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Short-term administration of common anesthetics does not dramatically change the endogenous peptide profile in the rat pituitary

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Abstract

Cell-cell signaling peptides (e.g., peptide hormones, neuropeptides) are among the largest class of cellular transmitters and regulate a variety of physiological processes. To identify and quantify the relative abundances of cell-cell signaling peptides in different physiological states, LC-MS-based peptidomics workflows are commonly utilized on freshly dissected tissue. In such animal experiments, the administration of general anesthetics is an important step for many research projects. However, acute anesthetic administration may rapidly change the measured abundance of transmitter molecules and metabolites, especially in the brain and endocrine system, which would confound experimental results. The aim of this study was to evaluate the effect of short-term (< 5 min) anesthetic administration on the measured abundance of cell-cell signaling peptides, as evaluated by a typical peptidomics workflow. To accomplish this goal, we compared endogenous peptide abundances in the rat pituitary following administration of 5% isoflurane, 200 mg/kg sodium pentobarbital, or no anesthetic administration. Label-free peptidomics analysis demonstrated that acute use of isoflurane changed the levels of a small number of peptides, primarily degradation products of the hormone somatotropin, but did not influence the levels of most other peptide hormones. Acute use of sodium pentobarbital had a negligible impact on the relative abundance of all measured peptides. Overall, our results suggest that anesthetics used in pituitary peptidomics studies do not dramatically confound observed results.

Graphical Abstract

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Author Contributions

J.W.C. conceived of the study. S.M., F.I.H., M.S.R.A., M.T.A., and J.W.C. performed the experiments. H.Q. performed statistical analysis. All authors analyzed the data. S.M. and J.W.C. wrote the initial draft of the manuscript. All authors contributed to writing the final manuscript.

Supporting Information

Supporting Note, Supporting Figures S1–S7, and Supporting Table S1, and Supporting Document.

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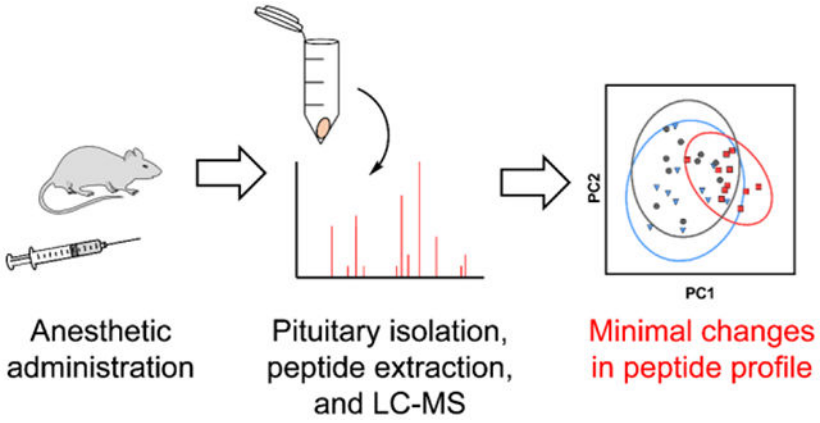

Keywords

peptidomics; anesthetics; mass spectrometry; pituitary; peptide hormones

Introduction

Neuropeptides and peptide hormones represent a large class of cell-to-cell signaling molecules that modulate critical functions in the central nervous and endocrine systems. Because of the central role cell-cell signaling peptides play in biology, identifying and quantifying these molecules is of great interest to understand both normal physiology and disease. Recent developments in liquid chromatography-mass spectrometry (LC-MS)-based "peptidomics" methodologies have enabled the thorough characterization and comparison of cell-cell signaling peptides in a variety of tissues and physiological states.[1–3] In contrast to antibody-based approaches (e.g., ELISA, Western blot), LC-MS-based peptidomics experiments do not require preselection of peptides of interest, allowing for non-targeted analysis of many endogenous peptide molecules simultaneously.

For peptidomics experiments involving animals, anesthetics may be implemented to facilitate specific experimental requirements. However, the use of anesthetics in peptidomics experiments is not standardized, and many protocols are adapted to meet experimental needs or conform with laboratory standards. Indeed, some peptidomics protocols call for use of volatile[3–10] or injectable anesthetics,[11–13] while others do not use any such anesthetic.[14–21] General anesthetics have been reported to alter the activity and the level of both proteins and small molecule neurotransmitters in the central nervous system (CNS).[22, 23] However, there is little data about the effects of these anesthetics on the full complement of cell-cell signaling peptides in brain and pituitary. Anesthetic use during a peptidomics experiment may alter the concentration or the enzymatic processing of peptides being measured, which may obscure changes resulting from experimental variables that are of real interest to researchers. Indeed, some prior studies suggest that common anesthetics like isoflurane, halothane, sevoflurane, or nitrous oxide can influence the levels of targeted cell-cell signaling peptides in the CNS, over either long-term or acute exposure.[24–33] However, how anesthetics change the measured peptide abundances over the course of a typical

peptidomics experiment, or what proportion of peptides may be affected, are unanswered questions.

Here, we performed label-free peptidomics analysis to evaluate the relative peptide levels from the rat pituitary under short-term (<5 min) administration of either isoflurane or sodium pentobarbital, two anesthetics commonly used in peptidomics studies prior to animal euthanasia. Our results demonstrate that anesthetic administration only minimally perturbs measured peptide abundances, suggesting that anesthetic use can be employed in peptidomics experimental designs without significant fear of confounding the results.

