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Mass Spectrometric Detection of Neuropeptides Using Affinity-Enhanced Microdialysis with Antibody-Coated Magnetic Nanoparticles

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Abstract

Microdialysis (MD) is a useful sampling tool for many applications due to its ability to permit sampling from an animal concurrent with normal activity. MD is of particular importance in the field of neuroscience, in which it is used to sample neurotransmitters (NTs) while the animal is behaving in order to correlate dynamic changes in NTs with behavior. One important class of signaling molecules, the neuropeptides (NPs), however, presented significant challenges when studied with MD, due to the low relative recovery (RR) of NPs by this technique. Affinity-enhanced microdialysis (AE-MD) has previously been used to improve recovery of NPs and similar molecules. For AE-MD, an affinity agent (AA), such as an antibody-coated particle or free antibody, is added to the liquid perfusing the MD probe. This AA provides an additional mass transport driving force for analyte to pass through the dialysis membrane, and thus increases the RR. In this work, a variety of AAs have been investigated for AE-MD of NPs *in vitro* and *in vivo*, including particles with C18 surface functionality and antibody-coated particles. Antibody-coated magnetic nanoparticles (AbMnPs) provided the best RR enhancement *in vitro*, with statistically significant ($p < 0.05$) enhancements for 4 out of 6 NP standards tested, and RR increases up to 41-fold. These particles were then used for *in vivo* MD in the Jonah crab, *Cancer borealis*, during a feeding study, with mass spectrometric (MS) detection. 31 NPs were detected in a 30 min collection sample, compared to 17 when no AA was used. The use of AbMnPs also increased the temporal resolution from 4–18 hrs in previous studies to just 30 min in this study. The levels of NPs detected were also sufficient for reliable quantitation with the MS system in use, permitting quantitative analysis of the concentration changes for 7 identified NPs on a 30 min time course during feeding.

Keywords

Microdialysis; *In Vivo* measurement; Affinity-enhanced microdialysis; Neuropeptides; Mass spectrometry; LC-MS; LC-ESI-QTOF; Crustacean; Hemolymph

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Supporting Information. Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Microdialysis (MD) is a sampling technique that allows collection of signaling molecules from an animal while it is alert and behaving, with minimal disturbance to the animal. In this technique, a MD probe is implanted into the tissue of interest and perfused with liquid at a flow rate in the range of 0.1–10 $\mu\text{L}/\text{min}$. The tip of this MD probe consists of a dialysis membrane, having pores with a defined molecular weight cutoff (MWCO). Molecules below this MWCO near the tip of the probe passively diffuse into the probe and are then carried by the slowly-moving liquid out of the probe, through a length of tubing, and finally to a sample collection vial or analysis system. This technique has been used successfully to collect a variety of different molecules from a number of tissues in several species, and has provided important insights into the action of compounds *in vivo* in a minimally perturbed animal.^{1,2}

MD is of great utility in neuroscience, in which time-resolved changes in neurochemistry during the performance of a behavior or exposure to a stimulus are of interest. Continual collection of neurochemicals without disturbing the animal to obtain the samples allows the experimenter to determine the molecular underpinnings of neuronal activity related to these events, in the absence of any sampling-induced neuronal changes. MD has been used successfully to monitor small molecule neurotransmitter (NT) changes in vertebrate animals under a variety of different conditions, and has contributed greatly to our understanding of the effects of NT release on behavior.^{1,3–5}

One area that is particularly challenging for MD sampling is the analysis of larger molecules, such as neuropeptides (NPs), which are below the MWCO of the probe but are in the mass range of 500–10,000.^{5–9} A number of complex factors make recovery of NPs difficult. One such reason is the lower relative recovery (RR) of NPs in comparison to small molecules due to their larger size hindering passage through the dialysis membrane. The RR is calculated by taking the concentration of an analyte collected through MD divided by the concentration outside the probe, and is usually expressed as a percentage. This RR is inversely related to the mass of the molecule, with larger molecules typically having RRs of less than 50%. Another challenge is the low endogenous concentration of these compounds. NPs are present *in vivo* at the nM - pM concentration range.² Therefore, the concentration collected, governed by the laws of passive diffusion, is reduced compared to NTs due not only to their low endogenous concentrations but also their reduced RR. Furthermore, RR is also governed by the amount of time the liquid is in contact with the membrane (the MD flow rate, FR), with lower flow rates leading to greater RRs.^{2,8,10} If increased amounts of analyte are desired, a longer collection time can be employed. If a short collection time is desired, an experiment will detect NPs reliably only if the RR is improved by other means,⁷ or a more sensitive detection technique is employed.

