HF mass spectrometer (Thermo Fisher Scientific, CA) coupled to a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific, CA). Mobile phase A was water with 0.1% FA, and mobile phase B was 80%ACN with 0.1% FA. Peptide samples were separated at a flow rate of 0.3 µL/min with a 142-min gradient: 0-18 min, 4 % phase B, and rises to 9 % phase B in 2 min, 9 % to 20 % phase B from 20 to 30 min, and ramped to 40 % B in 3 mins and the gradient was held at 40 % phase B for 79 mins, and ramped to 65 %B in 18 mins; the gradient was then ramped to 98 % phase B in 0.5 min and held at 98 % phase B for 6 mins and back to 4 % phase B and kept at 4 % for 6 mins for washing and equilibration. The following mass spectrometry parameters were used for data acquisition. Samples were analyzed on positive ion mode with a spray voltage of 2 kV, 275 °C capillary temperature, and S-lens RF level of 55. Full MS scans were acquired at m/z 200–2000 with a resolving power of 60,000 and AGC target of 1E6 with a maximum injection time of 120 ms. Top 20 precursors were selected for MS/MS data

acquisition. MS/MS acquisition was at a resolving power of 15,000, and AGC target of 5E4 with a maximum injection time of 150 ms. Only peaks with a charge state of 2-7 were fragmented through higher-energy collision dissociation (HCD) with a normalized collision energy (NCE) of 30. The dynamic exclusion of 30 s was used. Each sample was analyzed with one technical replicate because of the limited sample amount.

Data analysis

Two databases were used for the database search: a SignalP 6.0³³ filtered rat prohormone database and a self-curated neuropeptide database combining the rat neuropeptides from 4 open-source neuropeptide databases include NeuroPep³⁴, NeuroPred³⁵, NeuroPedia³⁶ and SwePep³⁷. Raw files were searched against both databases with PEAKS Studio (Version 10.6, Bioinformatics Solutions Inc, Waterloo, ON, Canada). For PEAKS analysis, the mass tolerance was set at 10ppm for precursor ions and 0.02 Da for fragment-ions. The following parameters were used for PEAKS *de novo* sequencing and database searches: no enzyme cleavage specified, instrument: Orbi-Orbi, HCD fragmentation, and precursor correction enabled. The following modifications were included in the search parameters: C-terminal amidation, pyroglutamation on glutamine and glutamic acid, N-terminal acetylation and oxidation. Peptides were searched with a maximum of three PTMs per peptide. No enzyme and unspecific cleavage were selected for enzymatic cleavage option with a max missed cleavage of 100. All other parameters were set to default. Statistical analysis for the resulting files was performed with Perseus³⁸ and R programming, including log₂(x) transformation, z-score normalization, Student's *t*-test, fold-change analysis, and principal component analysis. Gene ontology enrichment analysis was done with DAVID bioinformatics resources³⁹ with an FDR cutoff of 0.05.

Results and discussion

Experimental design: PTSD-like rat stress model and neuropeptide identification

A predator-exposure model of traumatic stress was used in this study, providing an ethologically relevant stimulus since natural predators of rats include foxes, ferrets, and cats; both live exposure and exposure to the predator's odor have been shown to induce a reliable stress response in rats^{30, 31, 40}. The stress responses caused by predator exposure are similar to PTSD symptoms, such as enhanced defensive behaviors, hypervigilance, stressed-induced anorexia, and exaggerated startle^{30, 31}. Rats were exposed to predator odor (the Stressed group) or no odor (the Ctrl group) as described above in the experimental section. We rigorously controlled for nonspecific variables that could alter the response to the stressor and thereby amplify potential individual differences between rats within each experimental group, since individual variability in stress reactions has been observed previously⁴¹⁻⁴³. Since rats can behave differently when exposed to predator stress, we took their individual behavioral response into consideration and monitored their activities after predator odor exposure. Rats' behaviors during the experiment were observed. **Figure 1** shows representative photos of the Ctrl (no odor) and Stressed (predator odor exposed) groups. Note that whereas the Ctrl group is asleep during the exposure phase, the Stressed group is showing multiple signs of stress (piloerection, hypervigilance, scanning the environment, and freezing behavior). After the designated exposure time, both Ctrl and Stressed groups of rats were sacrificed and the brain tissue punches of the BLA, PFC, HPC, PVN, and NAcc were collected for neuropeptide profiling; the workflow is shown in **Figure 2A**. These sites were selected on the basis of previous evidence indicating they were activated with predator exposure³². Briefly, tissue from each experimental group (Ctrl or Stressed) was processed as biological triplicates (N=3) followed by a thorough neuropeptide extraction prior to the LC-

MS/MS analysis. In order to minimize neuropeptide degradation, rats were perfused with chilled saline solution prior to brain dissection. Following decapitation and dissection, the brains were immediately heat stabilized⁴⁴. To retain most endogenous peptides from the crude sample, we used 8M urea and acidified methanol to extract both hydrophobic and hydrophilic neuropeptides⁴⁵. The peptide samples were also enriched using a 30 kDa MWCO filter and desalted before the LC-MS/MS analysis. Since we employed a data-dependent acquisition method for sensitive quantitation, a well-built rat neuropeptide database was important for the following analysis. Secher *et al.* showed that a combination of publicly available databases enabled higher identification numbers⁴⁵. As a result, we manually curated rat neuropeptide entries from 4 publicly available neuropeptide databases (NeuroPep³⁴, NeuroPred³⁵, NeuroPedia³⁶ and SwePep³⁷) and obtained a total of 322 neuropeptide sequences. In total, we observed 628 unique peptides from all brain regions collected (**Figure 2B, Supplementary Table S1**). In order to further review the peptides among the tissues, the identified peptides were plotted region-wise and 47% of the peptides were exclusive to a single brain region, while the remaining 53% of the peptides were colocalized in at least 2 brain regions (**Figure 2C**). 70 identified peptides were shared among all five regions, and this indicated that the 5 selected brain regions are closely related. However, there are still a large number of region-specific peptides identified, supporting their region-specific functionalities⁴⁶. For example, both neuropeptide Y (NPY) and the C-terminal flanking peptide of NPY were only discovered in BLA. A wealth of previous evidence indicates that NPY mitigates stress-induced effects¹⁹. NPY is an important biomarker in many neurological and psychological diseases, including PTSD^{47, 48}. Primeaux *et al.* used viral injection of NPY into amygdala and showed that increased NPY levels reduces stress responses for rodents in the elevated plus-maze paradigm⁴⁹. Our region-specific discovery

of the NPY family of peptides specifically in the BLA of Stressed rats is consistent with this result. Together these findings may suggest that trauma-like stress releases NPY family peptides, potentially to counteract stress-induced disease mechanisms that ultimately lead to PTSD.

