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Simultaneous oxytocin and arg-vasopressin measurements in microdialysates using capillary liquid chromatography-mass spectrometry

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Abstract

Oxytocin (OXT) and arg-vasopressin (AVP) are nonapeptides with many important functions both peripherally and centrally. Intracerebral microdialysis has helped characterize their importance in regulating complex social and emotional processes. Radioimmunoassay is the most commonly used analytical method used for OXT and AVP measurements in microdialysates. These measurements have several well-known issues including single peptide per assay limit, possible cross-reactivity between structurally related peptides, and laborious sample preparation with radioactive materials. Here we demonstrate the use of capillary LC-MS³ for measuring OXT and AVP simultaneously in dialysates at a 10 min sampling frequency. Microdialysate samples required no preparation and instrumentation was commercially available. Microdialysis probes made with polyacrylonitrile membranes were suitable for high level recovery of the peptides *in vitro* and *in vivo*. Responses were linear from 1 – 100 pM. Matrix effect was assessed by standard addition experiments and by comparing signal intensities of OXT and AVP standards made in aCSF or dialysate. It was determined that the online washing step used on this setup was adequate for removing contaminants which interfere with electrospray ionization efficiency. *In vivo*, both peptides were stimulated by high K⁺ (75 mM) aCSF perfusion in the paraventricular nucleus (PVN). Also, a systemic injection of high Na⁺ (2M) caused a rapid and transient increase in PVN OXT while AVP increased only after 1.5 h. Our findings suggest that Capillary LC-MS³ is a straightforward method for monitoring OXT and AVP simultaneously from complex samples such as dialysates.

Keywords

oxytocin; vasopressin; microdialysis; mass spectrometry; paraventricular nucleus; hypothalamus

Introduction

Oxytocin (OXT) and arginine vasopressin (AVP) are nonapeptides with a host of important biological functions. In the periphery OXT facilitates uterine contractions and mammalian milk let down reflex while AVP is linked to arterial blood pressure and plasma salt balance

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(Share, 1988; Borrow and Cameron, 2012). In the CNS both peptides are thought to mediate complex social behaviors such as pair bonding and instinctive maternal aggression (Winslow et al., 1993; Wang and Aragona, 2004; Bosch and Neumann, 2005). They are synthesized in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus (Swaab et al., 1975; Rhodes et al., 1981) where they are released locally from dendrites (Ludwig, 1998; Ludwig and Leng, 2006) and peripherally via the pituitary gland. PVN OXT/AVP containing neurons also project to brain nuclei involved in socialization, mood and cognition such as the nucleus accumbens, amygdala complex and frontal cortex (for review see Landgraf and Neumann, 2004). Because of their important effects on social behavior, these peptides and their receptors are currently being examined as treatments for a number of diseases including autism, depression, anxiety and schizophrenia (for reviews see Scantamburlo et al., 2007; Marazziti et al., 2008; Harony and Wagner, 2010; Ring, 2011).

The primary tool for measuring OXT and AVP concentrations in brain and plasma is radioimmunoassay (RIA; Hattori et al., 1990; Landgraf and Ludwig, 1991; Bosch et al., 2005). RIA can be coupled to microdialysis sampling to monitor *in vivo* release dynamics in response to behavioral or pharmacological stimuli; however, the assays have several shortcomings. They are laborious and time consuming since they require multiple reagent addition steps and incubation periods. RIA radioactive materials are potentially dangerous and their acquisition and disposal are tightly regulated, representing a substantial nonscientific hurdle to overcome. The mass limit of detection (LOD) for RIAs is typically around 100 amol/sample. As a result, relatively large sample volumes must be collected for microdialysis experiments (~100 µL) at high flow rates (3 µL/min) thus impairing temporal resolution. Finally, some RIAs may be nonspecific for peptides of interest. RIAs rely on competition between target peptide and a radiolabeled analogue. Both peptides compete for an antibody which may retain some degree of affinity for peptides with similar amino acid sequences. Thus, peptide cross-reactivity between target peptide, metabolites, or peptides with similar amino acid composition may create uncertainty in RIA measurements. Although highly specific RIAs have been reported (Ermisch et al., 1986; Neumann et al., 1993; Ludwig et al., 2005), commercially available RIAs for these peptides remain less than ideal.

