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Analysis of peptides secreted from cultured mouse brain tissue

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Abstract

Peptides represent a major class of cell-cell signaling molecules. Most peptidomic studies have focused on peptides present in brain or other tissues. For a peptide to function in intercellular signaling, it must be secreted. The present study was undertaken to identify the major peptides secreted from mouse brain slices that were cultured in oxygenated buffer for 3–4 hours. Approximately 75% of the peptides identified in extracts of cultured slices matched the previously reported peptide content of heat-inactivated mouse brain tissue, whereas only 2% matched the peptide content of unheated brain tissue; the latter showed a large number of postmortem changes. As found with extracts of heat-inactivated mouse brain, the extracts of cultured brain slices represented secretory pathway peptides as well as peptides derived from intracellular proteins such as those present in the cytosol and mitochondria. A subset of the peptides detected in the extracts of the cultured slices was detected in the culture media. The vast majority of secreted peptides arose from intracellular proteins and not secretory pathway proteins. The peptide RVD-hemopressin, a CB1 cannabinoid receptor agonist, was detected in culture media, which is consistent with a role for RVD-hemopressin as a non-classical neuropeptide. Taken together with previous studies, the present results show that short-term culture of mouse brain slices is an appropriate system to study peptide secretion, especially the non-conventional pathway(s) by which peptides produced from intracellular proteins are secreted.

Keywords

Neuropeptide; bioactive peptide; peptidomics

INTRODUCTION

The largest class of molecules involved in signaling between cells is that of peptides, with over 100 distinct peptides known to function as neuropeptides or peptide hormones [1]. Neuropeptides and peptide hormones function in a wide range of physiological systems including reproduction, feeding, circadian rhythms, sleep and arousal, pain, anxiety, depression, memory, metabolism, and the homeostatic control of body water, fat, sodium, potassium, and glucose [1, 2]. In addition to the known neuropeptides and peptide hormones, hundreds of other peptides have been identified from peptidomic analyses of

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brain and other neuroendocrine tissues or cell lines [3-11]. Some of these additional peptides are present in the secretory pathway and produced from known neuropeptide precursors or from other secretory pathway proteins [12]. Based on their presence in the secretory pathway, these peptides are presumably secreted from cells and potentially function as signaling molecules.

In addition to the secretory pathway peptides, other peptides identified in previous peptidomic studies are derived from cytosolic, mitochondrial, or nuclear proteins [10, 13-15]. The function of these intracellular peptides is not clear. Some intracellular peptides may participate in the regulation of protein-protein interactions [14, 16, 17]. Other peptides derived from intracellular proteins have been found to bind to cell surface receptors as either agonists or antagonists. For example, the hemoglobin-derived peptide named hemopressin is a 9 residue peptide which binds to CB1 cannabinoid receptors and functions as an inverse agonist, while longer N-terminally extended hemopressin peptides (such as RVD-hemopressin and VD-hemopressin) bind to CB1 cannabinoid receptors and act as agonists [18-20].

In order to make predictions of the function of newly discovered peptides, it is important to know which peptides are secreted from cells. For example, the hemopressin peptides can play a role in cell-cell signaling only if they are secreted [20]. Therefore, a fundamental question is whether the peptides derived from intracellular proteins are secreted from cells. There are numerous previous reports of intracellular peptides and proteins functioning outside of the cell. In yeast, the peptide α -mating factor is produced in the cytosol and secreted where it serves as a mating pheromone [21]. In mammals, a number of cytosolic or mitochondrial proteins and peptides have been previously found to be secreted from cells; examples include basic fibroblast growth factor, thymosin, interleukins, and others [22-24]. Various mechanisms of secretion of intracellular peptides have been proposed [25], and while this is an important area for further studies, it is necessary to first know which of the cellular peptides are secreted.

The present study was aimed at identifying peptides secreted from cultured slices of mouse brain. Before considering secretion, it was important to validate the slice cultures as being representative of peptides previously found in mouse brain. Initial peptidomics studies on mouse brain used approaches developed decades earlier for studies examining peptides using radioimmunoassays [9]. For many of these analyses, mice were sacrificed by decapitation, the brains were removed from the skull, dissected into regions, and quickly frozen on dry ice until subsequent processing which typically involved homogenization of the frozen tissue in dilute acid and heat inactivation of proteases [26]. While this general procedure was used for many studies employing radioimmunoassays to detect peptides, there were two problems with this approach for peptidomic studies. First, heating in dilute acid caused the breakdown of some peptides and proteins at bonds adjacent to Asp residues, with pronounced cleavage of Asp-Pro bonds [26, 27]. Second, a large number of protein fragments were detected that appeared to arise from postmortem changes in the brain, which were independent of the acid-induced extraction artifacts [3, 27, 28]. Together, these extraction-induced and postmortem artifacts caused changes in the brain peptidome that complicated the interpretation of the results.