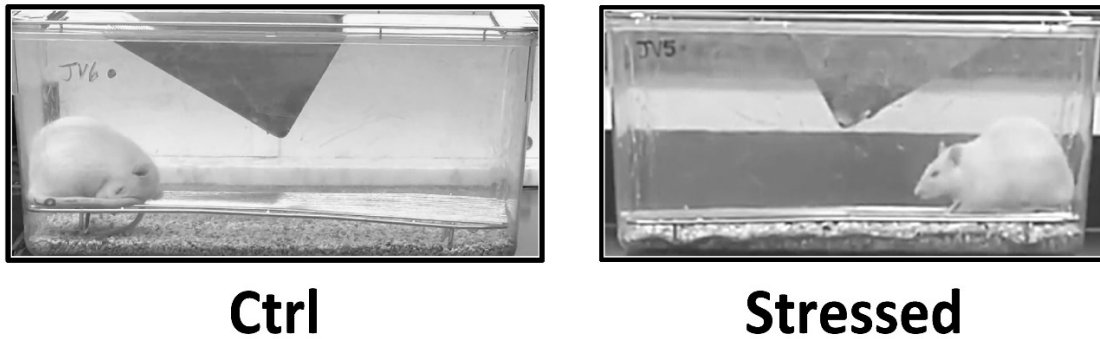


Figure 1. Representative photos of no stress (Ctrl) and Stressed rats during exposure. Note that Ctrl rat is asleep, indicating low/no stress, but Stressed rat is freezing, indicating hypervigilant defensive behavior in the presence of predator odor.

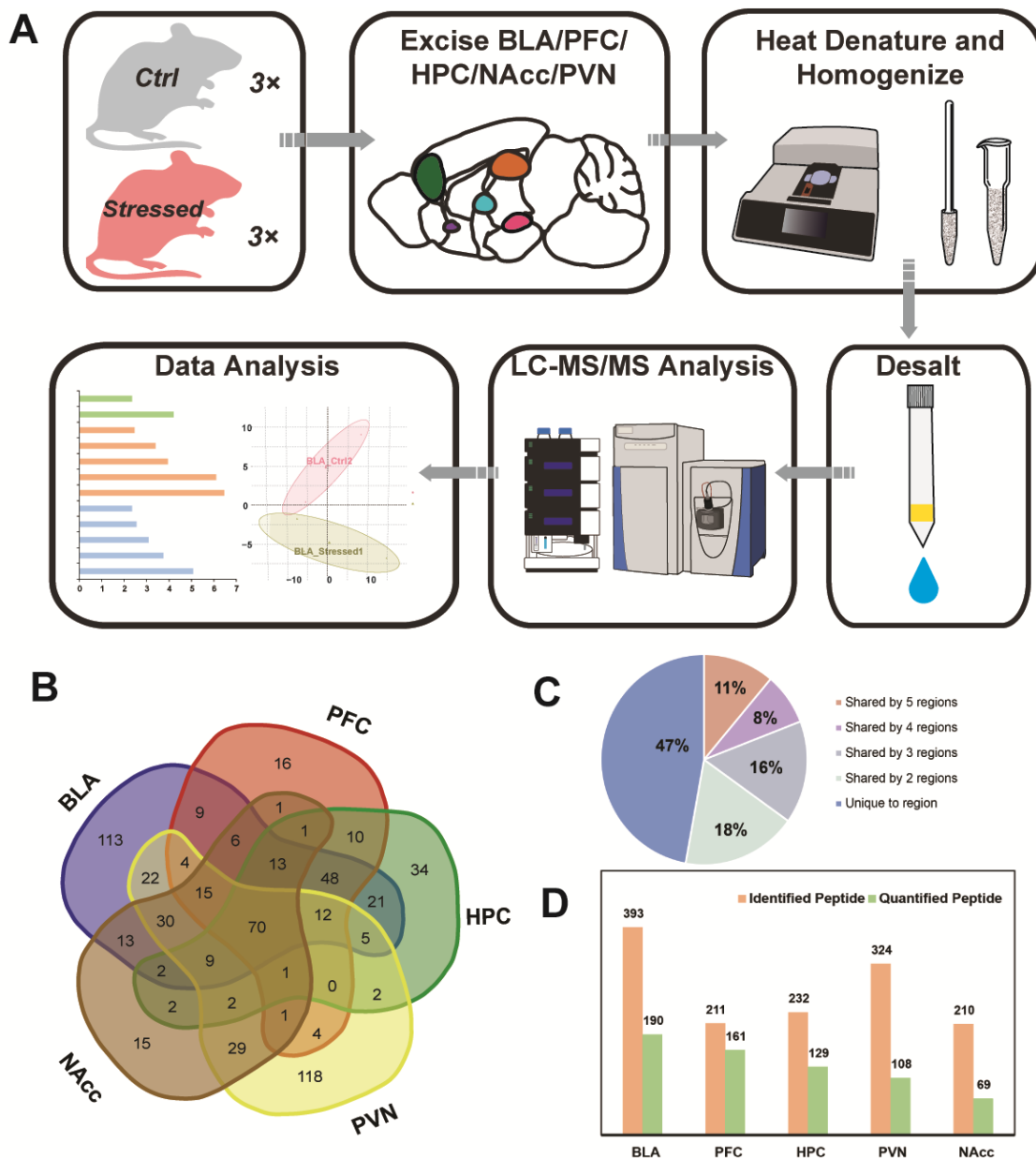


Figure 2. Experimental workflow and peptide identification. (A) Workflow for the LC-MS/MS analysis of neuropeptides extracted from rat brain regions. (B) Identified neuropeptides in five brain regions represented by a Venn diagram. (C) Localization of the identified peptides. (D) Number of identified and quantified peptides from 5 brain regions visualized by a bar graph (orange represented identified peptides, green represented quantified peptides).