Progress has been made in using highly sensitive and specific detection methods for NPs in samples obtained via microdialysis, relying mainly on mass spectrometry (MS).^{1,3,4,11–13} Some of these studies use MS for surveys of NP content and identity.^{14–23} Other studies use MS for quantitation of identified NPs in microdialysate, mostly with selected reaction monitoring (SRM) of daughter or granddaughter ions.^{2,10,24–30} Finally, microdialysates can be analyzed via MS-based techniques for NP discovery combined with less precise quantification methods commonly used in proteomics.^{31–36} In addition to MS-based analysis of dialysates, other sensitive techniques, including those that rely on immunochemical or spectrophotometric detection, have been used for quantitation of NPs in dialysates, but these methods lack specificity.^{1,3,13} Although MS instruments are highly sensitive, not all perform adequately in the concentration range at which NPs are present *in vivo*, and other methods to increase sensitivity must be investigated.

An important method to increase the sensitivity of NP detection in MD is to increase the RR. The relative recovery can be increased by adding affinity agents (AAs) to the liquid perfusing the probe. The analytes form interactions with the AAs and lead to reduction of free concentration of analytes in the dialysate, thus increasing the concentration gradient for analytes which drives mass transport to allow additional analytes to diffuse into the probe.⁷ This technique is termed affinity-enhanced microdialysis (AE-MD), and has been used by a number of different researchers with a wide variety of compounds.^{5–7,17,34,35,37–52} Stenken and colleagues, among others, have achieved success in improving the recovery of cytokines *in vitro* and *in vivo*^{7,39,51,53,54} and neuropeptides *in vitro*,⁴³ using free antibody, cyclodextrins, and micron-sized beads coated with antibodies or heparin.

AE-MD is not yet optimal, as saturation of the beads can occur, leading to non-linear recovery enhancement. Clogging or settling of the beads in solution is also a major concern. The technique has also not yet been applied to study NPs *in vivo* (although the cytokine CCL2 has been studied in rats using AE-MD⁵⁴), nor have smaller beads been employed as affinity agents. In this work, several AAs are tested for enhancement of NP recovery. Nanoscale magnetic beads are developed for use as AAs, with the advantages of reduced settling rate and greater binding capacity. They enhance recovery of 4 out of 6 NP standards tested *in vitro*. They are also employed *in vivo* to study the time course of NP release following feeding in the Jonah crab, *Cancer borealis*. This new affinity agent for AE-MD greatly increases the utility of this technique for monitoring peptide secretion during behavior.

Materials and Methods

Reagents

Peptide standards (bradykinin (BK), somatostatin-14 (SMT), substance P (SP), *Homarus americanus* FMRFamide-like peptide I (FLP I), *H. americanus* FMRFamide-like peptide II (FLP II), and FMRFamide) were purchased from American Peptide (Sunnyvale, CA, USA) and used without further purification. C18 silica microparticles (C18S μ P) were purchased from Varian (now Agilent Technologies, Santa Clara, CA, USA) and were 5 μ m in size with 300Å pore size. They were used in perfusate at a concentration of 0.2mg/mL, or 3.04×10^3 beads/ μ L. C18 magnetic microparticles of 1 μ m diameter (C18M μ P) were purchased from Varian at a stock concentration of 2×10^6 beads/ μ L and used in perfusate at 3.3×10^4 beads/ μ L. Magnetic microparticles pre-coated with protein G were purchased from New England Biolabs (Ipswich, MA, USA), with a stock concentration of 3.11×10^4 beads/ μ L and a final perfusate concentration of 518 beads/ μ L. Magnetic nanoparticles of 100nm diameter pre-coated with protein G were purchased from Chemicell GmbH (Berlin, Germany) with a stock concentration of 1.8×10^{10} beads/ μ L, and thus perfusate concentrations of 3.0×10^8 beads/ μ L and 1.8×10^9 beads/ μ L as indicated below. Bovine serum albumin (BSA) and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal rabbit anti-FMRFa antibody was purchased from Abcam (Cambridge, MA, USA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) at ACS reagent-grade and used without further purification. ACS reagent-grade solvents and Milli-Q water were used for sample preparation. Optima grade solvents were used for operation of the UPLC-QTOF. C18-coated magnetic beads and antibody-linked beads were prepared and used as recommended by the manufacturers. Details of preparation, unbinding, and *in vitro* bead binding assays can be found in the supplementary information.