A solution to the postmortem complications was identified by the Andren laboratory, and involved sacrificing mice with focused microwave irradiation to rapidly raise the temperature of the brain and inactivate all cellular processes [3]. Subsequently, our laboratory found that decapitation followed by microwave irradiation in a conventional microwave oven could achieve the same result and suppress the postmortem production of peptides [27]. Furthermore, the use of microwave-induced heat inactivation eliminated the need for

homogenizing the tissue in hot acid, thus greatly reducing the problem with the extraction-induced cleavages. While heat inactivation using microwave irradiation or other methods was useful for peptidomic analyses of brain peptides, this approach would not permit studies on secreted peptides; once slices are heat inactivated all cellular processes stop, including secretion. The Sweedler laboratory examined secretion of peptides from brain slices prepared using methods similar to those used for electrophysiology, and which typically remain electrically active for many hours [5]. In this approach, mice are sacrificed by decapitation and the brains rapidly removed from the skull, sliced into 500 micron sections, and cultured for 3-4 hours in oxygenated artificial cerebrospinal fluid (aCSF) prior to processing for peptide extraction. This culture period is thought to allow the cells to recover from the injury of slice preparation and hypoxic conditions that occur after decapitation. However, previous studies did not compare the peptidome of cultured slices to that of the mouse brain. Therefore, the first goal of this study was to assess whether the peptides detected in cultured slices more closely resembles those in unheated or heat-inactivated brains. Once it was established that peptides present in the cultured slices were generally free of the postmortem changes seen in unheated brains, the second goal was to examine peptides secreted from the slices and compare to those peptides found in slices but not secreted. The finding that RVD-hemopressin is secreted from slices supports the proposal that this peptide functions as a cell-cell signaling molecule, and the finding of many additional secreted peptides raises the possibility of other cytosol-produced peptides functioning in cell-cell communication.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma/Aldrich unless indicated. Acetonitrile was obtained from Fisher. Hydrochloric acid and trifluoroacetic acid mass spectroscopy grade were obtained from Pierce Thermo Scientific. The isotopic labeling reagents 4-trimethylammoniumbutyryl-N-hydroxysuccinimide (TMAB-NHS) containing either 0, 3, 6, or 9 atoms of deuterium (D0-, D3-, D6-, and D9-, respectively) or 9 atoms of deuterium and three ¹³C atoms (D12-) were synthesized as described [29].

Animals and the preparation of slices

All studies involving mice were carried out in accordance with the standards described in the European Commission policy on laboratory animals Directive 2010/63/EU (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). A total of 24 mice were used for the preparation of slices described in this report. Both males and females were used, ranging in age from 8 weeks to several months. Mice were sacrificed by cervical dislocation followed by decapitation. The brain was removed from the skull and three coronal sections (each of 500 μ m thickness) from each mouse were prepared with a McIlwain Tissue Chopper. The slices were immediately transferred to aCSF medium and incubated for 3 hours at 36°C. Oxygenation was sustained throughout the incubation period by bubbling 95% O₂/5% CO₂ into the aCSF. Following this incubation, the slices were transferred to a 12-well plate (three slices per well) and incubated in 800 μ l of oxygenated aCSF for 1 hour. After incubation, the three slices and media from each well were transferred into 2 ml low-retention microcentrifuge tubes, followed by the immediate addition of hot water to the slices. The slices and media were then incubated in a hot water bath at 80°C for 20 minutes to inactivate proteases and preclude enzymatic degradation of the peptides. The slices and media were stored at -80°C until further processing.

Peptide extraction and labeling

The slices were homogenized by sonication, as described previously for analysis of mouse brain tissue [30, 31]. The homogenates were incubated in a hot water bath at 70°C for 20 minutes followed by immediate transfer of the microcentrifuge tubes to ice. After cooling, appropriate volumes of 0.1 M HCl were added to achieve a final concentration of 10 mM HCl. Each sample was thoroughly mixed and allowed to remain on ice for another 15 minutes. The samples were centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatants were combined with 0.4 M sodium phosphate buffer to adjust the pH of the peptide extracts to 9.5 and stored at –80°C until labeling.

Peptides were labeled with TMAB-NHS as described [29-31]. For the present study, 4-5 labels were used, depending on the number of mice in the group. The label was solubilized in DMSO to a concentration of 400 µg/µl. Typically, 15 mg of TMAB-NHS label was used per three slices of brain tissue from one animal. Similarly, 15 mg of label was used for media from one animal. The 15 mg of label was dissolved in 37.5 µl of DMSO and 4.7 µl of the label added eight times over several hours. The first five rounds of labeling involved additions of 4.7 µl of the TMAB label to the sample, incubation at room temperature for 10 minutes, checking and re-adjusting of the pH to 9.5 using 1 M NaOH, and incubation for 10 minutes before the next round of labeling. The sixth and seven rounds consisted only of addition of the label, minus the step for pH adjustment. The eighth round followed the same routine as the first five. After the final round of labeling and pH adjustment, the samples were incubated at room temperature for another 1.5 hours. Any unreacted TMAB-NHS label in the samples was quenched by the addition of 30 µl of 2.5 M glycine solution in water and incubation at room temperature for 40 minutes. Samples were stored at –80°C prior to purification.

Peptide purification

Samples labeled with different isotopic tags were pooled and filtered through Amicon Ultra-4ml Ultracel filters (Millipore) with a molecular weight cut-off of 10 kDa. To hydrolyze any TMAB-labeled tyrosines, the pH was adjusted to 9.0 using 1 M NaOH and 10 µl of 2 M hydroxylamine in DMSO was added followed by incubation at room temperature for 10 minutes. The pH was re-adjusted to 9.0 and the addition of hydroxylamine and incubation were repeated twice more. Peptides were desalted on Pierce^R C-18 spin columns (Thermo Scientific). Resin from two C-18 columns was combined for each sample and used according to the manufacturer's instructions. In brief, the sample was mixed with sample buffer (0.5% trifluoroacetic acid in 5% acetonitrile) and applied to the resin bed. The sample bound resin was washed with 0.5% trifluoroacetic acid in 5% acetonitrile solution, after which the peptides were eluted from the column using 160 µl of 0.5% trifluoroacetic acid in 70% acetonitrile in two steps. Finally, the peptides were freeze-dried in a vacuum centrifuge and stored at –80°C until mass spectrometric analysis.