Quantitative analysis of neuropeptides exhibiting significant changes

To assess the results with more confidence, we filtered the identified peptides with stringent criteria. Quantifiable peptides were selected only when the peptide could be relatively quantified based on their MS1 spectra in at least 2 biological replicates ($N \geq 2$). Hence, there were 190, 161, 129, 108, and 69 quantifiable peptides from BLA, PFC, HPC, PVN, NAcc, respectively (**Figure 2D, Supplementary Table S2**). The principal component analysis (PCA) of the brain regions especially for BLA, PVN, and NAcc showed that the Ctrl group and the Stressed group were clearly separated with little overlap (**Figure 3A, Figure S1A-D**). Although we strictly controlled for nonspecific variables for our rat model, the PCA figure for HPC and PFC showed some individual variability of rat peptidomes within each experimental group; this existence of individual differences is normal with predator stress paradigms⁴¹⁻⁴³. In fact, this is seen clinically with trauma exposure in humans⁵⁰; not every individual that experiences trauma goes on to develop PTSD. To identify the candidate neuropeptide markers in relation to predator stress, we then did a Student's *t*-test and further filtered the data with a fold change larger than 1.5, and a *p*-value smaller than 0.05. The significantly changed peptides in the five brain regions were visualized as a bar plot, where the upper panel indicated upregulation, and the bottom panel indicated downregulation (**Figure 3B, Supplementary Table S3**). Peptides derived from ProSAAS, granin and 7B2 were all downregulated in the Stressed group BLA region. The granin family and ProSAAS are both involved in the regulation of neuropeptide release, feeding, and locomotion^{51, 52}. Both big SAAS (ARPVKEPRSLSA) and little SAAS (SLSAASAPLAETSTP) were discovered to be downregulated, consistent with the decreased ingestive behavior and locomotion that are observed with predator stress exposure⁵³. As a protease convertase 2 (PC2) chaperone, the downregulation of 7B2 could result in an inactivation of PC2 and lead to an

increase in the adrenocorticotrophic hormone (ACTH), that could induce more stress reactions via increasing cortisol production^{54, 55}. Upregulations in substance P (RPKPQQFFGLMamide, tachykinin family, **Figure 4A**) and ACBP (KTYVEKVEELKKKYGI) levels in odor exposed rats were also observed; both peptides are involved in stress and appetite regulation^{21, 56}. A study done by Ebner *et al.* found that substance P was elevated in the amygdala after immobilization stress and that blocking its receptors reduced anxiety-like effects in the elevated plus-maze²¹. Thus, with an increased level of substance P, our neuropeptide results were in line with literature suggesting amygdala involvement in PTSD, and also our previous observation of exaggerated startle and enhanced defensive behaviors after predator stress and in PTSD^{30, 31, 57, 58}. Unlike BLA, studies have shown that PFC might be involved in stress alleviation (extinction of conditioned fear); PTSD patients have demonstrated a decrease in cortex volume⁹. A decrease in cortex volume could indicate not only a decrease in total NP production but also a reduced capacity to actively recruit the PFC stress modulation mechanism. We found a significant decrease of the Met-enkephalin peptide (YGGFM(O)RGL, opioid family, **Figure 4B**) in the odor-treated rats' PFC region. During acute stressors, the activation of BLA and HPC can suppress the function of PFC⁵⁹, and in this case, we indeed found more significantly changed neuropeptides in BLA and HPC compared to PFC. This could indicate that increased changes in neuropeptide abundance could be correlated to stress-induced hypersensitivity of those brain regions. Hippocampal function is also really important in stress regulation and has been found to play a role in consolidating memories and flashbacks in PTSD⁶⁰. Most peptides discovered in the HPC region were derived from CCK, ProSAAS, granin family, cocaine- and amphetamine-regulated transcript protein (CART), and vasoactive intestinal peptide (VIP), and all of which are related to stress regulation^{51, 52, 61, 62}. Interestingly, peptides from ProSAAS, unlike in the BLA,

were upregulated in the HPC region. PEN (AVDQDLGPEVPPENVLGALLRV), little SAAS (SAASAPLAETSTPL, **Figure 4C**), and big SAAS (ARPVEPRSLSAAPLAETSTPL) were discovered. However, their change in expression within HPC (~2.8 fold) is not as large as the change that is seen in BLA (~ 5-fold). Likewise, all granin peptides discovered were derived from secretogranin-I and secretogranin-II, and they also show differentiated expressions in the BLA region (downregulated) versus HPC and PVN regions (upregulated) in the Stressed group. These results indicated that neuropeptides regulate behavioral measures in a more complex manner whereby peptides from the same family have different expressions and potentially different functional roles in different brain regions.

We then performed gene ontology (GO) enrichment analysis using DAVID functional annotation bioinformatics microarray analysis³⁹ to examine the functional profile of all the peptides identified (**Figure 3C**). The two molecular function classifiers discovered were peptide hormone receptor binding and neuropeptide hormone activity. For the cellular component, the classifiers were extracellular space, secretory granule, and neuronal cell body. Both the molecular function and cellular component results underscored that our method for peptide profiling successfully and accurately identified the neuropeptides. For the biological processing classifiers, regulation of sensory perception of pain, negative regulation of appetite, and positive regulation of epinephrine secretion all conformed well to our stress-triggered Stressed group of rats.