Results and Discussion

Analysis of peptide changes as a result of anesthetic administration

In this study, we sought to understand the influence of short-term anesthetic administration on peptide levels in the pituitary using label-free peptidomics (Figure 1).[2] We chose to evaluate isoflurane and sodium pentobarbital because these anesthetics are commonly used in peptidomics studies.[3, 4, 6, 12, 13] We chose to investigate the pituitary because it is a major producer of cell-cell signaling peptides that function in the hypothalamus-pituitary-adrenal (HPA) axis, and thus a common tissue analyzed for peptidomics experiments.[13, 15, 17, 34–37] Prior studies using immunoassays have suggested that some peptide hormone levels in the pituitary may be altered by long-term (>1 hr) anesthetic administration,[25, 26, 29] which make this an excellent tissue to examine for our study.

Thirty-two Sprague-Dawley rats were assigned to one of three groups: “No Anesthesia” group, “5% Isoflurane” group, or “200 mg/kg Sodium Pentobarbital” group. Animals in the “No Anesthesia” group were sacrificed by rapid decapitation, while animals in the “5% Isoflurane” or “200 mg/kg Sodium Pentobarbital” groups were administered the indicated anesthetic, allowed time for the anesthetic to take effect (negative assessment by toe pinch), and then sacrificed by rapid decapitation. The total time from initiation of anesthetic to negative toe pinch was comparable in both anesthetic groups (166 ± 43 sec for “5% Isoflurane”, 180 ± 23 sec for “200 mg/kg Sodium Pentobarbital”, Figure S1a). Likewise, the total time from initiation of anesthetic to sacrifice was similar between both anesthetic groups (181 ± 43 sec for “5% Isoflurane”, 197 ± 26 sec for “200 mg/kg Sodium Pentobarbital”, Figure S1b). Following sacrifice, the pituitary was rapidly isolated and flash-frozen in liquid nitrogen. The average pituitary dissection times following decapitation were not statistically distinguishable between any of the three groups (195 ± 42 sec for “No Anesthesia”, 186 ± 24 sec for “5% Isoflurane”, 204 ± 36 sec for “200 mg/kg Sodium Pentobarbital”, Figure S2). The high similarity in dissections times is critical to ensure that any similarities/differences in peptide levels between groups are due to anesthetic treatment and not due to peptide degradation post-mortem by endogenous proteases.[15, 18, 20]

Peptides were extracted from pituitaries using a two-stage protocol consisting of homogenization in acidified acetone[38, 39] followed by acidified water,[34] removal of high-molecular-weight proteins using a centrifuge-based filtration device, and desalting via solid-phase extraction (SPE). The approximate total peptide concentration was determined by bicinchoninic acid (BCA) assay, and 200 ng of each peptide extract was analyzed by LC-MS

and LC-MS/MS. Over 500 individual peptides were identified from all samples at the 1% false discovery rate (FDR) threshold. To facilitate downstream statistical analysis, data were filtered to remove peptides present in <50% of samples, absent from pooled quality control (QC) runs, or with <5 total MS2 identifications across runs, leaving a total of 164 peptides for statistical analysis (Table 1). As expected for the pituitary,[15, 17, 34, 40] the majority of these peptides arose from the pro-opiomelanocortin (POMC) precursor protein (COLI_RAT). Peptides were also identified from other known pituitary proteins, such as somatotropin (also known as growth hormone), ProSAAS, proenkephalin-B (also known as prodynorphin), vasopressin-neurophysin 2-copeptin, and others. Data were then processed, imputed using k-nearest neighbors algorithm,[41] and normalized using EigenMS (Figure S3).[42, 43]

Principal component analysis (PCA) of the resulting data showed considerable overlap between the three experimental conditions (Figure 2), suggesting little difference between the three groups based on peptide profile when using an unsupervised multivariate analysis technique. There was also no separation between female and male rats in the PCA (Figure S4), indicating that we detected no major differences in peptide profile due to biological sex. Separation between groups could be visualized when the data were analyzed using partial least squares-discriminant analysis (PLS-DA, Figure S5). Peptides with the highest VIP scores in PLS-DA (See Supporting Document) include peptides from somatotropin, pro-opiomelanocortin, and secretogranin-1, indicating the abundance profiles of these peptides contribute to the separation using this supervised multivariate method. Perhaps not surprisingly, many of the peptides with the highest VIP scores from the PLS-DA were the peptides that also were found to significantly differ in our univariate approaches, as described below.