Mass spectrometry (MS) analysis—Liquid chromatography (LC) and MS analysis was performed on a Synapt G1 (Waters), as previously described [32]. In brief, the peptide mixture was resuspended in 10 µl of water and 2-5 µl injected onto a Symmetry C18 trapping column (5 µm particles, 180 µm i.d. × 20 mm, Waters, USA). The trapping column was desalted for 15 min and eluted into a BEH 130 - C18 column (1.7 µm particles, 100 µm i.d. × 100 mm, Waters, USA) using a water/acetonitrile/ 0.1% formic acid gradient with a flow rate of 600 nL/min [13]. Data were acquired in data-dependent mode and selected peptides dissociated by collisions with argon to provide tandem mass spectrometry (MS/MS) spectra. The liquid chromatography and electrospray ionization conditions included nanoflow capillary voltage of 3.5 kV, block temperature of 100°C, and cone voltage of 100 V.

The MS spectra were analyzed using the MassLynx software (Waters) and groups of peaks representing peptides labeled with the different isotope tags were detected (Figure 1). Peptides were identified from MS/MS data using the Mascot search engine (Matrix Science Ltd, UK) and the IPI_mouse data base. Search parameters included with variable modifications of N-terminal acetylation, methionine oxidation, and the isotopic D0-, D3-, D6-, and D9-TMAB tags used in our study (listed on Mascot as GIST-Quat). The D12-TMAB tag used for one of the replicates in our study is not available as a search option in Mascot. Results were manually analyzed using criteria previously described in order to eliminate false positives [29-31, 33]. In brief, these criteria include the following nine points: (i) the isotopic form of TMAB selected by Mascot corresponds to the isotopic form determined from analysis of the peak set. For example, if Mascot matched a spectrum to the D3-TMAB-labeled form of a peptide, then this peptide must correspond to the D3-TMAB peak and not the D0, D6, D9, or D12 peak observed in MS mode. This criterion eliminated 80% (4 out of 5) of the false positives for peptides labeled with a single tag, 96% (24 out of 25) of the false positives for peptides labeled with two TMAB tags, and >99% (124 out of 125) of the false positives of peptides labeled with 3 TMAB tags. (ii) The number of tags found to be incorporated into the peptide (based on the mass difference between peaks) matches the number of free amines in the peptide (i.e. the side chain amines of Lys and the N-terminus, if not blocked by modification). (iii) For those peptides that incorporate multiple tags (due to more than one free amine), all tags must be the same isotopic form on a particular peptide. False positives will rarely have consistent forms of the tags, while true positives always have the same isotopic form of the tag. (iv) The Mascot score is either the top score of all potential peptides, or the other peptides with comparable scores can be excluded by the above criteria. (v) More than 80% of the major observed MS/MS fragment ions match predicted a, b, or y ions, internal ions, or precursor ions with loss of trimethylamine. (vi) At least five fragment ions match b- or y-series ions. (vii) The mass accuracy of the fragment ions is within the accepted specification for the q-TOF instrument used for the analysis, usually within 20 parts per million. (viii) The observed charge state of the peptide matched the predicted charge state, usually corresponding to the number of isotopic tags (which are positively charged) plus the number of Arg and His residues (although His residues are not always positively charged and often exist with two different charge states). Some peptides pick up an additional proton to give a charge state one higher than the maximum predicted from the number of amines, and some lose a proton (from –COOH groups on Glu, Asp, and the C-terminus) to give a charge state lower than expected. However, the ion with the correct charge state is always found. (ix) The fragment ions match the expected ions based on the peptide sequence. For example, fragmentation of Xaa-Pro bonds is favored, while fragmentation of Pro-Xaa bonds is rarely observed [26]. The b2 and a2 ions are usually very strong if the N-terminus has a TMAB-label attached, unless there is an N-terminal Pro or a basic residue in positions 1 or 2.

The results of the peptide analysis of slices (described above) were compared to peptides our laboratory identified in previous studies of unheated mouse brain [27] and those found in heat-inactivated mouse brain which were summarized in a recent review [10]. Both of these previously published datasets were slightly modified for the present analyses. First, only peptides found in wild-type mouse brain were included, and not those specific to mutant mice (such as *Cpe^{fat/fat}* mice, which contain a large number of C-terminally extended neuropeptides). Secondly, peptides previously detected in the MS analysis but not identified by MS/MS analyses at the time were subsequently considered “identified” if their mass, charge, number of TMAB tags incorporated, and elute time from the LC column matched those of peptides identified by MS/MS analysis of the present study on brain slices.