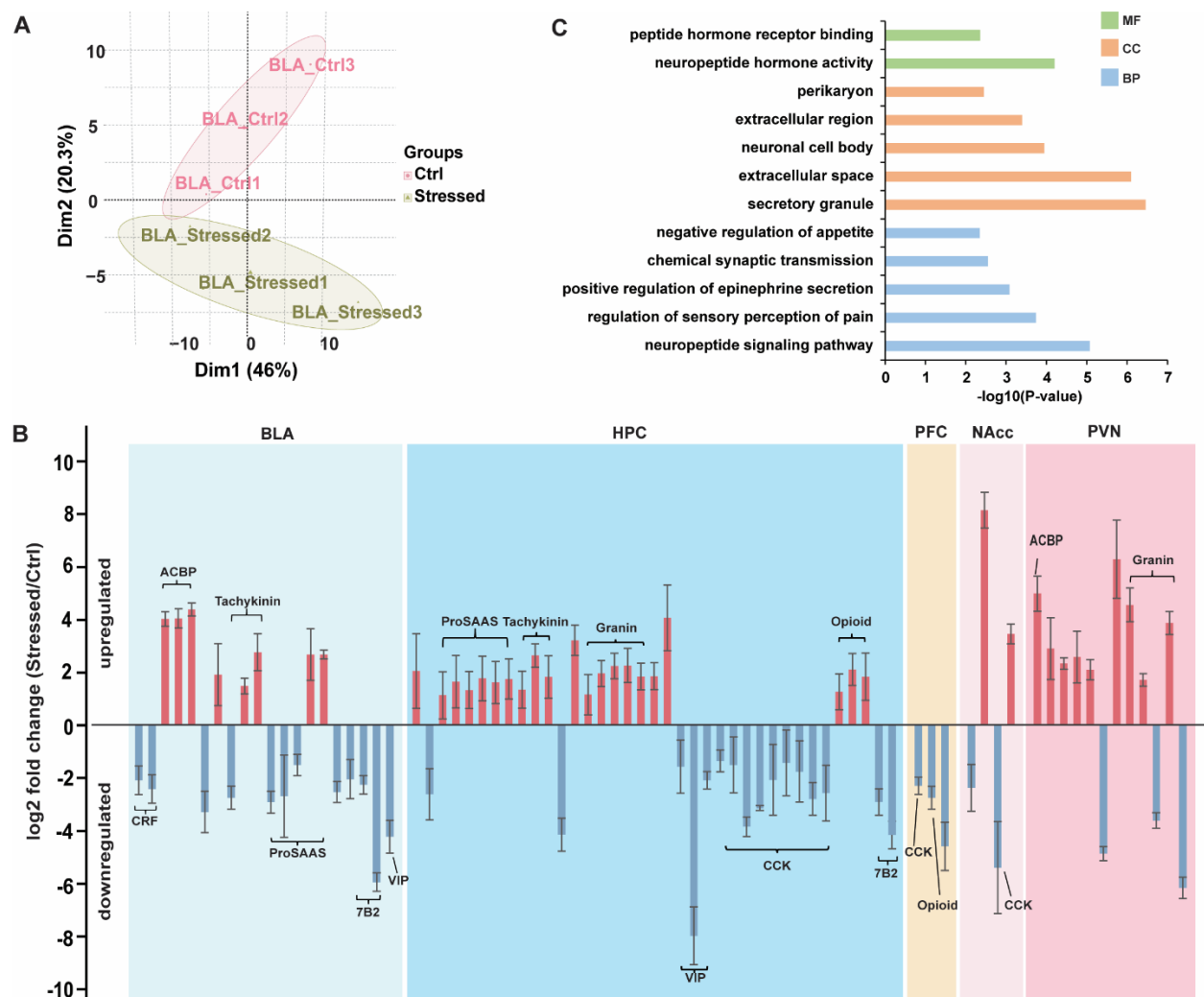


Figure 3. Data analysis for quantified peptide sequences. (A) PCA for BLA (pink indicated no-stress (Ctrl) samples, and green indicated Stressed samples). (B) Fold change analysis for significantly changed neuropeptide sequences. The upper panel indicated upregulated peptides and their peptide families. The bottom panel indicated downregulated peptides and their peptide families. Different brain regions were categorized using different color blocks. (C) GO analysis for the quantified peptides. MF: molecular function; CC: cellular component; BP: biological process.

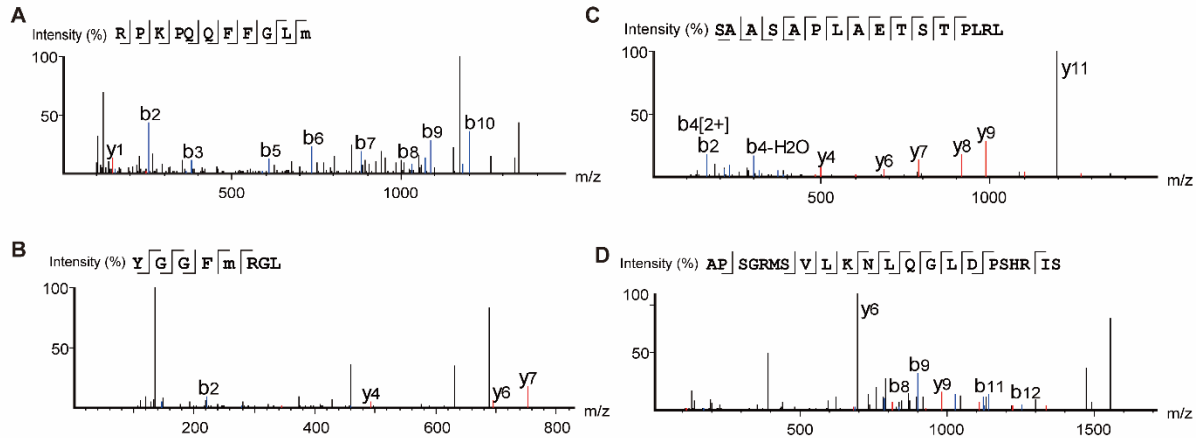


Figure 4. Representative MS/MS spectra. (A) RPKPQQFFGLMamide (substance P, tachykinin family) from BLA. (B) YGGFM(O)RGL (Met-enkephalin, opioid peptide) from PFC. (C) SAASAPLAETSTPL (little SAAS, ProSAAS) from HPC. (D) APSGRMSVLKLNQLGLDPSHRIS (CCK) from HPC.

Neuropeptide colocalization analysis

A lot of colocalizations of the peptides from the same family were also observed. All of the peptides detected from the CCK family in the HPC (**Figure 4D**), PVN, and NAcc regions were downregulated in the Stressed group. CCK peptides and their relation to anxiety are well characterized in the literature; they have a beneficial effect on behavioral fear responses and take part in fear memory formation⁶³. The reduction in CCK we observed here thus could indicate dysfunction of stress regulation and memory formation with our PTSD-like stressor^{61, 64}. The peptides from VIP in all brain regions detected (BLA, PFC, HPC) also showed a significant down-regulation (~ -16 fold). Ivanova *et al.* showed that VIP infusion into the CA1 region of the HPC reduces anxiety-like behavior in the elevated plus-maze in a rodent model of depression-like symptoms⁶⁵. A decrease of VIP level also accounts for numerous negative effects on the circulatory system and neuroendocrine system, including altering insulin release in the endocrine

system, and decreasing feeding⁶⁶. The increased level of CART that we found in the HPC and PVN region of Stressed rats could lead to a negative effect on feeding⁶⁷. When undergoing a stress condition, especially predator stress, studies have shown that rats exhibit a decrease in feeding⁵³, and predator odor was often used as a crop repellent since 1985⁶⁸. In this case, our neuropeptidome profiling result is concordant with previous studies in the literature.

Protease cleavage preference analysis

We also looked at the N-terminus and C-terminus amino acid patterns for the identified peptides searched with the neuropeptide database. By performing a linear sequence motif analysis, we discovered that hydrophobic amino acids tend to reside at the end of both N- and C-termini (**Figure 5A**). In order to look at the proteolytic cleavage pattern of the peptides, we also searched the raw files against SignalP 6.0 filtered³³ rat neuropeptide prohormone database. In total, we identified more peptides (both identified and quantified) from most brain regions (**Figure 5B**), but many of the peptides identified are prohormone related peptides but not functional neuropeptides (**Supplementary Table S4 and S5**). For the cleavage pattern of the neuropeptide in the N-terminus (**Figure 5C**), the most prevalent amino acids are arginine (R) and lysine (K), in line with the neuropeptide dibasic and monobasic cleavage pattern⁶⁹. In the C-terminus, except for the K and R, glycine (G) is also present in relatively high abundance (**Figure 5D**). Glycine is usually present at the C-terminus of amidated neuropeptides and is an essential component of neuropeptide functionalities⁷⁰. This result indicated that we discovered more mature neuropeptides than prohormone peptide cleavage products or peptide fragments, proving our sample preparation procedure did not induce further enzymatic cleavages.