Animals

Jonah crabs (*Cancer borealis*) were purchased from Ocean Resources, Inc. (Sedgwick, ME, USA) and The Fresh Lobster Company (Gloucester, MA, USA). These crabs were wild-caught and shipped overnight packed on ice. The crabs were then maintained in an artificial seawater tank at 10–12°C, with crushed gravel as a substrate. Details of animal housing procedures are included in the supplementary information.

Microdialysis Supplies

CMA/20 Elite probes with 4 mm membranes of polyarylether sulfone (PAES) were purchased from CMA Microdialysis (Harvard Apparatus, Holliston, MA, USA). All MD probes were rinsed with water prior to use. Several pumps were used, including a CMA/102, a KD Scientific 100 (KD Scientific Inc., Holliston, MA, USA), and a Harvard 22 (Harvard Apparatus, Holliston, MA, USA). When required, additional FEP (CMA) or PEEK (Upchurch-Scientific, IDEX Health and Science, Oak Harbor, WA, USA) tubing was connected to the tubing of the probe by flanged connectors from CMA, and BASi (West Lafayette, IN, USA). BD (Franklin Lakes, NJ, USA) plastic syringes were typically used. Flanged connectors were used to connect 21 gauge Luer-lock needles (included with CMA 20 series probes) blunted by grinding with a rotary tool (Dremel, Robert Bosch LLC, Farmington Hills, MI, USA) to the probe tubing.

In vitro MD Experiments

For *in vitro* experiments, the tip of the probe was immersed into a vial with a home-built apparatus to hold the probe in place. Typically, 3 different probes' tips were immersed in the solution in the vial concurrently to provide multiple experimental replicates. The vial contained microdialysis medium, which consisted of a phosphate buffered saline (PBS) solution with neuropeptide standards of interest dissolved in it at known concentrations, in the 1–5 micromolar range, except for C18 silica particles, which used 50 μ M. The vial with probe holder was placed on an orbital rotating platform to produce constant mixing. A sample of the medium was taken prior to starting microdialysis and following the experiment. The probe was allowed to equilibrate at the flow rate of the experiment (0.5 μ L/min) for 30 min before starting dialysate collection. Technical replicates were taken as consecutive 30 min samples of the liquid flowing out of the tubing. Samples from the medium taken before and after the experiment were used to determine the relative recovery percentage. A minimum of three technical replicates were obtained per experiment, and a minimum of three experimental replicates were obtained, each coming from either a different probe or a different instance of setting up and conducting the experiment. Medium samples and samples containing no AA were placed immediately in a 96-well sample plate for UPLC-QTOF analysis. For AE-MD, NPs were unbound from AAs as recommended by the manufacturer (see Supplemental Information for details) and combined with the liquid portion of the sample in a 96-well plate for analysis. The percent of beads passing through the probe was determined by counting on a hemacytometer for micron-sized beads, and by comparing the dry mass of particles for nanoscale beads.

When affinity agent was used, a clean steel ball bearing of appropriate size (1/8 inch, Wheels Manufacturing, Louisville, CO, USA) was added inside the barrel of the syringe delivering microdialysate. The pump was placed into a rocking platform shaker with the syringe placed at an angle to the axis of rotation of the shaker. The rolling of the ball bearing inside the syringe kept the affinity agent in solution.⁵⁵ For the affinity agent perfusate, the equivalent of 50 μ L of bead solution was diluted to 3 mL with PBS (a dilution of 1:60), with one exception. Although the concentrations of beads in mg/mL varied, it was determined that they had equal activity per mL, as the manufacturers' protocols recommended the same ratio of beads to sample, i.e. 50 μ L beads with 0.5 mL cell lysate, a 1:10 ratio. In one set of

experiments, a higher concentration of affinity agent was used, as it was possible to increase bead concentration without adverse experimental effects. This trial is noted as 6× AbMnP, containing six times as many nanoparticles per unit volume (50μL of bead solution diluted to 0.5mL of perfusate).