RESULTS

Altogether, 24 mice were individually examined for peptide content of cultured slices, and 20 of these were also examined for peptides secreted into the media during the last 60 minutes of the 4 hour culture period. To reduce the number of LC/MS runs needed to analyze this number of samples, TMAB isotopic tags were used so that samples of similarly-processed slices from 4-5 distinct mice could be combined into a single LC/MS run. This reduced the number of LC/MS runs to 12, with 7 runs for the peptides in slices and 5 runs for peptides secreted into the media. A typical scheme for the preparation and labeling of peptides is shown in Figure 1 along with representative data from LC/MS runs of slices and media for samples from 4 mice labeled with D0, D3, D9 and D12-TMAB tags. These data show a single MS scan over a narrow m/z range (800-880); this allows for resolution of the peptides in the four different mice. The full m/z range (300 to 2000) and elute time (~30 minutes) revealed hundreds of additional peptides (not shown). In the representative spectrum shown in Figure 1, three distinct peptides are detected, each of which is present in all four biological replicates labeled with the different isotopic forms of TMAB. One peptide (m/z 817 to 826) is detected in the slice extracts and is not detectable in the media. Another peptide (m/z 837-841) is present in both slice extracts and media, and a third peptide (m/z 860-870) is detected in the media but not the slice extracts. In addition to allowing for mouse-to-mouse variation, the TMAB labels also assisted with the identification of peptides by MS/MS sequencing; as described in the Materials and Methods section, the majority of false positives could be eliminated by comparison of the expected and observed number(s) of tags incorporated into each peptide and other related criteria.

Although the focus of these analyses is a comparison of the slices and media, it was first necessary to validate the slice cultures and test whether the detected peptides were similar to those found in heat-inactivated brain tissue and not unheated brain tissue; the latter contains many peptides that arise from postmortem changes. A total of 344 peptides were identified by MS/MS sequence analysis of peptides in cultured brain slices. In order for a peptide to be considered identified, it needed to satisfy a rigorous set of criteria that required manual verification of each peptide. The sequences of all 344 peptides and the proteins from which they are derived are included in supplemental Table S1 (this table also includes 28 peptides found only in media, discussed below, for a total of 372 entries). In a previous study evaluating the effect of heat inactivation by microwave irradiation [27], 158 peptides were identified in unheated brain extracts (Table S2). The vast majority of these peptides were only found in the unheated brain extracts, and not in heat-inactivated brain extracts included in these studies as a control. Since this study was published in 2005 [27], many additional studies have been performed in our laboratory using microwave-irradiated heat-inactivated brain tissue [34-38](also see [10] for review of studies prior to 2010). Of the 158 peptides originally found in the unheated brain extracts, 36 were subsequently detected in heat-inactivated tissue, whereas 122 peptides were not; these latter peptides represent those that appear to be unique to the unheated brain extracts (see Table S2, column G). A comparison of the peptides identified in extracts of cultured brain slices versus all 158 peptides identified in unheated brains revealed only 8 peptides in common between these two groups (Figure 2). Furthermore, all 8 of these peptides were among the 36 peptides also found in heat-inactivated tissue, and none matched the 122 peptides unique to unheated brain tissue (Table S2). In contrast, 259 of the 344 peptides found in the cultured brain slices were also found in the heat-inactivated brain data set (Figure 2 and supplemental Table S3). Thus, whereas only 2% of the peptides identified in cultured brain slices matched peptides previously detected in extracts of unheated brain tissue (and none matched peptides unique to unheated brain extracts), 75% of the peptides identified in cultured slices matched those previously detected in extracts of heat-inactivated brains.

The 85 peptides detected in extracts of brain slices but not previously identified in extracts of heat-inactivated brain were further considered. Of these, the majority (58 peptides, or 68% of the total) were observed in only one LC/MS run (out of 7 LC/MS runs for slices), and the rest (27 peptides, or 32% of the total) were seen in 2-4 LC/MS runs. None of these 85 peptides were seen in all LC/MS runs of slices. In contrast, there were 37 peptides observed in 5 or more of the LC/MS runs of slices, and of these, 30 were also frequently seen in LC/MS runs of brain extracts. Therefore, the 85 peptides seen in slices but not detected in previous studies in brain extracts appear to represent low abundance or difficult to detect peptides, and not the major peptides and/or easily detected ones.

To further characterize the peptides found in brain slices, we examined the intracellular location of the protein from which the peptide was derived. Approximately one half (46%) of the peptides detected in extracts of heat-inactivated wild-type mouse brains were derived from cytosolic proteins, 29% were derived from secretory pathway proteins, and 18% from mitochondrial proteins (Figure 3). For the slices, 57% of the peptides represent fragments of cytosolic proteins, 25% fragments of mitochondrial proteins, and 13% pieces of secretory pathway proteins (Figure 3). Previously, it was noted that ~45% of the peptides derived from cytosolic and mitochondrial proteins represented the N- or C-terminus of the protein [10]. Considering that there are many more possible internal peptides than N- or C-terminal peptides, this observation was unexpected. In the present analyses, ~40% of the peptides identified in brain slices were found to represent the N- or C-terminus of the intracellular precursor proteins (Figure 4). In contrast, peptides derived from secretory pathway proteins largely represented the internal parts of the proteins, with 11% of the peptides detected in heat-inactivated brain extracts and 17% of the peptides found in slices representing the N- or C-terminus (Figure 4).