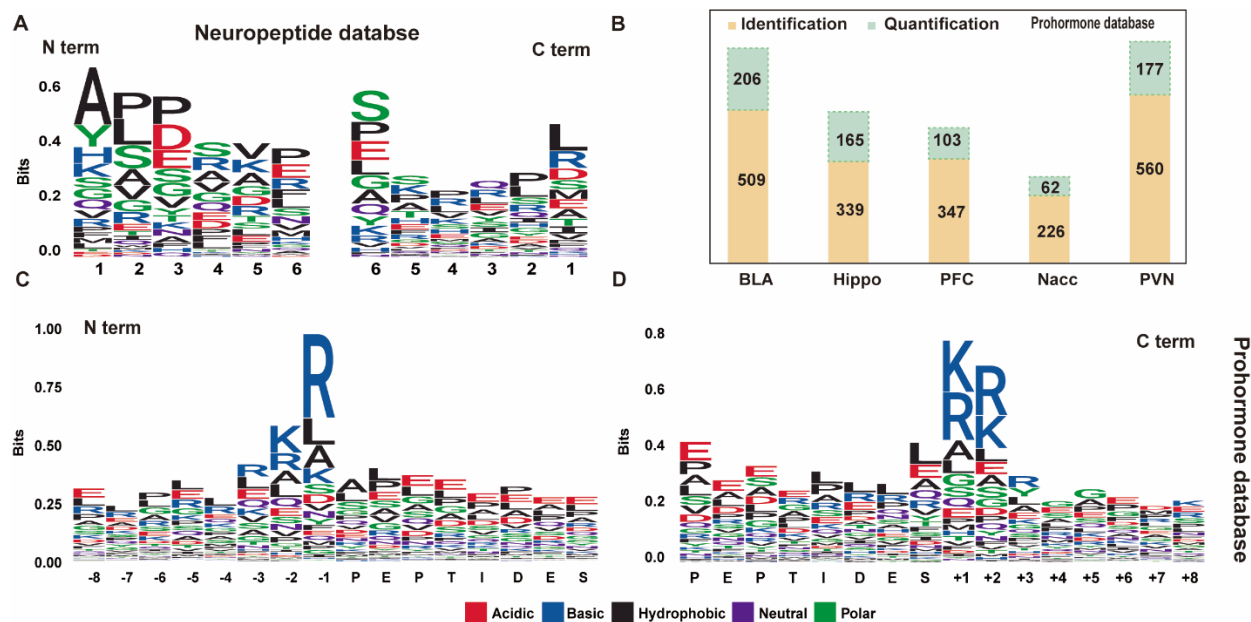


Figure 5. Prohormone protease cleavage preference analysis. (A) End sequence analysis for quantified neuropeptide sequences (resulting from the neuropeptide database). (B) Identified and quantified peptide sequences obtained from prohormone database searching. (C) N terminus and (D) C terminus protease cleavage preference. The “PEPTIDES” indicates the peptide sequence, whereas -1 to -8 indicate the N-terminal flanking peptides, and +1 to +8 indicate the C-terminal flanking peptide. (A), (C), and (D) color coding: red is acidic amino acids; blue is basic amino acids; black is hydrophobic amino acids; purple is neutral amino acids; green: polar amino acids.

Conclusions

This study examined the neuropeptidome across 5 separate brain regions and how it changed under a rodent analog of trauma-like stress, which can lead to PTSD. We successfully profiled the regions that are most closely related to stress regulation with label-free high-resolution mass spectrometry. In total, 628 unique peptide sequences were identified from the brain regions collected and 190, 161, 129, 108, and 60 peptides were quantified from BLA, PFC, HPC, PVN,

and NAcc respectively. Our results showed that traumatic stress (predator odor exposure) triggered peptidomic changes both qualitatively and quantitatively. Peptides from CCK, ProSAAS, CART, VIP, opioid family, and granin family were detected, and they are closely related to stress regulation, pain perception, and appetite. Our peptidome result was accordant with behavioral observations during the stress exposure as well as previous literature that demonstrate robust defensive behaviors including freezing in response to predator stress. We also took advantage of the specific sequence information provided by mass spectrometry and found that peptide fragments from the same protein precursor can have different changes in different brain regions. Collectively, our global neuropeptide profiling results paved the way for further targeted research and advanced the understanding of neuropeptide mechanisms that may underlie the process by which traumatic stress ultimately could lead to PTSD.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publication website (<http://pubs.acs.org>).

Figure S1. PCA for PFC, HPC, PVN, NAcc;

Table S1. Identified neuropeptide from five brain regions of rat;

Table S2. Quantified Neuropeptide from five brain regions of rat;

Table S3. T-test of the neuropeptide results;

Table S4. Identified unique modification specific prohormone peptide from five brain regions of rat;

Table S5. Quantified unique modification specific prohormone peptide from five brain regions of rat

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Author Contributions

W.W. and M.M. contributed equally to this work. M.M., W.W., V.P.B., and L.L. designed the study and conceived the experiments. V.P.B. did the live rodent work and collected rat brain tissue samples. M.M. and W.W. conducted the MS experiments and analyzed the data. W.W. wrote the first draft of the manuscript. I.A., L.G. assisted with the sample preparation and preparation of some figures. W.W., M.M., V.P.B. and L.L. prepared and revised the manuscript and all authors provided editorial feedback and input.

Notes

The authors declare no competing financial interest.

Data availability

Data are available via ProteomeXchange with identifier PXD038470.