***In Vivo* Microdialysis**

The procedure for implantation of a MD probe was adapted from previous publications.^{14,15} A detailed description of the implantation and modifications are presented in the supplementary information. The probe was surgically implanted in the crab 2 days prior to the first feeding experiment, and the last feeding experiment was conducted 8 days after surgery. This time window was chosen to avoid effects from surgery (stress of anesthesia and being out of water, trauma to the hypodermis) and tissue growth over the probe's active membrane. Artificial crab saline (440 mM NaCl; 11 mM KCl; 13 mM CaCl₂; 26 mM MgCl₂; 10 mM HEPES acid, pH 7.4, adjusted with NaOH) was used as the basis for perfusate. The flow rate was 0.5 μL/min, supplied by a programmable syringe pump (KD Scientific Model 100, Holliston, MA, USA) and samples were collected every 30 min with a refrigerated fraction collector (BASi Honeycomb, Bioanalytical Systems, Inc. Indianapolis, IN, USA). For each feeding trial, a 30min sample was acquired prior to feeding the animal but after allowing the probe to equilibrate for 30min, and this was used as the baseline sample. This experiment was conducted 3 times on the crab under normal MD conditions. For AE-MD *in vivo*, a 1:10 dilution of nanobeads was used (equal to the high AbMnP concentration for *in vitro* AE-MD studies). Upon collection, 1.5 μL of formic acid was added to each sample to improve NP stability² and unbind NPs from the antibody-coated nanoparticles, and an internal standard (bradykinin, 1μM) was added for quantitation. Samples collected without affinity agent were directly injected onto the UPLC-MS system, and magnetic beads were removed from AE-MD samples prior to addition of internal standard and MS analysis.

UPLC-MS and UPLC-MS/MS Analysis and Data Processing

In vitro MD samples were analyzed via a UPLC-MS approach. A Waters nanoAcquity UPLC system (Waters, Millford, MA, USA) was used in conjunction with a home-packed capillary column (360 μm OD, 75 μm ID, 10cm long, Magic C18 particles (Michrom, Auburn, CA, USA), 3 μm diameter, 100Å pore size) with integrated laser-pulled (approx. 7 μm diameter, with a Sutter Instruments P-2000 (Novato, CA, USA)) ESI emitter tip. Details of the UPLC-MS parameters and data analysis methods can be found in the supplemental information, including a Supplemental Table S1, which enumerates the retention times of the peptides using a reversed phase separation (H₂O/ACN/0.1% formic acid) with gradient from 95% aqueous to 95% organic over 30 min. Statistical significance was determined using JMP statistical software (Version 9.0.2 SAS Institute, Inc., Cary, NC, USA). Graphs illustrating this data were generated in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

In vivo MD samples were analyzed using the same instrumentation platform but with different MS and LC specifications. The LC gradient was 60 min long and a larger MS window was monitored for quantitative analyses. Data analysis is detailed in the supplemental section.

Results and Discussion

***In vitro* recovery enhancement**

Due to previous work using column packing materials as AAs,^{34,35,38,39,43,45,49} initial experiments employed C18 silica microparticles as a generic, easily obtained AA

(Supplemental Figure S1). These experiments led to a modest increase in NP recovery, but problems in their implementation, including bead settling/clogging (only ~25% pass through the probe and tubing) and the need to use additives (BSA) to improve bead dispersion made them impractical for *in vivo* use. In order to obtain non-specific affinity enhancement of NPs, another type of particles that had C18 surface functionality but also magnetic cores for simplified sample handling and other surface modifications for increased water solubility were employed, C18 magnetic microparticles (C18M μ P). These 1 μ m diameter particles are commonly employed for removal of salts from biological samples prior to analyses that are sensitive to salt, such as mass spectrometry. Results obtained using C18M μ P as affinity agents are presented in Fig. 1 and Tables 1 and S2. The C18M μ P significantly enhanced the recovery of 4 peptides—FLP I, FLP II, SP, and SMT. Recovery was at least doubled, with final RRs of several compounds at 50% or higher. However, settling and clogging were still observed due in part to the propensity of the particles to attract each other via magnetism. The settling observed was less than the C18 silica particles due to surface modifications of these particles for improved aqueous solubility.

Based on previous work employing antibody-coated microspheres^{6,7,37,39,41–43} to improve the recovery of cytokines and neuropeptides, antibody-coated microparticles were also employed for AE-MD. Commercial magnetic immunoprecipitation (IP) kits and a commercially available anti-FMRFa antibody were used to create antibody-coated beads. Traditional agarose bead-based IP kits contain beads of ~140 μ m diameter, which is unsuitable for passage through the probe and tubing. Magnetic IP kits employ smaller beads (diameters 1–5 μ m), and have the advantage of simpler separation. Magnetic microparticles coated with protein G were linked to rabbit polyclonal anti-FMRFa as recommended by the manufacturer to create antibody-coated magnetic microparticles (AbM μ P) and added to the perfusate.