We next examined the abundances of individual peptides using univariate statistical analysis to identify if any peptide abundances were altered due to experimental conditions (Figure 3, Table 2, Figure S6). Overall, the abundances of most peptides in both the “5% Isoflurane” and “200 mg/kg Sodium Pentobarbital” conditions were similar to those in the “No Anesthesia” group, suggesting that neither anesthetic has a major impact on peptide profile. When comparing “5% Isoflurane” treatment to “No Anesthesia”, a total of 15 peptides out of 164 analyzed were found in significantly different abundances as a result of anesthesia treatment with log2 fold-change >0.6 or <−0.6 (Table 2). The majority of these peptides (11/15) were degradation products of the N-terminus of mature somatotropin (also known as growth hormone), a pituitary hormone which plays a major role in growth.[44] Each of these somatotropin fragments were found to decrease as a result of 5% isoflurane administration ~2–5-fold. This result may indicate a change in somatotropin synthesis or its release from vesicle stores, both of which would lead to changes in the abundances in degradation products. Consistent with this observation, 15 min isoflurane administration has previously been shown to cause an increase in plasma somatotropin levels in humans, likely indicating increased release from the pituitary.[45] Unfortunately, our peptidomics approach cannot detect the full-length somatotropin because this mature protein is relatively large (190 residues) and contains two disulfide bonds, making it difficult to sequence by tandem mass spectrometry without enzymatic digestion. As a result, it is not clear if the mature protein changes in abundance in the pituitary as a

result of isoflurane administration similar to its degraded peptides. In contrast, two related peptides from secretogranin-1 (also known as chromogranin-B) increased as a result of 5% isoflurane administration, LLDEGHDPVHESPVDTA and LDEGHDPVHESPV, which were found to increase ~1.9- and ~2.3-fold, respectively. LLDEGHDPVHESPVDTA is a mature peptide hormone generated by the endogenous machinery responsible for the processing neuropeptides and peptide hormones[46, 47], while LDEGHDPVHESPV is a partially degraded form of this peptide likely generated after cellular release by proteases in extracellular space. Secretogranin-1, along with other granin-related proteins, plays roles in the sorting and processing of prohormones into neuropeptides and peptide hormones in dense core vesicles.[48] Secretogranin-1 also contains canonical basic cleavage sites which are hydrolyzed by prohormone processing machinery to generate smaller peptides, some of which may be biologically active.[49–51] Little is known about the biological functions of LLDEGHDPVHESPVDTA, but this peptide was previously demonstrated to increase in the rat hippocampus as a result of acute stress[52] and was found to decrease in the mouse hypothalamus as a result of chronic imipramine use.[53] Thus, changes in the levels of this secretogranin-1 peptide may reflect changes in processing or release of these peptides due to stress resulting from isoflurane administration. Consistent with this hypothesis, isoflurane has previously been shown to increase blood/serum corticosterone levels in some studies.[54, 55] One peptide each from glyceraldehyde-3-phosphate dehydrogenase and histone H4 were also found in slightly lower levels in the “5% Isoflurane” condition, although it is unclear if this observation has any biological relevance. Despite the changes described above, the majority of detected peptides, including the mature and truncated peptide hormones from the pro-opiomalanocortin, vasopressin-neurophysin 2-copeptin, proSAAS, and proenkephalin B precursors, did not show any significant changes as a result of 5% isoflurane administration relative to no anesthesia.

When comparing “200 mg/kg Sodium Pentobarbital” treatment to “No Anesthesia”, no peptides were found to be significantly different by pairwise comparison. This result demonstrates that sodium pentobarbital injection has negligible impact on the measured abundance of detected peptides over the time period studied. When compared directly with the result of isoflurane administration described above, sodium pentobarbital appears to impact measured peptide levels to a lesser extent than isoflurane.

We also performed a pairwise comparison of the “200 mg/kg Sodium Pentobarbital” and “5% Isoflurane” conditions. Here, we identified 16 peptides that were significantly different between the two anesthetic treatments (Figure S7 and Table S1), including several peptides that were not identified in previous pairwise comparisons with the “No Anesthesia” group. Significant peptides found in higher abundance in the “5% Isoflurane” group relative to “200 mg/kg Sodium Pentobarbital” group include the C-terminal peptide from neuroendocrine protein 7B2 (also known as secretogranin-5), additional internal processed forms of secretogranin-1, and a truncated form of α-melanocyte-stimulating hormone (α-MSH) from pro-opiomalanocortin. Neuroendocrine protein 7B2 is known to generate an extended C-terminal peptide (SVNPYLQGKRLDNVVAKKSVPHFSEEEKEPE) that acts as a potent inhibitor of prohormone convertase 2 (PC2).[56] Cleavage of this peptide at an internal dibasic site to generate the peptide seen in our experiments (SVPHFSEEEKEPE) by PC2 itself is a known method by which the full-length C-terminal peptide loses inhibitory activity.[50, 51]

Thus, the increase in 7B2 C-terminal peptide may indicate altered PC2 activity in the isoflurane condition, although there may be alternative explanations for this observation.