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References

- (1) Yehuda, R. Post-traumatic stress disorder. *New England journal of medicine* **2002**, *346* (2), 108-114.
- (2) Davidson, J. R.; Hughes, D.; Blazer, D. G.; George, L. K. Post-traumatic stress disorder in the community: an epidemiological study. *Psychol Med* **1991**, *21* (3), 713-721. DOI: 10.1017/s0033291700022352
- (3) McFarlane, A. C.; Atchison, M.; Rafalowicz, E.; Papay, P. Physical symptoms in post-traumatic stress disorder. *J Psychosom Res* **1994**, *38* (7), 715-726. DOI: 10.1016/0022-3999(94)90024-8
- (4) Sherin, J. E.; Nemeroff, C. B. Post-traumatic stress disorder: the neurobiological impact of psychological trauma. *Dialogues in clinical neuroscience* **2022**, *13* (3), 263-278.
- (5) Harris, B. N.; Carr, J. A. The role of the hypothalamus-pituitary-adrenal/interrenal axis in mediating predator-avoidance trade-offs. *Gen Comp Endocrinol* **2016**, *230-231*, 110-142. DOI: 10.1016/j.ygcen.2016.04.006
- (6) Jankord, R.; Herman, J. P. Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. *Ann N Y Acad Sci* **2008**, *1148* (1), 64-73. DOI: 10.1196/annals.1410.012
- (7) Gunnar, M.; Quevedo, K. The neurobiology of stress and development. *Annu Rev Psychol* **2007**, *58*, 145-173. DOI: 10.1146/annurev.psych.58.110405.085605
- (8) Fenster, R. J.; Lebois, L. A. M.; Ressler, K. J.; Suh, J. Brain circuit dysfunction in post-traumatic stress disorder: from mouse to man. *Nat Rev Neurosci* **2018**, *19* (9), 535-551. DOI: 10.1038/s41583-018-0039-7
- (9) Shin, L. M.; Rauch, S. L.; Pitman, R. K. Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Ann N Y Acad Sci* **2006**, *1071* (1), 67-79. DOI: 10.1196/annals.1364.007
- (10) Anapindi, K. D. B.; Romanova, E. V.; Checco, J. W.; Sweedler, J. V. Mass Spectrometry Approaches Empowering Neuropeptide Discovery and Therapeutics. *Pharmacol Rev* **2022**, *74* (3), 662-679. DOI: 10.1124/pharmrev.121.000423.
- (11) van den Pol, A. N. Neuropeptide transmission in brain circuits. *Neuron* **2012**, *76* (1), 98-115. DOI: 10.1016/j.neuron.2012.09.014
- (12) Crown, A.; Clifton, D. K.; Steiner, R. A. Neuropeptide signaling in the integration of metabolism and reproduction. *Neuroendocrinology* **2007**, *86* (3), 175-182. DOI: 10.1159/000109095
- (13) Saper, C. B.; Chou, T. C.; Elmquist, J. K. The need to feed: homeostatic and hedonic control of eating. *Neuron* **2002**, *36* (2), 199-211. DOI: 10.1016/s0896-6273(02)00969-8
- (14) Hokfelt, T.; Broberger, C.; Xu, Z. Q.; Sergeev, V.; Ubink, R.; Diez, M. Neuropeptides--an overview. *Neuropharmacology* **2000**, *39* (8), 1337-1356. DOI: 10.1016/s0028-3908(00)00010-1
- (15) Dubowy, C.; Sehgal, A. Circadian Rhythms and Sleep in *Drosophila melanogaster*. *Genetics* **2017**, *205* (4), 1373-1397. DOI: 10.1534/genetics.115.185157
- (16) An, S.; Harang, R.; Meeker, K.; Granados-Fuentes, D.; Tsai, C. A.; Mazuski, C.; Kim, J.; Doyle, F. J., 3rd; Petzold, L. R.; Herzog, E. D. A neuropeptide speeds circadian entrainment by reducing intercellular synchrony. *Proc Natl Acad Sci U S A* **2013**, *110* (46), E4355-4361. DOI: 10.1073/pnas.1307088110
- (17) Arnison, P. G.; Bibb, M. J.; Bierbaum, G.; Bowers, A. A.; Bugni, T. S.; Bulaj, G.; Camarero, J. A.; Campopiano, D. J.; Challis, G. L.; Clardy, J.; et al. Ribosomally synthesized and post-

translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Nat Prod Rep* **2013**, 30 (1), 108-160, 10.1039/C2NP20085F. DOI: 10.1039/c2np20085f

(18) Ramos-Cejudo, J.; Genfi, A.; Abu-Amara, D.; Debure, L.; Qian, M.; Laska, E.; Siegel, C.; Milton, N.; Newman, J.; Blessing, E.; et al. CRF serum levels differentiate PTSD from healthy controls and TBI in military veterans. *Psychiatr Res Clin Pract* **2021**, 3 (4), 153-162. DOI: 10.1176/appi.prcp.20210017

(19) Schmeltzer, S. N.; Herman, J. P.; Sah, R. Neuropeptide Y (NPY) and posttraumatic stress disorder (PTSD): A translational update. *Exp Neurol* **2016**, 284 (Pt B), 196-210. DOI: 10.1016/j.expneurol.2016.06.020

(20) Hokfelt, T.; Ljungdahl, A.; Terenius, L.; Elde, R.; Nilsson, G. Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: enkephalin and substance P. *Proc Natl Acad Sci U S A* **1977**, 74 (7), 3081-3085. DOI: 10.1073/pnas.74.7.3081

(21) Ebner, K.; Rupniak, N. M.; Saria, A.; Singewald, N. Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats. *Proc Natl Acad Sci U S A* **2004**, 101 (12), 4280-4285. DOI: 10.1073/pnas.0400794101

(22) DeVane, C. L. Substance P: a new era, a new role. *Pharmacotherapy* **2001**, 21 (9), 1061-1069. DOI: 10.1592/phco.21.13.1061.34612

(23) Ebner, K.; Rjabokon, A.; Pape, H. C.; Singewald, N. Increased in vivo release of neuropeptide S in the amygdala of freely moving rats after local depolarisation and emotional stress. *Amino Acids* **2011**, 41 (4), 991-996. DOI: 10.1007/s00726-011-1058-0

(24) Pomrenze, M. B.; Giovanetti, S. M.; Maiya, R.; Gordon, A. G.; Kreeger, L. J.; Messing, R. O. Dissecting the Roles of GABA and Neuropeptides from Rat Central Amygdala CRF Neurons in Anxiety and Fear Learning. *Cell Rep* **2019**, 29 (1), 13-21 e14. DOI: 10.1016/j.celrep.2019.08.083

(25) Guan, X. M.; Yu, H.; Van der Ploeg, L. H. Evidence of altered hypothalamic pro-opiomelanocortin/ neuropeptide Y mRNA expression in tubby mice. *Brain Res Mol Brain Res* **1998**, 59 (2), 273-279. DOI: 10.1016/s0169-328x(98)00150-8

(26) Fricker, L. D. Analysis of mouse brain peptides using mass spectrometry-based peptidomics: implications for novel functions ranging from non-classical neuropeptides to microproteins. *Mol Biosyst* **2010**, 6 (8), 1355-1365. DOI: 10.1039/c003317k

(27) Neilson, K. A.; Ali, N. A.; Muralidharan, S.; Mirzaei, M.; Mariani, M.; Assadourian, G.; Lee, A.; van Sluyter, S. C.; Haynes, P. A. Less label, more free: approaches in label-free quantitative mass spectrometry. *Proteomics* **2011**, 11 (4), 535-553. DOI: 10.1002/pmic.201000553

(28) Sivanich, M. K.; Gu, T. J.; Tabang, D. N.; Li, L. Recent advances in isobaric labeling and applications in quantitative proteomics. *Proteomics* **2022**, 22 (19-20), e2100256. DOI: 10.1002/pmic.202100256