The relative recoveries obtained with and without AbM μ P in the perfusate are enumerated in Fig. 2 and Tables 1 and S2. No significant increases in RR were obtained for the NP standards, with the exception of SMT, whose RR was enhanced significantly ($p=0.002$) by about 2.5-fold. These results do not mirror findings of *in vitro* bead-binding assays (Supplemental Figure S2), in which SMT bound poorly to AbM μ P and other NPs had a high degree of binding. Bead settling and tube clogging were observed to a similar extent as observed with the magnetic C18 particles, and this could explain these contrary results—beads with bound NPs may have remained stuck in the tubing. Approximately half of the AbM μ P passed through the probe and tubing.

Magnetic nanoparticles of 100 nm diameter were the final affinity agent tested in this study. These particles are also coated with protein G and were conjugated to the same anti-FMRFamide antibody (AbMnP). Particles of this size do not have permanent magnetic fields and thus are not attracted to each other in the absence of an external magnetic field.⁵⁶ This greatly reduces settling of the beads in the syringe and tubing. The smaller size of these particles also reduces settling, and it was observed that around 100% of the particles pass through the tubing. Two different concentrations of nanoparticles were used; one that was equal to that used for the magnetic microparticles and C18 beads, and one that was 6 times concentrated (6 $\times$ AbMnP), possible due to the reduced settling and lack of magnetic interaction between nanoparticles.

RR enhancements are shown in Fig. 3 and Tables 1 and S2. Statistically significant ($p<0.05$) recovery enhancements were obtained with AbMnP for 3 of the 6 NPs. When the concentration of nanobeads was increased, 4 out of 6 NPs had significantly enhanced RRs. FMRFa showed a strong trend toward enhanced recovery, but this trend did not meet the statistical significance threshold. The recovery of bradykinin was not enhanced by any of the

affinity agents, likely due to its small, hydrophilic character and lack of an amidated C-terminus. From the data obtained here for several NPs with different sequences, it is fair to assume that the antibody used primarily recognizes C-terminal amidation, followed by hydrophobic and basic amino acids. As a side note, the promiscuity of this antibody provides additional support for the use of mass spectrometry as an unbiased technique for NP analysis.

One concern that thus becomes important due to non-specific binding to antibodies is saturation of the beads' binding capacity. High-concentration components of a complex biological sample (or fragments thereof), such as albumin in mammals and cyanin proteins in crustaceans, could fully occupy these sites *in vivo*, and thus binding of low-concentration biological molecules of interest to the beads will be non-linear and unreliable for accurate representation of *in vivo* concentration changes. Thus, antibody-linked beads in microdialysis perfusate should be used with caution when attempting to accurately determine the concentration changes of analytes in the extracellular environment.

***In vivo* recovery enhancement**

Several proof-of-principle tests to determine the suitability of affinity-enhanced microdialysis for *in vivo* application were conducted. Jonah crabs (*Cancer borealis*) were implanted with microdialysis probes following a modified version of a published technique.¹⁴ The probes of several crabs were perfused with crab saline or antibody-linked nanobeads in crab saline solution.

Representative data for baseline NP content in microdialysis samples obtained with and without AbMnP in the perfusate is shown in Fig. 4. Here, extracted ion chromatograms (XICs) for two FMRFamide-like peptides (FLPs) in samples obtained under baseline conditions with and without AbMnP are displayed. These samples were obtained from the same crab at the same time period on different days, with the AbMnP experiment conducted on the last day, so probe fouling is not a factor. They were also analyzed on the same day after storage under acidic conditions, which have been shown to stabilize MD samples². XICs are plotted with the same y-axis scale after smoothing and baseline subtraction. FLP peaks from samples obtained without AbMnP have intensities that are 20 to 40% as intense as those obtained with AbMnP. Only very slight retention time shifts are observed, likely due to changes in ambient temperature. The UPLC column was kept at room temperature, which varies several degrees throughout the day. A similar qualitative enhancement of peak intensity was observed in two other AE-MD experiments, conducted on different crabs, the results of which are not presented here.