Recently, Price and colleagues evaluated the turnover rate of a large number of mouse brain proteins using isotopic incorporation of ^{15}N , and found the average half-life of mouse brain proteins to be 9.0 days [39]. To examine if the protein precursors of the peptides detected in slices represent unstable proteins with high turnover rates, the 103 distinct proteins giving rise to the 344 peptides detected in brain slices were compared to the Price et al dataset of protein turnover. Altogether, two thirds of the protein precursors of peptides detected in the brain slices corresponded to proteins reported in the turnover study. The half-lives of these proteins ranged from 1.5 to ~400 days, with a median of 21 days (Figure 5). Thus, the peptides detected in slices are largely derived from relatively stable proteins, and represent the N- and C-terminal of cytosolic and mitochondrial proteins at a higher frequency than predicted; these properties are similar to peptides found in extracts of heat-inactivated brain.

After establishing that the peptides present in brain slices cultured for 3-4 hours in oxygenated aCSF are representative of those found in heat-inactivated brain, the next objective was to examine peptides secreted from the slices and compare them to those found in slices. For these studies, the culture media from the last 60 minutes of incubation was used for peptidomic analysis so that the secreted peptides represented those from the stabilized slices, and not from the slices immediately after preparation. Altogether, 78 peptides were identified from the MS/MS analysis of secreted material. Unexpectedly, only 3 of these 78 peptides were derived from secretory pathway proteins; the vast majority was derived from cytosolic or mitochondrial proteins (Table S1). Of the 78 peptides detected in the media, 50 were also observed in the extracts from cultured brain slices, while 28 were not seen in the analysis of slice extracts (Figure 6). Of the 344 peptides identified in extracts of cultured brain slices, 294 were not detected in the media (Figure 6). The failure to detect a peptide is difficult to interpret; the peptide may truly not be present, or it may be present at low levels below the detection limit of the assay or otherwise difficult to detect due to inherent properties of the peptide. To address this, we examined the number of times each

peptide was observed in the various analyses. Approximately one half of the peptides found in slices but not in the media (156 out of 294) were seen in a single LC/MS run of the slice extracts, indicating that they are low abundance or otherwise difficult to detect by MS. However, 114 peptides of the 294 peptides were detected in 2-4 of the LC/MS runs of slices, and 24 peptides were detected in 5 or more of the LC/MS runs of slices, but never in the LC/MS runs of media. These peptides are likely to represent those present in slices and not secreted into the media at appreciable levels. Similarly, of the 28 peptides seen only in the media and not in slices, 14 of these were seen only a single time and thus are likely to represent peptides that are more difficult to detect by MS. However, the other half of the 'media-only' peptides were detected multiple times in the media; three of these peptides were detected in five LC/MS runs of media but never in the LC/MS runs of extracts of brain slices.

Based on the number of times some peptides were found in media samples, they appear to be readily detected by LC/MS analysis and so the failure to detect them in the extracts of brain slices raises the possibility that they are enriched in the media, either by selective secretion or selective production by extracellular peptidases. In support of this latter possibility, it was observed that many of the peptides detected in media samples but not in slices are shorter fragments of peptides detected in the slices. For example, the alpha-hemoglobin derived peptide named RVD-hemopressin was detected in seven LC/MS runs of slice extracts and four LC/MS runs of media samples (Table 1). A shorter form lacking two C-terminal residues was found in one LC/MS run of the media, and a form lacking four C-terminal residues was found in three LC/MS runs of media samples; neither peptide was detected in any of the LC/MS runs of slice extracts (Table 1). Similarly, peptides representing the N-terminal regions of N-myc downstream regulated gene 2 and 3 were detected in extracts of slices and in media, whereas peptides lacking two or four C-terminal residues were found only in LC/MS runs of media samples (Table 1). Internal fragments of tubulin alpha-1 isoforms were found in LC/MS runs of slices and media samples, whereas shorter forms lacking three, four, or five N-terminal residues were detected only in the LC/MS runs of media samples (Table 1). Other examples of shorter peptides detected in the media and longer forms detected in the slices were also observed (see Supplemental Table S1 for all data). Taken together, these results support the possibility that some of the peptides detected only in the media samples represent post-secretion cleavage products of extracellular peptidases.

A comparison of the peptides detected only in the LC/MS runs of cultured brain slices with those found in the media (either in media and not slices, or both in media and in slices) did not reveal any major differences in amino acid composition among groups. In all three groups, highly charged residues represent ~25% of all amino acids, with the basic residues Lys and Arg comprising ~10-12% of all amino acids and the acidic residues Asp and Glu comprising ~13-15% of all amino acids. Hydrophobic residues (Met, Ala, Val, Leu, Ile, Trp, Tyr, and Phe) represented 34-39% of the residues in all three groups of peptides. Although the amino acid composition of the secreted peptides was not substantially different than the composition of peptides detected only in slices, it was noted that the presence of N-terminal acetyl groups was more frequently found in the secreted peptides, as compared to those detected only in extracts of slices. Altogether, 21 of the 78 peptides found to be secreted were N-terminally acetylated (27%) whereas only 36 of the 294 peptides detected in slices (12%) were acetylated (supplemental Table S1). However, this may reflect the greater number of secreted peptides that represent the N-terminus of the precursor protein; 35% of the secreted peptides are N-terminal fragments of the protein, as opposed to only 19% of those found in the slices. It is possible that this reflects the greater stability of N-terminally acetylated peptides to extracellular aminopeptidases.