Discussion

Several prior studies have suggested that the administration of anesthetics can alter the levels of metabolites and signaling molecules in mammals. For example, short-term exposure (~2–3 min) to 100–150 mg/kg sodium pentobarbital in rats led to significant changes in the abundance of *Hif1a* and *Tnfa* mRNA and in the abundance of several metabolites and oxidative markers in serum and tissues.[57] A separate study identified changes in serum glucose, cholesterol, and fatty acid levels upon 120 mg/kg pentobarbital exposure.[58] Likewise, short-term exposure to isoflurane has been shown to alter plasma levels of a number of metabolites.[59, 60] Some studies also suggest that neuropeptide/peptide hormone abundance can be affected by anesthetic treatment. For example, the neuropeptides somatostatin, substance P, and cholecystokinin were found to be altered in several brain regions within 30 min of ketamine injection, while the levels of thyrotropin-releasing hormone did not change during this same time period.[61] Similarly, 15 min isoflurane administration has previously been shown to alter levels of Met-enkephalin and Leu-enkephalin in the brain.[27] Changes in the concentration of cholecystokinin immunoreactivity in the rat brain were noted for anesthetics such as halothane (after 15 min of inhalation), chloral hydrate (30–120 min after injection), and pentobarbital (30–60 min after the injection).[62] However, general anesthetics were not found to affect the release of cholecystokinin from isolated rat nerve terminals.[63] Several studies have also demonstrated that the long-term use of anesthetics (hours-days) causes significant changes in neuropeptide transcription, abundance, or release.[25, 26, 29–33]

One important prior peptidomics study has examined the effects of general anesthetic conditions on neuropeptide levels.[24] In this study, a mixture of isoflurane and nitrous oxide anesthesia was administered to tree shrews for 12 hours prior to euthanasia, peptide extraction, and LC-MS-based peptidomics analysis. The result of this study showed significant changes in several neuropeptides, most notably a >600% increase in somatostatin-14 abundances in the hypothalamus. However, the conditions of this experiment do not mimic a traditional peptidomics experiment, in which the animal would be subjected to the anesthetic for a much shorter period prior to euthanasia and would only receive isoflurane to induce anesthesia.

Our study differs from prior studies examining anesthetic effects on peptides in several important ways. First, many prior studies in this field measure transcript (mRNA) level changes resulting from anesthetic administration,[57, 64, 65] rather than directly measuring the peptide/protein products. Although such mRNA measurements provide insight into changes in gene expression resulting from anesthetic administration, total peptide levels also depend highly on rates of translation, post-translational processing and modifications, cellular release, and signal degradation. Thus, final peptide levels often cannot be inferred from transcriptomic measurements, but must be directly measured, as we have done here. Second, because our study utilized a non-targeted LC-MS-based approach, we were able to monitor the changes in peptide modifications and degradation that

would not be possible with traditional transcriptomic or antibody-based approaches. For example, the peptidomics approach easily allowed for evaluating the relative levels for multiple peptides from a single prohormone, including their individual degradation products, which would not be feasible using commonly used antibody-based approaches like radioimmunoassay.[25–27, 29, 30, 54, 61, 62] Finally, we specifically examined peptide changes on a very short time-scale (<5 min total). This contrasts with many prior studies, which examine extended periods of anesthetic administration,[24–26, 29–31, 65] or measure the changes at time points long after administration (≥30 min).[54, 61, 64] Our study thus provides valuable insight into any rapid changes in pituitary peptides that may occur in a typical peptidomics study.

Changes in measured peptide levels resulting from anesthetic exposure could influence study conclusions, including for studies that measure target peptides to understand physiology or for diagnostic purposes. Additionally, there are also certain experimental protocols in which anesthetic use cannot be applied consistently to all treatment groups. For example, our group and others are interested in studying changes in the cell-cell signaling peptides of hibernating mammals, such as the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*).[66] While anesthetic administration may be necessary for such wild-caught animals during the active season, the effectiveness of anesthetics is expected to change dramatically during the hibernation season when breathing, heart rate, and metabolism decrease dramatically. As a result, if anesthetic administration did significantly affect peptide levels, the animal's inconsistent metabolism may lead to artificial changes that may be mistaken as related to natural physiology. Interestingly, some previous studies on mammalian hibernators used different anesthetic conditions on active animals versus torpid animals.[67, 68] When embarking on such studies, it is important to understand the influence anesthetic treatment may have on levels of such molecules. However, it should be noted that other regions of the brain, particularly the hypothalamus, are also major producers of cell-cell signaling peptides. It will be of great interest for future efforts to examine the effect of anesthetics on neuropeptide levels in other brain regions using peptidomics approaches, and our study sets the stage for such research.

In conclusion, our results show that the use of short-term anesthetics (isoflurane or sodium pentobarbital) immediately prior to euthanasia only minimally perturbs the measured abundance of peptides in the rat pituitary. Although some differences were observed resulting from anesthetic administration, particularly for 5% isoflurane administration, these differences were limited to modest changes in a small number of peptides. Our results should be useful for future researchers in the area of peptidomics research, directly aiding in experimental design and interpretation of results.

Methods

Materials

LC-MS-grade solvents were used for all sample preparation prior to LC-MS analysis. Unless otherwise specified, all other reagents and solvents were purchased from ThermoFisher Scientific or MilliporeSigma.

Animals and tissue collection

All animal methods were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska (Project #2113).