Quantitative analysis also indicates that AbMnP improve NP recovery *in vivo*. Table S3 enumerates NPs previously detected in MD and whether these compounds were also detected in samples obtained during a feeding experiment with or without AbMnP in the perfusate. Compared with previous work in which only 35 NPs were detected in samples collected over 4–18 hours and concentrated ~100-fold prior to analysis,¹⁴ the detection of 31 NPs in a sample collected over only 30 min and analyzed without preconcentration is a great enhancement in sensitivity. Microdialysate from the same crab obtained with simple crab saline as perfusate only contained an average of 25 NPs, with 17 of those detected in all three feeding trials. Therefore, the increased NP detection sensitivity is due not only to enhancements in UPLC-MS sensitivity that have occurred since the previous work was published, but also to the affinity-enhanced recovery by AbMnPs.

For many trials without affinity agent in the perfusate, it was possible to detect NPs with reasonable reproducibility across trials, but their abundance was so low that reliable quantity changes could not be observed. In other words, the NPs were present at their lower limit of

detection (LLOD), which is below their lower limit of quantification (LLOQ). Addition of affinity agents increased the concentration of the NPs for collection and detection to a higher level, and thus reliable quantitation could be conducted. While concerns about AA active site saturation should still be addressed by validation of observed NP trends by more sensitive techniques, affinity enhancement improves MD from a mostly qualitative technique to a quantitative technique by increasing the concentration of NPs to above their LLOQ. The LLOQ, LLOD, and linear range of this detection method when used with BK as an internal standard are illustrated in Fig. S3. For the LC-MS system employed, the LLOQ of these peptides is around 45 μM , and the LLOD is $\sim 5 \mu\text{M}$. Thus, NPs must be enriched for reliable quantitation.

AE-MD was employed to enrich NPs collected *in vivo*. Results are indicated in Figs. 5 and S4, which describe relative concentration changes in seven identified crab NPs following feeding. All data points were obtained from the same crab; three feeding trials were conducted without AA, and one was conducted with AbMnP. The means and SEMs are plotted for the no AA trials. The NPs shown had low variance between the three no AA trials, and were detected in all time points. However, their concentrations do not appreciably change as they cannot be reliably quantified. Dynamic changes in these peptides are observed when AbMnPs are added to the perfusate, increasing the concentration of the NPs to a level at which they can be quantitated. This result has been replicated in a separate *C. borealis* feeding trial (unpublished data).

Most of the NPs appear to increase in concentration following feeding; reaching peak levels around 105 min after feeding. It is possible, therefore, that these NPs are neurochemicals involved in feeding. In the authors' experience, crabs start feeding immediately after food is presented and stop 15–30 min. later, while still holding on to the food. They then recommence eating a few minutes later, around 60–90 min. after food is presented. This may occur as a result of the crab filling its stomach with food, allowing that food to be processed by the stomach and passed on to more distal parts of the digestive tract, and followed by repetition of the process. Since some of the NPs gradually increase before reaching peak concentration (RYLPT, SDRNFLRFa, YSFGLa, and pERPYSFGLa), these peptides might be continually released into the hemolymph while the animal is eating, and reaching a certain peak level could be an indicator of satiety. Other NPs that have a more dramatic spike in concentration levels (GPRNFLRFa, I/LNFTHKFa, and NFDEIDRSFGFN) could be released after satiety is reached. Although these results are preliminary and further investigation will be required, they demonstrate how AE-MD can be used to observed dynamic changes in NPs that would otherwise appear unchanged in typical MD experiments, due to their presence below their LLOQ.

Conclusions

AE-MD has been conducted with a variety of AAs. AbMnP provided the greatest enhancement in neuropeptide recovery and could be used at higher concentrations than AbM μ P due to their non-aggregation. This permitted statistically significant recovery enhancements for 4 out of 6 NP standards tested. The recovery enhancement was not specific to compounds with sequence similarity to FMRFamide, the compound against which the antibody was generated, and thus the mechanism of recovery enhancement is non-specific. This is likely due to the high cross-reactivity of most NP antibodies, and although it could be detrimental if one desires to enrich a single NP for analysis due to specific interest in that compound, it will provide greater success in NP discovery and survey experiments, in which multiple and/or unknown NPs are of interest. These AbMnPs were used in experiments in the crab to determine the roles of circulatory NPs in feeding. They increased NP identification and made quantitation of NPs possible during a dynamic process. This will