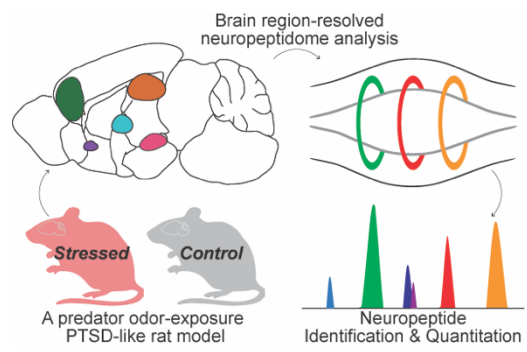
(29) Greer, T.; Lietz, C. B.; Xiang, F.; Li, L. Novel isotopic N,N-dimethyl leucine (iDiLeu) reagents enable absolute quantification of peptides and proteins using a standard curve approach. *J Am Soc Mass Spectrom* **2015**, 26 (1), 107-119. DOI: 10.1007/s13361-014-1012-y

(30) Bakshi, V. P.; Alsene, K. M.; Roseboom, P. H.; Connors, E. E. Enduring sensorimotor gating abnormalities following predator exposure or corticotropin-releasing factor in rats: a model for PTSD-like information-processing deficits? *Neuropharmacology* **2012**, 62 (2), 737-748. DOI: 10.1016/j.neuropharm.2011.01.040

- (31) Rajbhandari, A. K.; Baldo, B. A.; Bakshi, V. P. Predator Stress-Induced CRF Release Causes Enduring Sensitization of Basolateral Amygdala Norepinephrine Systems that Promote PTSD-Like Startle Abnormalities. *Journal of Neuroscience* **2015**, *35* (42), 14270-14285. DOI: 10.1523/Jneurosci.5080-14.2015.
- (32) Baisley, S. K.; Cloninger, C. L.; Bakshi, V. P. Fos expression following regimens of predator stress versus footshock that differentially affect prepulse inhibition in rats. *Physiology & Behavior* **2011**, *104* (5), 796-803. DOI: 10.1016/j.physbeh.2011.08.001.
- (33) Teufel, F.; Armenteros, J. J. A.; Johansen, A. R.; Gislason, M. H.; Pihl, S. I.; Tsirigos, K. D.; Winther, O.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology* **2022**, *40* (7), 1023-+. DOI: 10.1038/s41587-021-01156-3.
- (34) Wang, Y.; Wang, M.; Yin, S.; Jang, R.; Wang, J.; Xue, Z.; Xu, T. NeuroPep: a comprehensive resource of neuropeptides. *Database (Oxford)* **2015**, *2015*, bav038. DOI: 10.1093/database/bav038
- (35) Southey, B. R.; Amare, A.; Zimmerman, T. A.; Rodriguez-Zas, S. L.; Sweedler, J. V. NeuroPred: a tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides. *Nucleic Acids Res* **2006**, *34* (Web Server issue), W267-272. DOI: 10.1093/nar/gkl161
- (36) Kim, Y.; Bark, S.; Hook, V.; Bandeira, N. NeuroPedia: neuropeptide database and spectral library. *Bioinformatics* **2011**, *27* (19), 2772-2773. DOI: 10.1093/bioinformatics/btr445
- (37) Falth, M.; Skold, K.; Norrman, M.; Svensson, M.; Fenyo, D.; Andren, P. E. SwePep, a database designed for endogenous peptides and mass spectrometry. *Mol Cell Proteomics* **2006**, *5* (6), 998-1005. DOI: 10.1074/mcp.M500401-MCP200
- (38) Tyanova, S.; Temu, T.; Cox, J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc* **2016**, *11* (12), 2301-2319. DOI: 10.1038/nprot.2016.136
- (39) Dennis, G., Jr.; Sherman, B. T.; Hosack, D. A.; Yang, J.; Gao, W.; Lane, H. C.; Lempicki, R. A. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* **2003**, *4* (5), P3.
- (40) Takahashi, L. K.; Nakashima, B. R.; Hong, H.; Watanabe, K. The smell of danger: a behavioral and neural analysis of predator odor-induced fear. *Neurosci Biobehav Rev* **2005**, *29* (8), 1157-1167. DOI: 10.1016/j.neubiorev.2005.04.008
- (41) Sapolsky, R. M. Individual differences and the stress response. *Seminars in Neuroscience* **1994**, *6* (4), 261-269. DOI: 10.1006/smns.1994.1033.
- (42) COHEN, H.; ZOHAR, J. An Animal Model of Posttraumatic Stress Disorder: The Use of Cut-Off Behavioral Criteria. *Annals of the New York Academy of Sciences* **2004**, *1032* (1), 167-178. DOI: 10.1196/annals.1314.014.
- (43) Cohen, H.; Zohar, J.; Matar, M. A.; Zeev, K.; Loewenthal, U.; Richter-Levin, G. Setting apart the affected: the use of behavioral criteria in animal models of post traumatic stress disorder. *Neuropsychopharmacology* **2004**, *29* (11), 1962-1970. DOI: 10.1038/sj.npp.1300523
- (44) Yang, N.; Anapindi, K. D. B.; Romanova, E. V.; Rubakhin, S. S.; Sweedler, J. V. Improved identification and quantitation of mature endogenous peptides in the rodent hypothalamus using a rapid conductive sample heating system. *Analyst* **2017**, *142* (23), 4476-4485. DOI: 10.1039/c7an01358b