Sprague Dawley rats, aged ~2 months, weight = 225 ± 24 g, were obtained from Charles River Laboratories (001CD). A total of 32 animals (17 females, 15 males) were assigned to one of three groups: “No Anesthesia” group (10 animals, 50% female/male), “5% Isoflurane” group (11 animals, 55% female/male), or “200 mg/kg Sodium Pentobarbital” group (11 animals, 55% female/male). Animal sacrifices were performed over the course of 4 consecutive days, with 2–3 animals from each group sacrificed per day. For animals in the “No Anesthesia” group, animals were sacrificed by rapid decapitation and the pituitary was immediately extracted, flash-frozen in liquid nitrogen, and stored on dry ice or −80 °C until processing. For animals in the “5% Isoflurane” group, animals were placed in a 3.2 L anesthesia chamber and administered 5% isoflurane in oxygen at 1.5 L/min. For animals in the “200 mg/kg Sodium Pentobarbital” group, animals were administered 200 mg/kg Fatal-Plus (stock concentration = 390 mg/mL, diluted to 39 mg/mL for animal injection) by intraperitoneal injection. For both anesthetic groups, animals were monitored for loss of righting reflex and a toe pinch test was used to confirm the loss of consciousness. Upon negative toe pinch, animals were sacrificed by rapid decapitation and the pituitary was immediately extracted, flash-frozen in liquid nitrogen, and stored on dry ice or −80 °C until processing.

Peptide extraction and sample preparation

Tissues were processed on the same day as isolation. For peptide extraction, frozen tissues were homogenized in 400 μL of ice-cold acidified acetone (40:6:1 acetone: water: con. HCl) using manual homogenization with a plastic pestle (Bel-Art) in microcentrifuge tubes, followed by sonication for 10 min and vortexing. Samples were then centrifuged at 15,000 × g, 10 min, 4 °C, and the supernatant was transferred to a clean tube and stored on ice. The remaining pellet was then subjected to the second stage of peptide extraction by adding 400 μL of acidified water (0.25% acetic acid in water). The tissue was again subjected to manual homogenization with a plastic pestle followed by sonication for 10 min and vortexing. Samples were centrifuged at 15,000 × g, 10 min, 4 °C, and the supernatant was combined with the acidified acetone extracts. The combined extracts were dried via a vacuum concentrator for at least 4 h to remove both water and acetone. Dried samples were redissolved in 300 μL of 5% acetonitrile (ACN)/water with 0.1% formic acid (FA) and centrifuged at 15,000 × g, 10 min, 4 °C to remove insoluble material. The resulting supernatant was passed through an Amicon Ultra-0.5 mL centrifugal filter unit with a 30 kDa molecular weight cutoff (Millipore Sigma, UFC503024), centrifuging at 14,000 × g for 15 min at 4 °C. The device was washed with 2 × 200 μL 5% ACN/water with 0.1% FA to ensure low molecular weight species passed through the filter. The resulting filtrate containing low molecular weight compounds was dried by a vacuum concentrator. The dried peptide extracts were then redissolved in 200 μL of 5% ACN/water with 0.1% FA and desalted via C18 SPE columns (Thermo Scientific, 889870). The resulting desalted peptide extracts were dried via vacuum concentration and stored at −20 °C until further analysis.

LC-MS and LC-MS/MS analysis

To prepare samples for LC-MS and LC-MS/MS analysis, dried and desalted peptide extracts were dissolved in 12 μL of 3% ACN/water with 0.1% FA, and the total protein concentration was estimated by BCA assay (Micro BCA Protein Assay Kit, Thermo Scientific, 23235). Each sample was diluted with 3% ACN/water with 0.1% FA to 33 ng/μL total protein. To account for batch effects, sample injection order was randomized, and injections of pooled quality control (QC) samples were included at regular intervals.

Prepared peptide extracts (6 μL of the above solution, ~200 ng total protein) were injected onto a Waters ACQUITY UPLC M-Class system coupled to a Waters Xevo G2-XS quadrupole time-of-flight mass spectrometer with Waters Z spray NanoLockSpray nano-electrospray ionization source. For nano-LC separations, the mobile phase Solvent A was composed of water and 0.1% FA, and Solvent B was composed of ACN and 0.1% FA. Samples were first loaded onto a Waters nanoEase M/Z symmetry C18 trap column (180 μm × 20 mm, product #186008821) for online desalting, using a loading solvent of 1% B and a flow rate of 5 μL/min for 10 min. Separations were then performed using a Waters nanoEase-C18 column (75 μm × 250 mm, product# 186008818) with a flow rate of 0.35 μL/min, column compartment temperature of 35 °C, and the autosampler compartment temperature of 8 °C. Peptides were separated using the following gradient: 0 – 40 min, 3 – 40% B; 40 – 44 min, 40 – 85% B; 44 – 48 min, 85% B; 48 – 50 min, 85 – 3% B; 50 – 60 min, 3% B. MS and MS/MS (collision-induced dissociation fragmentation) analysis was performed in positive ion mode with a mass range of 100–2000 *m/z*. For each MS scan, 5 precursor ions were selected for MS/MS using data-dependent acquisition based on a peak intensity threshold of 5000. The MS scan time was 0.5 s, and the MS/MS scan time was 1 s.