DISCUSSION

Over the past decade, advances in peptidomic approaches have led to the discovery of hundreds of novel peptides in cells and tissues [3, 10, 11, 40-42]. Some of these peptides are produced within the secretory pathway, based on the presence of an N-terminal signal peptide sequence on the peptide precursor that drives the translocation of the nascent protein into the lumen of the endoplasmic reticulum. These secretory pathway peptides may play a role in cell-cell signaling as neuropeptides and peptide hormones. An unexpected finding from the various peptidomic studies has been the detection of peptides derived from cytosolic, mitochondrial, and other non-secretory pathway proteins. While proteins are known to be processed by the proteasome into peptides, the resulting peptides were thought to be highly unstable and rapidly degraded by intracellular aminopeptidases [43]. Studies using human cell lines have shown that the majority of peptides detected upon peptidomic analyses are produced from the proteasome [32, 44], but are not derived from the most abundant or least stable proteins, suggesting that the observed peptides do not simply reflect protein turnover [15]. Instead, it is likely that the observed peptides are either selectively produced or selectively retained; either of these possibilities implies a function. In order to gain insights towards potential roles for these peptides, it is important to know if any of them are secreted and thus potentially able to serve as cell-cell signaling molecules.

Short term cultures of brain slices are commonly used for electrophysiology studies, and have been used to examine the secretion of peptides from specific regions [5, 45]. However, it was not established if short term cultures of brain slices showed postmortem changes similar to those seen in mouse brain. Previous studies found that mouse tissues needed to be rapidly heat-inactivated to prevent changes in the cellular peptidome [3, 27, 28, 46, 47]. These postmortem changes may be a result of the hypoxia that tissues undergo after death of the animal, prior to the dissection and subsequent freezing of the tissue. In addition to hypoxia, brain slices also undergo the stress of tissue injury caused during sectioning. For these reasons, slices were cultured for several hours prior to collection of media for analysis of the secretome and subsequent processing of the media and slice tissue for peptide analysis. The observation that the peptidome of brain slices is more similar to the peptidome of heat-inactivated brain than to the peptidome of unheated brain suggests that the injuries incurred upon preparation of slices are largely resolved within 3-4 hours of culture in oxygenated aCSF. However, there were fewer secretory pathway peptides detected in brain slices or media than found in previous studies on heat-inactivated brain, relative to the number of peptides derived from intracellular proteins. One possible explanation is that the coronal slices used in the present study included brain regions not enriched in neuropeptides. Most of the previous peptidomic analyses of mouse brain focused on neuropeptide-rich brain regions such as the hypothalamus, amygdala, striatum, and hippocampus. In contrast, the coronal sections used in the present study included a high ratio of cortex in addition to the hypothalamus. Another possible explanation is that the 3-4 hour recovery time of the slices was not sufficient to allow for new synthesis of neuropeptides, which requires production of the prohormone and processing by specific peptidases into the bioactive forms [1]. Ischemic conditions cause the exocytosis of peptide-containing secretory vesicles [48]. A comparison of heat-inactivated and unheated brains showed that the heat-inactivated tissue had higher levels of neuropeptides, presumably because the peptides in unheated brains were secreted and subsequently degraded by extracellular peptidases during the ischemic postmortem period [27]. When secretory vesicles in rat adrenal were substantially depleted by hyperglycemic shock, a marker of secretory vesicle content did not return to normal until 2 days after depletion of vesicles [49]. Thus, if massive secretion of neuropeptides were triggered by the ischemic conditions after death, a 3-4 hour incubation would not likely be sufficient to restore neuropeptides to their normal levels. Still, the slices were found to contain a number of neuropeptides, and because their intracellular peptide

content was similar to that of heat-inactivated mouse brain, the cultured slices represent a model system to study secretion, especially of non-secretory pathway peptides.

A major finding of the present study is that RVD-hemopressin is secreted from slices, and thus fits an important criterion of neuropeptides [20]. This peptide, an agonist of the CB1 cannabinoid receptor, was previously found in peptidomic analyses of mouse brain [19]. RVD-hemopressin is derived from hemoglobin, a well-known red blood cell protein which has recently been shown to be expressed in neurons and glia [50, 51] as well as other tissues [52-56]. Levels of RVD-hemopressin in brain were altered in an overweight mouse model, indicating that the production of this peptide is under dynamic regulation [35]. In the present study, 20 different peptides derived from alpha hemoglobin and 3 peptides from beta hemoglobin were identified in the brain slices and/or media (Table S1). Some of the hemoglobin-derived peptides were only detected in media, such as smaller forms of RVD-hemopressin, which may reflect products of extracellular peptidase activity. Further studies are needed to test if these other forms of RVD-hemopressin interact with the CB1 cannabinoid receptor.

A caveat in peptidomic studies is that the failure to detect a peptide in a sample does not necessarily imply that the peptide is not present. Some peptides are difficult to detect due to inherent properties with ionization while others are easily detected but not readily identified from MS/MS analysis. In the present study, several hundred peptides were detected in MS mode but could not be conclusively identified from MS/MS analysis. Often, peptides that are very large do not produce easily interpreted MS/MS spectra, while very short peptides usually do not produce enough fragment ions for unequivocal identification. To reduce the influence that MS/MS sequencing had on the interpretation of the results, we performed a cross comparison of the peptides identified in the extracts of slices and those identified in the media, and considered that if a peptide was identified in only one of the two samples but was also detected in the other (with the same mass, charge, number of TMAB tags incorporated, and elute time from the LC column) that the peptides were identical. By comparing the number of times each peptide was seen in the various samples, we found that some peptides frequently detected in slices were never detected in media samples, and vice versa. While it is possible that this represents a detection problem, it is likely that this reflects the fact that some peptides in slices are not secreted in appreciable levels, and other peptides found in media are either selectively secreted or produced after secretion by the action of extracellular peptidases.