enable assignment of putative function for several NPs in feeding. AE-MD with AbMnP is a technique that has great potential to enrich NPs in microdialysate for correlation of function with molecular identity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AA	affinity agent
AE-MD	affinity-enhanced microdialysis
AbMnP	antibody-coated magnetic nanoparticles
AbMμP	antibody-coated magnetic microparticles
ANOVA	analysis of variance
BK	bradykinin
C18SμP	C18 silica microparticles
CabPK	<i>Cancer borealis</i> pyrokinin
CabTRP	<i>Cancer borealis</i> tachykinin-related peptide
CCAP	crustacean cardioactive peptide
C18MμP	C18 magnetic microparticles
FLP	FMRFa-like peptide
FLP I	<i>Homarus americanus</i> FMRFa-like peptide I
FLP II	<i>Homarus americanus</i> FMRFa-like peptide II
FR	flow rate
LLOD	lower limit of detection
LLOQ	lower limit of quantitation
MD	microdialysis
MS	mass spectrometry
MWCO	molecular weight cutoff
NP	neuropeptide
NT	neurotransmitter
RPCH	red pigment concentrating hormone
RR	relative recovery

SEM	standard error of the mean
SMT	somatostatin-14
SP	substance P

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Figure 1.

Relative recovery enhancement for six different NP standards by the addition of C18 magnetic microparticles to the perfusate. Values indicated are the means, with error bars showing the SEM. NP names are abbreviated as follows: *Homarus americanus* FMRFamide-like peptide I (FLP I), *Homarus americanus* FMRFamide-like peptide II (FLP II), substance P (SP), somatostatin-14 (SMT), and bradykinin (BK). C18 magnetic microparticles are abbreviated C18M μ P, and no affinity agent is written as No AA. Significant differences ($p < 0.05$) from the No AA condition are indicated with an asterisk (*).

Figure 2.

Relative recovery enhancement for 6 NP standards by the addition of antibody-coated magnetic microparticles to the probe perfusate. Values indicated are the means, with error bars showing the SEM. NP abbreviations are indicated in the legend to Fig. 1. Antibody-coated magnetic microparticles is abbreviated AbM μ P, and no affinity agent is written as No AA. Significant differences ($p < 0.05$) from the No AA condition are indicated with an asterisk (*).

Figure 3.

Relative recovery enhancement for 6 NP standards by the addition of antibody-linked magnetic nanoparticles at a concentration equivalent to those used with previous affinity agents and at a concentration six times higher. Values indicated are the means, with error bars showing the SEM. NP abbreviations are indicated in the legend to Fig. 1. Antibody-coated magnetic nanoparticles is abbreviated AbMnP, the higher concentration is noted as 6× AbMnP, and no affinity agent is written as No AA. Significant differences ($p < 0.05$) from the No AA condition are indicated with an asterisk (*). Significant differences ($p < 0.05$) from the AbMnP condition are indicated with a dagger (†).

Figure 4.

Ultrahigh performance liquid chromatography-time-of-flight (UPLC-TOF) extracted ion chromatograms (XICs) for two peptides of interest (A. GPRNFLRFamide, B. ENRNFLRFamide, in their +2 charge states) in microdialysis samples obtained from a single *Cancer borealis* under baseline conditions. The highest intensity peak (red) represents a sample collected with AE-MD, with antibody-linked magnetic nanoparticles (AbMnPs) in the perfusate, and the two lower intensity peaks (maroon and black) represent samples collected without affinity agent from the same crab. Samples were all run on UPLC-TOF on the same day with the same chromatographic and mass spectrometric conditions. Chromatograms are smoothed, baseline subtracted, and plotted with the same Y axis scale, which is represented as raw ion counts. Retention times are indicated at the top of the peak, and the masses selected for generation of the XIC are indicated in the top right corners.

Figure 5.

Affinity-enhanced microdialysis permits observation of changes in NP levels following feeding. The x-axis crosses the y-axis at a value of one, which would indicate no change from baseline conditions. Without affinity agent, the NPs are present at levels too low for changes to be observed. For No AA data, 3 feeding trials from the same crab are pictured as an average with standard error as the error bars. Some error bars are obscured by the size of the marker at each data point. The AE-MD data was obtained from the same crab in a feeding trial. AE-MD data shows dynamic changes in NPs after feeding for several peptides, whereas the No AA data shows little variation.