Data processing and statistics

The LC-MS data were processed using PEAKS Studio X Pro software (Bioinformatics Solutions Inc.)[69, 70] with the rat SwissProt reference proteome from UniProt[71] used as a database for peptide identifications. For peptide identifications, the parent mass error tolerance was set to 10 ppm, the fragment mass error tolerance was set to 0.1 Da, and the false discovery rate (FDR) threshold was set to 1%. The following PTMs were included as variable modifications: acetylation (N-terminus), amidation (C-terminus), oxidation (M), pyroglutamylation (E or Q), and phosphorylation (S, T, or Y). To calculate peptide peak areas, PEAKS Q Module for label-free quantification was used, and the PEAKS Q Module's ID-transfer was used to reduce false missing values using a mass error tolerance of 20.0 ppm and a retention time shift tolerance of 0.5 minutes. All identified peptides and peak areas (sum of peak areas for each charge state) were normalized to total ion current (TIC) and exported from the PEAKS Q Module. Peptides were then manually inspected to identify any ambiguous identifications during the ID-transfer step for peptide peaks with similar *m/z* and retention times. Peptide peaks with ambiguous assignments or peptides with <5 total MS2 identifications were filtered out.

Data were batch corrected using the statTarget R package,[72] where a peptide was considered present if it was measured in at least 50% of the samples per group, and was present in pooled QC samples (See note in Supporting Information). Peptides deemed absent were

omitted after batch correction, leaving 164 peptides “present” for analysis. Signal imputation was performed using k-nearest neighbor (knn; k=10) method.[41] Present peptides were then log2 transformed and normalized using EigenMS.[42, 43] Data preprocessing was prepared using the R statistical software version 4.0.3.[73]

Differential peptides were assessed using parametric (t-test) and non-parametric (Wilcoxon rank-sum) statistical tests to account for the degree, direction, and difference between two groups. For both statistical tests, the Benjamini-Hochberg (BH) procedure was applied to correct for multiple hypothesis testing.[74] Peptides were considered differential when they passed with a BH-corrected p-value less than 0.05 in both statistical tests. The fold change (FC) was calculated for each peptide to quantify the magnitude difference between the two groups. In this statistical analysis, three pairs of comparisons were performed: “5% Isoflurane” vs “No Anesthesia”, “200 mg/kg Sodium Pentobarbital” vs “No Anesthesia”,, and “200 mg/kg Sodium Pentobarbital” vs “5% Isoflurane”. Principal component analysis (PCA) was performed using R package pcaMethods[75] and partial least squares-discriminant analysis (PLS-DA) was performed used R package ropls.[76]

The mass spectrometry peptidomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[77] partner repository with the dataset identifier PXD034821 and 10.6019/PXD034821.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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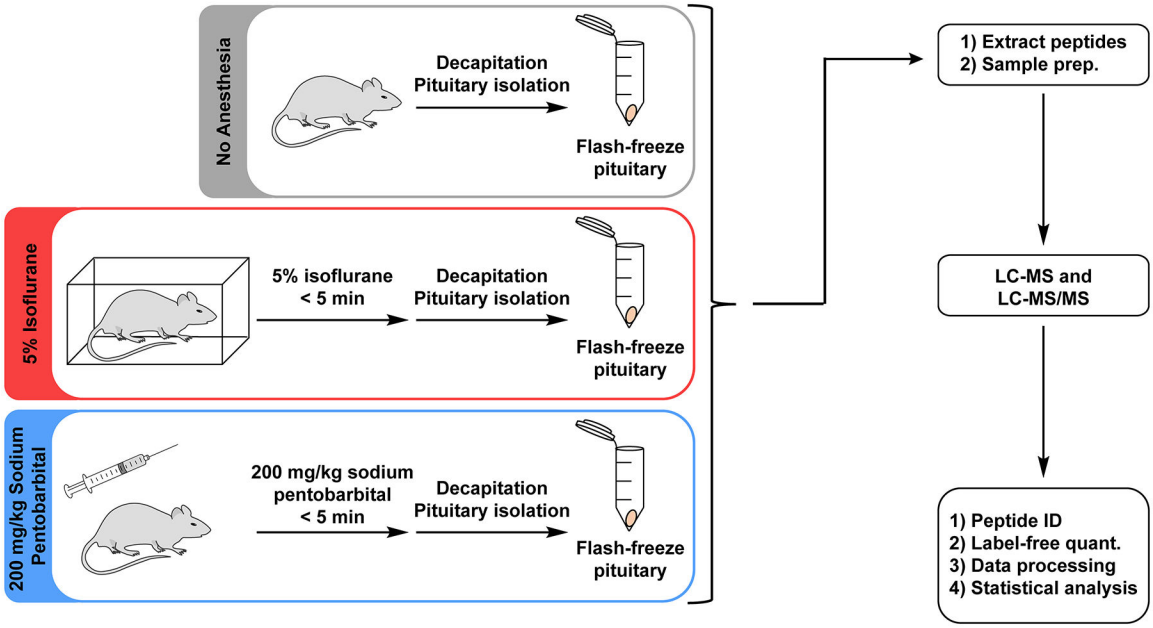

Figure 1.

Overview of the experimental design. Rats were separated into “No Anesthesia”, “5% Isoflurane”, or “200 mg/kg Sodium Pentobarbital” groups. For each group, animals were subjected to the indicated condition prior to rapid decapitation and pituitary isolation, after which pituitaries were flash frozen. Peptides were then extracted from pituitary samples and analyzed using an LC-MS and LC-MS/MS peptidomics workflow.