Variability was an issue when comparing LC/MS runs; most peptides were not detected in every LC/MS run performed for slices or media samples. By using isotopic tags to label slices or media samples from 4-5 different mice that were combined into a single LC/MS run, we were able to evaluate mouse-to-mouse variation. The variation between peptides found in different mice in a particular experiment (i.e. a single LC/MS run) was usually much less than that observed while comparing peptides found in different experiments. Most peptides were either seen in all 4-5 mice in a single LC/MS run, or were not observed at all in that run. Furthermore, there were typically only minor differences in the relative levels of peptides among the different mice used for each LC/MS run.

Many of the peptides found to be secreted from slices are produced from cytosolic or mitochondrial proteins, which is consistent with a role for these peptides in cell-cell communication as “non-classical neuropeptides” [10]. This term is analogous to non-classical neurotransmitters such as nitric oxide and anandamide; compounds produced in the cytosol by a regulated process and then constitutively secreted [57, 58]. This is in contrast to classical neurotransmitters and classical neuropeptides, which are secreted via a regulated process. The mechanism of secretion of the peptides from the cytosol needs to be

determined. Several mechanisms are possible based on secretion of peptides from other systems. In yeast, the cytosol-produced a-mating factor is secreted via a transmembrane transporter that uses ATP to drive the secretion of the peptide [21]. Homologous transmembrane proteins are found in mammals, although it is not known if they function in the secretion of peptides. Cytosolic peptides may also be secreted via connexin hemichannels, as found for the secretion of glutathione [59]. Another potential mechanism is through the lysosomal system; the contents of some lysosome are secreted through a poorly understood process [60, 61]. It is also possible that intracellular peptides have unique mechanisms of secretion, like the cytosol-produced basic fibroblast growth factor which is secreted through a complex mechanism involving polymerization and a number of other cellular proteins [22]. Further studies are needed to address the mechanism of secretion, and the present findings show that cultured brain slices are a useful system for further analysis of the mechanism.

While those peptides that were not found to be secreted from the slices are unlikely to participate in cell-cell signaling (unless the failure to detect them in media was a technical problem, as discussed above), these peptides may still have important functions within the cell. Ferro and colleagues proposed that intracellular peptides could function in gene regulation, metabolism, cell signaling and protein targeting [62]. Consistent with this hypothesis is the observation that small peptides introduced into the cytosol of a cell can affect protein interactions by competing for binding sites within proteins [14, 63-65]. For example, agonist-mediated signal transduction of G-protein coupled receptors was influenced by the addition of synthetic peptides found to exist in the HEK293 and CHO cells [14], and the insulin-induced glucose uptake in 3T3-L1 adipocytes was affected by the addition of peptides found to exist in adipocytes [16]. Peptides corresponding to mitochondrial protein degradation fragments influenced nuclear-encoded mitochondrial chaperone genes, indicating the stress of mitochondrial protein misfolding in *C. elegans* [66]. Epidermal differentiation in *Drosophila* was affected by peptides produced by the translation of small open reading frame genes [67]. Taken together, it appears that intracellular peptides have functional roles in a variety of cellular processes, and are not merely intermediates in the protein degradation pathway.

In conclusion, short-term culture of mouse brain slices for 3-4 hours in oxygenated medium results in a peptide profile that generally matches the peptidome of heat-inactivated mouse brain and does not show the postmortem changes previously observed in extracts of unheated brain tissue. Although secretory pathway-derived peptides are not as abundant in the extracts of cultured brain slices as compared to extracts of heat-inactivated hypothalamus and other neuropeptide rich brain regions, a subset of the intracellular peptides derived from cytosolic or mitochondrial proteins was found to be secreted. The cultured slice system represents an excellent model system to further explore mechanisms of secretion as well as potential functions of the intracellular peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

aCSF	artificial cerebrospinal fluid
DMSO	Dimethyl Sulfoxide
LC/MS	liquid chromatography / mass spectrometry
MS/MS	Tandem Mass Spectrometry
m/z	mass/charge ratio
TMAB	4-trimethylammoniumbutyrate
TMAB-NHS	4-trimethylammoniumbutyryl-N-hydroxysuccinimide

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Highlights

- Mouse brain slices cultured 3-4 hours in oxygenated buffer contain many peptides.
- These peptides resemble those found in extracts of heat-inactivated mouse brain.
- A subset of peptides in the slices was secreted into the culture media.
- Secreted peptides included those derived from cytosolic and mitochondrial proteins.
- RVD-hemopressin, a peptide CB1 cannabinoid receptor agonist, was secreted.

Figure 1.