- (45) Secher, A.; Kelstrup, C. D.; Conde-Frieboes, K. W.; Pyke, C.; Raun, K.; Wulff, B. S.; Olsen, J. V. Analytic framework for peptidomics applied to large-scale neuropeptide identification. *Nat Commun* **2016**, *7* (1), 11436. DOI: 10.1038/ncomms11436
- (46) Godoy, L. D.; Rossignoli, M. T.; Delfino-Pereira, P.; Garcia-Cairasco, N.; de Lima Umeoka, E. H. A Comprehensive Overview on Stress Neurobiology: Basic Concepts and Clinical Implications. *Front Behav Neurosci* **2018**, *12*, 127. DOI: 10.3389/fnbeh.2018.00127
- (47) Lin, S.; Boey, D.; Herzog, H. NPY and Y receptors: lessons from transgenic and knockout models. *Neuropeptides* **2004**, *38* (4), 189-200. DOI: 10.1016/j.npep.2004.05.005
- (48) Sah, R.; Geraciotti, T. D. Neuropeptide Y and posttraumatic stress disorder. *Mol Psychiatry* **2013**, *18* (6), 646-655. DOI: 10.1038/mp.2012.101
- (49) Primeaux, S. D.; Wilson, S. P.; Cusick, M. C.; York, D. A.; Wilson, M. A. Effects of altered amygdalar neuropeptide Y expression on anxiety-related behaviors. *Neuropsychopharmacology* **2005**, *30* (9), 1589-1597. DOI: 10.1038/sj.npp.1300705
- (50) Sapolsky, R. M. Stress and the brain: individual variability and the inverted-U. *Nature Neuroscience* **2015**, *18* (10), 1344-1346. DOI: 10.1038/nn.4109.
- (51) Bartolomucci, A.; Possenti, R.; Mahata, S. K.; Fischer-Colbrie, R.; Loh, Y. P.; Salton, S. R. The extended granin family: structure, function, and biomedical implications. *Endocr Rev* **2011**, *32* (6), 755-797. DOI: 10.1210/er.2010-0027
- (52) Morgan, D. J.; Wei, S. W.; Gomes, I.; Czyzyk, T.; Mzhavia, N.; Pan, H.; Devi, L. A.; Fricker, L. D.; Pintar, J. E. The propeptide precursor proSAAS is involved in fetal neuropeptide processing and body weight regulation. *Journal of Neurochemistry* **2010**, *113* (5), 1275-1284. DOI: 10.1111/j.1471-4159.2010.06706.x.
- (53) Jochman, K. A.; Newman, S. M.; Kalin, N. H.; Bakshi, V. P. Corticotropin-releasing factor-1 receptors in the basolateral amygdala mediate stress-induced anorexia. *Behav Neurosci* **2005**, *119* (6), 1448-1458. DOI: 10.1037/0735-7044.119.6.1448
- (54) Braks, J. A. M.; Martens, G. J. M. 7b2 Is a Neuroendocrine Chaperone That Transiently Interacts with Prohormone Convertase Pc2 in the Secretory Pathway. *Cell* **1994**, *78* (2), 263-273. DOI: Doi 10.1016/0092-8674(94)90296-8.
- (55) Aguilera, G. Regulation of pituitary ACTH secretion during chronic stress. *Front Neuroendocrinol* **1994**, *15* (4), 321-350. DOI: 10.1006/frne.1994.1013
- (56) Bravo-San Pedro, J. M.; Sica, V.; Martins, I.; Pol, J.; Loos, F.; Maiuri, M. C.; Durand, S.; Bossut, N.; Aprahamian, F.; Anagnostopoulos, G. Acyl-CoA-binding protein is a lipogenic factor that triggers food intake and obesity. *Cell metabolism* **2019**, *30* (4), 754-767. e759.
- (57) McGaugh, J. L. The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annu Rev Neurosci* **2004**, *27*, 1-28. DOI: 10.1146/annurev.neuro.27.070203.144157
- (58) Liu, L.; Dai, L.; Xu, D.; Wang, Y.; Bai, L.; Chen, X.; Li, M.; Yang, S.; Tang, Y. Astrocyte secretes IL-6 to modulate PSD-95 palmitoylation in basolateral amygdala and depression-like behaviors induced by peripheral nerve injury. *Brain Behav Immun* **2022**, *104*, 139-154. DOI: 10.1016/j.bbi.2022.05.014
- (59) McEwen, B. S. Protection and damage from acute and chronic stress: allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. *Ann N Y Acad Sci* **2004**, *1032* (1), 1-7. DOI: 10.1196/annals.1314.001

- (60) Bannerman, D. M.; Rawlins, J. N. P.; McHugh, S. B.; Deacon, R. M. J.; Yee, B. K.; Bast, T.; Zhang, W. N.; Pothuizen, H. H. J.; Feldon, J. Regional dissociations within the hippocampus - memory and anxiety. *Neurosci Biobehav R* **2004**, *28* (3), 273-283. DOI: 10.1016/j.neubiorev.2004.03.004.
- (61) Bhatnagar, S.; Viau, V.; Chu, A.; Soriano, L.; Meijer, O. C.; Dallman, M. F. A cholecystokinin-mediated pathway to the paraventricular thalamus is recruited in chronically stressed rats and regulates hypothalamic-pituitary-adrenal function. *Journal of Neuroscience* **2000**, *20* (14), 5564-5573. DOI: Doi 10.1523/Jneurosci.20-14-05564.2000.
- (62) Keita, A. V.; Carlsson, A. H.; Cigehn, M.; Ericson, A. C.; McKay, D. M.; Soderholm, J. D. Vasoactive intestinal polypeptide regulates barrier function via mast cells in human intestinal follicle-associated epithelium and during stress in rats. *Neurogastroenterol Motil* **2013**, *25* (6), e406-417. DOI: 10.1111/nmo.12127
- (63) Ballaz, S. J.; Bourin, M. Cholecystokinin-Mediated Neuromodulation of Anxiety and Schizophrenia: A "Dimmer-Switch" Hypothesis. *Curr Neuropsychopharmacol* **2021**, *19* (7), 925-938. DOI: 10.2174/1570159X18666201113145143
- (64) Feng, H.; Su, J.; Fang, W.; Chen, X.; He, J. The entorhinal cortex modulates trace fear memory formation and neuroplasticity in the mouse lateral amygdala via cholecystokinin. *Elife* **2021**, *10*, e69333. DOI: 10.7554/eLife.69333
- (65) Ivanova, M.; Belcheva, S.; Belcheva, I.; Stoyanov, Z.; Tashev, R. Modulatory effect of VIP injected into hippocampal CA1 area on anxiety in olfactory bulbectomized rats. *Acta neurobiologiae experimentalis* **2014**, *74* (3), 317-327.
- (66) Vu, J. P.; Larauche, M.; Flores, M.; Luong, L.; Norris, J.; Oh, S.; Liang, L. J.; Waschek, J.; Pisegna, J. R.; Germano, P. M. Regulation of Appetite, Body Composition, and Metabolic Hormones by Vasoactive Intestinal Polypeptide (VIP). *J Mol Neurosci* **2015**, *56* (2), 377-387. DOI: 10.1007/s12031-015-0556-z
- (67) Koylu, E. O.; Balkan, B.; Kuhar, M. J.; Pogun, S. Cocaine and amphetamine regulated transcript (CART) and the stress response. *Peptides* **2006**, *27* (8), 1956-1969. DOI: 10.1016/j.peptides.2006.03.032
- (68) Sullivan, T. P.; Nordstrom, L. O.; Sullivan, D. S. Use of predator odors as repellents to reduce feeding damage by herbivores. *J. Chem. Ecol.* **1985**, *11* (7), 903-919.
- (69) Veenstra, J. A. Mono- and dibasic proteolytic cleavage sites in insect neuroendocrine peptide precursors. *Arch Insect Biochem Physiol* **2000**, *43* (2), 49-63. DOI: 10.1002/(SICI)1520-6327(200002)43:2<49::AID-ARCH1>3.0.CO;2-M
- (70) Eipper, B. A.; Stoffers, D. A.; Mains, R. E. The biosynthesis of neuropeptides: peptide alpha-amidation. *Annu Rev Neurosci* **1992**, *15* (1), 57-85. DOI: 10.1146/annurev.ne.15.030192.000421



TOC Figure