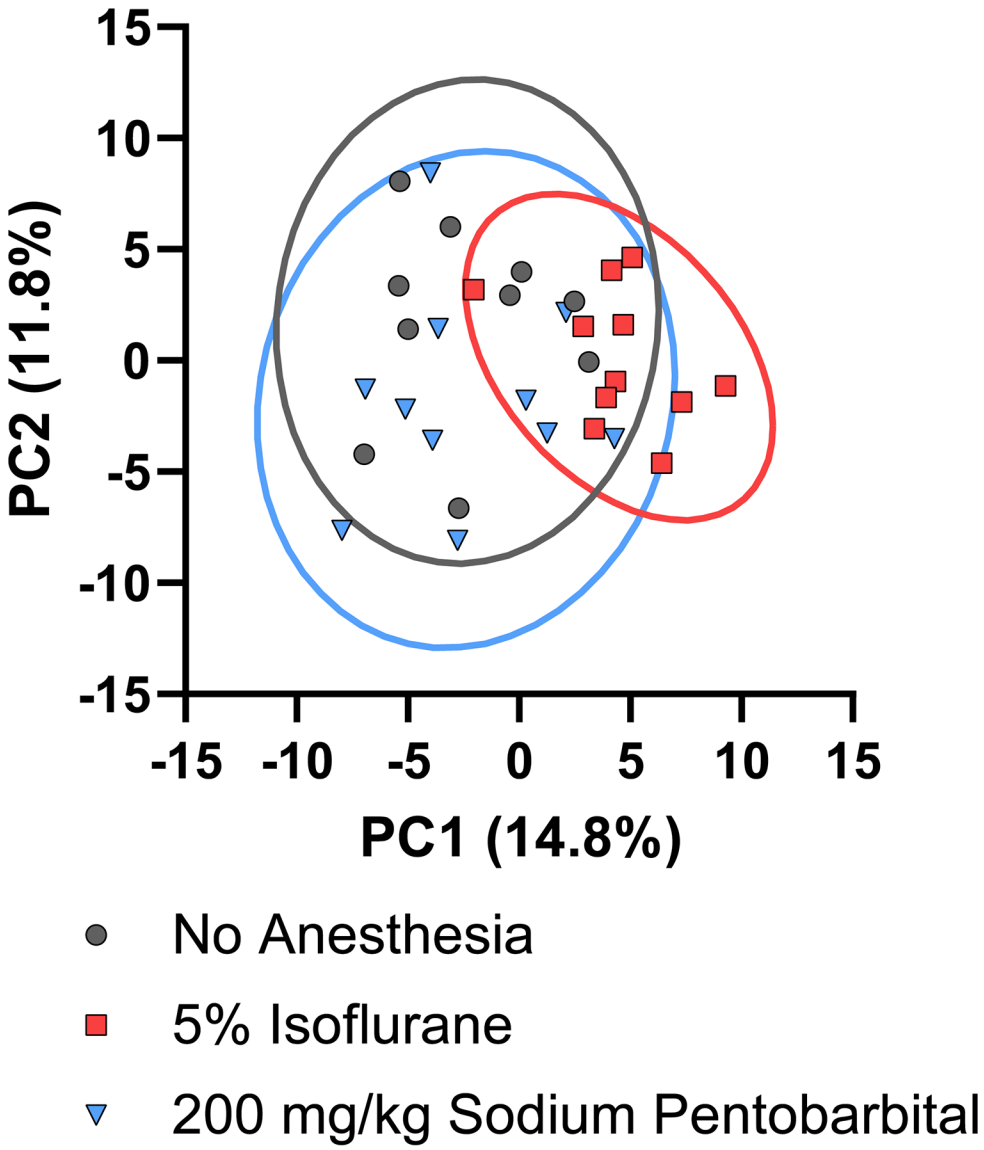

Figure 2.
PCA scores plot for peptide abundance data after preprocessing and normalization by EigenMS shows no separation of the three experimental conditions based on peptide profile.