Schematic diagram of a typical experiment and representative MS data. A. Individual mice were sacrificed, the brains removed, and rapidly cut into coronal slices. Three slices from each animal were cultured together for ~3 hours in oxygenated aCSF. The media were removed, slices were washed, and then incubated for another hour in fresh aCSF. Following the incubation, these media were removed and slices were heat inactivated prior to extraction of peptides. B. Peptides in the extracts of slices or media were labeled with an isotopic form of TMAB and then combined prior to purification and analysis of the peptides on LC/MS. C. Each of the 7 LC/MS runs of extracts of slices and the 5 LC/MS runs of media samples were scanned over the m/z range from 300 to 2000. Representative spectra from a single scan of slice (top) and media (bottom) over the m/z range from 800 to 880 is shown. For these runs, slices or media from 4 animals were individually labeled with D0-, D3-, D9-, or D12-TMAB and then combined and analyzed in a single LC/MS run for slices and another LC/MS run for media samples. The four peak sets with m/z of 816-827 represent the 4+ ion of a peptide that incorporated 3 TMAB tags and has a monoisotopic mass of 2881.46 (without TMAB tags or protons). This peptide, which was not identified by MS/MS sequence analysis, is detected in extracts of slices (top panel) but not in the LC/MS run of media (bottom panel). The four peak sets with m/z of 836-842 represent the 3+ ion of

a peptide that incorporated 1 TMAB tag and has a monoisotopic mass of 2380.04 (without tags or protons). This peptide was also not identified from MS/MS sequence analysis, and was detected in both slice extracts and media samples. The four peak sets with m/z of 860-871 represent the 4+ ion of a peptide that incorporated 3 TMAB tags with a monoisotopic mass of 3056.54 (without tags or protons). This peptide was identified by MS/MS analysis as a C-terminal fragment of brain acid soluble protein 1, and is detected in this LC/MS run of media samples, and all other LC/MS runs of media samples (not shown). Although not detected in the representative LC/MS run of slice extracts that is shown in this figure, the peptide was detected in a single one of the 7 LC/MS runs of slice extracts (not shown).

Figure 2.

Comparison of peptides identified to be present in extracts of brain slices with peptides in extracts of unheated brain (left) and heat-inactivated brain (right). The dataset for unheated mouse brain was previously reported [27] and includes 158 identified peptides. See supplemental Table S2. The dataset for heat-inactivated brain extracts was previously published [10], and represents a number of different experiments using TMAB tags to measure peptide levels in various regions of heat-inactivated wild-type mouse brains. Additional peptides found in heat-inactivated mouse brain and sequenced in the past three years have been included with the previously published data; see Supplemental Table S3.

Figure 3.

Intracellular location of protein precursors of peptides found in heat-inactivated wild-type mouse brain or in cultured brain slices. Approximately 46% of the peptides detected in heat-inactivated brain arise from precursors present in the cytosol, 18% from proteins found in mitochondria, 29% from secretory pathway proteins, and 2% from proteins found in the nucleus, lysosomes, or membranes. In the cultured slices, approximately 57% of the peptides arise from cytosolic proteins, 25% from mitochondrial proteins, 13% from secretory pathway proteins, and 2% from proteins found in the nucleus, lysosomes, or membranes.

Figure 4.

Location of peptide within precursor protein. Of the 192 secretory pathway peptides detected in heat-inactivated wild-type mouse brain, 89% arise from internal cleavages, and only 11% represent the C-terminus or N-terminus (after signal peptide removal) of the peptide precursor. In contrast, 42% of the 464 peptides that arise from cytosolic, mitochondrial, and other intracellular proteins are the N- or C-terminal fragment of the protein. Of the 43 secretory pathway peptides detected in cultured brain slices, 83% arise from internal cleavages, 12% represent the C-terminal peptide, and 5% represent the N-terminal peptide. The 301 intracellular peptides detected in cultured slices represent the N-terminal peptide 24% of the time, the C-terminal peptide 16% of the time, and internal peptides 59% of the time.

Figure 5.

Protein half-life of peptide precursors found in brain slices. All of the peptides identified in the slices arise from 103 distinct proteins, and of these, 73 were reported in a previous study on the turnover of proteins in mouse brain by Price et al [39]. However, the half-life of six of the proteins reported in this previous study could not be accurately determined due to inconsistencies within the data, and so 67 proteins in common were found between the precursors of peptides found in slices and the proteins reported in the half-life study. The half-life was either calculated from Price et al Table S1 (when listed) or estimated from Price et al Table S2. These half-life values (in days) were ranked from shortest (1.5 d) to longest (~400 d) and then sorted by rank and plotted. The median half-life of all proteins representing precursors of peptides identified in the slices is 21 days. See supplementary Table S4 for data.

Figure 6.

Comparison of peptides identified in cultured brain slices versus those identified in media from the final 60 minutes of culture. Slices were cultured for three hours and then transferred to tissue culture plates containing fresh media for one hour incubation. The media from the last hour of incubation were removed and slices and media were processed for peptidomics as described in Materials and Methods.

Table 1

Examples of peptides present in media and/or extracts of slices

Precursor	Peptide	Detected in	
		Slices	Media
Hemoglobin alpha (RVD-Hemopressin)	RVDPVNFKLLSH	yes	yes
	RVDPVNFKLL	no	yes
	RVDPVNFKL	yes	yes
	RVDPVNF	no	yes
N-myc downstream regulated gene 2	Ac-AELQEVQITEEKPLLPG	yes	yes
	Ac-AELQEVQITEEKP	no	yes
N-myc downstream regulated gene 3	Ac-MDELQDVQLTEIKPLL	yes	yes
	Ac-MDELQDVQLTEIKP	no	yes
Tubulin alpha 1A/B/C	AVFVDLEPTVIDEVR	yes	yes
	VDLEPTVIDEVR	no	yes
	DLEPTVIDEVR	no	yes
	LEPTVIDEVR	no	yes