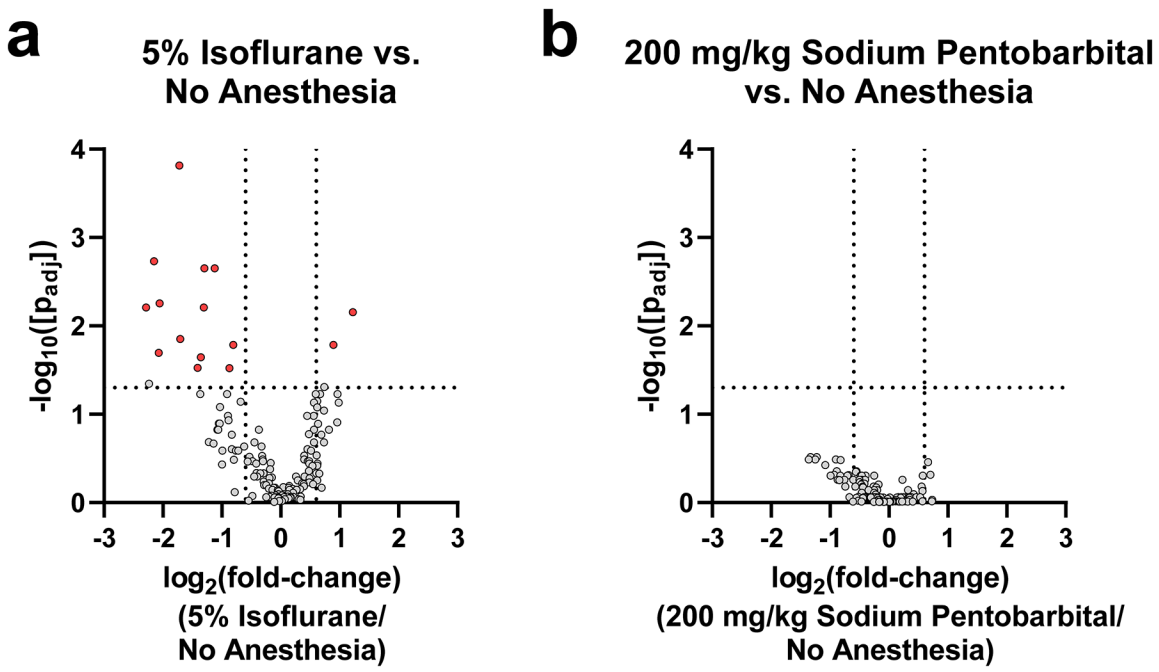

Figure 3.

Volcano plots comparing pituitary peptide changes for (a) “5% Isoflurane” or (b) “200 mg/kg Sodium Pentobarbital” compared to “No Anesthesia” condition. Red data points indicate significantly changing peptides (passing both parametric and non-parametric tests) with a $\log_2$(fold-change) > 0.6 or < −0.6. The two vertical lines are demarcation points for the $\log_2$(fold-change) > 0.6 or < −0.6 between the groups. The horizontal line marks a corrected p-value of 0.05. Grey points above this horizontal line did not pass both the parametric and non-parametric tests.

Table 1.

Protein distribution of rat pituitary peptides analyzed in this study.

Protein ID	Gene name	Protein name	Peptides
COLI_RAT	POMC	Pro-opiomelanocortin	46
SOMA_RAT	GH1	Somatotropin	25
SCG1_RAT	CHGB	Secretogranin-1	16
NEU2_RAT	AVP	Vasopressin-neurophysin 2-copeptin	12
HBB1_RAT/HBB2_RAT	HBB	Hemoglobin subunit beta-1/2	9
HBA_RAT	HBA1	Hemoglobin subunit alpha-1/2	6
PCS1N_RAT	PCSK1N	ProSAAS	6
PDYN_RAT	PDYN	Proenkephalin-B	5
7B2_RAT	SCG5	Neuroendocrine protein 7B2	5
CMGA_RAT	CHGA	Chromogranin-A	4
G3P_RAT	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	3
SCG2_RAT	SCG2	Secretogranin-2	3
H4_RAT	H4C2	Histone H4	3
LSHB_RAT	LHB	Lutropin subunit beta	2
CBPE_RAT	CPE	Carboxypeptidase E	2
PGAM1_RAT	PGAM1	Phosphoglycerate mutase 1	2
SCG3_RAT	SCG3	Secretogranin-3	2
NEU1_RAT	OXT	Oxytocin-neurophysin 1	1
PRL_RAT	PRL	Prolactin	1
ALBU_RAT	ALB	Albumin	1
MDHM_RAT	MDH2	Malate dehydrogenase, mitochondrial	1
CLUS_RAT	CLU	Clusterin	1
NMU_RAT	NMU	Neuromedin-U	1
NEC2_RAT	PCSK2	Neuroendocrine convertase 2	1
MIF_RAT	MIF	Macrophage migration inhibitory factor	1
CX7A2_RAT	COX7A2	Cytochrome c oxidase subunit 7A2, mitochondrial	1
COF1_RAT	CFL1	Cofilin-1	1
TPIS_RAT	TPI1	Triosephosphate isomerase	1
UBB_RAT	UBB (and others)	Ubiquitin	1
GNAS3_RAT	GNAS	Neuroendocrine secretory protein 55	1

Table 2.

Peptides found to significantly change as a result of “5% Isoflurane” administration relative to the “No Anesthesia” condition. A “fold-change” value >0 indicates the peptide was measured in higher abundance in the “5% Isoflurane” condition, while a “fold-change” value <0 indicates the peptide was measured in lower abundance in the “5% Isoflurane” condition.

Peptide	Protein ID	Fold-change (log2)
LDEGHDPVHESPV	SCG1_RAT	1.22
LLDEGHDPVHESPVDTA	SCG1_RAT	0.89
VKVGVNGF	G3P_RAT	−0.81
RTLYGFGG	H4_RAT	−0.87
FPAMPLSSLFANAVLRAQHLHQ	SOMA_RAT	−1.12
FPAMPLSSLFANAVLRAQHLH	SOMA_RAT	−1.30
FPAMPLSSLF	SOMA_RAT	−1.31
FPAMPLSSLFANAVLRAQHLHQLAAD	SOMA_RAT	−1.36
FPAMPLSSLFANAVLRAQHLHQLA	SOMA_RAT	−1.41
FPAMPLSSLFANAVLRA	SOMA_RAT	−1.71
FPAMPLSSLFANAVLRAQH	SOMA_RAT	−1.72
FPAMPLSSLFAN	SOMA_RAT	−2.06
FPAMPLSSLFANA	SOMA_RAT	−2.07
FPAMPLSSLFANAVLR	SOMA_RAT	−2.15
FPAMPLSSLFA	SOMA_RAT	−2.29