Capillary liquid chromatography (LC) with electrospray ionization-mass spectrometry (ESI-MS) has been shown to be a sensitive method for determining neuropeptides in dialysate (Emmett et al., 1995; Haskins et al., 2001; Behrens et al., 2008; Li et al., 2009; Mabrouk et al., 2011). This method is advantageous since structurally similar peptides are chromatographically separated and further resolved with multi-stage MS (MS^n) by identifying unique ion fragmentation patterns. As a result, the method can measure multiple peptides in one assay and does not suffer from problems of cross-reactivity since mass to charge (m/z) ratios and fragmentation patterns are sequence specific. Mass LODs are in the low amol range (at least for some peptides) allowing smaller samples to be used and better temporal resolution for measurements.

Although LC- MS^n has proven useful for some neuropeptide measurements in dialysates such as enkephalins, dynorphin (DYN), neurotensin and angiotensin IV (for review see Van Eeckhaut et al., 2011), it has yet to be applied to OXT or AVP detection. Given the importance of central OXT and AVP release, we aimed to develop a capillary LC-MS method to monitor these peptides simultaneously in dialysates from the hypothalamus.

Materials and Methods

Chemicals

OXT, AVP and dynorphin (DYN) peptides were all purchased from Phoenix Pharmaceuticals (Burlingame, CA). Water and methanol for mobile phases are Burdick & Jackson HPLC grade purchased from VWR (Radnor, PA). All other salts and chemicals were from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Microdialysis

Adult male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing between 250 and 350 g were used for all experiments. Rats were housed in a temperature and humidity controlled room with 12 h light/dark cycles with food and water available *ad libitum*. All animals were treated as approved by the University of Michigan Unit for Laboratory Animal Medicine (ULAM) and in accordance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Prior to surgery, rats were anesthetized with an intraperitoneal (i.p.) injection of a ketamine (65 mg/kg) and dexdormitor (0.25 mg/kg) mixture prepared in an isotonic salt solution. Concentric microdialysis probes with 1 mm long active membrane (AN69 from Hospal, Bologna, Italy) were implanted. Microdialysis probes consisted of silica capillary (Polymicro, Phoenix, AZ) inlets and outlets measuring 50/150 inner diameter (i.d.) and outer diameter (o.d.), respectively. Inlet and outlet was threaded through a 12 mm long, 25G stainless steel tube. Inlet of probe protruded from the stainless steel 1 mm while the outlet was recessed in the stainless steel 5 mm. Hollow fiber membrane was then threaded into the stainless steel tube and glued with Loctite 5 min epoxy (Henkel, Dusseldorf, Germany). Epoxy was also used to plug the tip of the dialysis probe. Inlet and outlet adapters were made of 150/360 i.d./o.d. capillary at the far end of the probe to connect to fluid lines.

Probes were implanted during under anesthesia using an ultra precise model 963 stereotaxic instrument (David Kopf Instruments, Tujunga, CA), into the PVN according to the following coordinates from bregma and top of the skull: AP -1.6 , ML ± 1.8 , DV -9.1 at a 10° angle to avoid sagittal sinus damage (Paxinos and Watson, 2007). Probes were secured to the skull by acrylic dental cement and metallic screws. Following surgery, rats were allowed to recover and experiments were run 24 h after probe implantation. Microdialysis probes were flushed at a flow rate of $1.5 \mu\text{L}/\text{min}$ with a modified Ringer solution (composition in mM: CaCl_2 1.2; KCl 2.7, NaCl 148 and MgCl_2 0.85) for 2 h using a Chemyx Fusion 400 syringe pump (Chemyx, Stafford, TX). Perfusion flow rate was then reduced to $0.9 \mu\text{L}/\text{min}$ and samples were collected every 10 min into tubes containing $0.5 \mu\text{L}$ acetic acid to preserve peptide stability as previously described (Li et al., 2009). Samples were immediately injected on the LC-MS system following collection.

For high K^+ experiments, aCSF was modified to contain (in mM) CaCl_2 1.2; KCl 75, NaCl 72.7 and MgCl_2 0.85. In another set of experiments, hypertonic saline (2 M NaCl) was injected i.p. ($600 \mu\text{L}/100 \text{ g}$ animal weight) as described elsewhere (Ludwig et al., 2005). When experiments were completed, animals were euthanized and brains were extracted and submerged in a 10% paraformaldehyde solution at 4°C until histology. Probe position was verified by cresyl violet staining of $35 \mu\text{m}$ sections taken via microtome (Leica, SM2000R, Bannockburn, IL).

For *in vitro* recovery experiments, a 1 mm microdialysis probe was lowered into a 100 pM OXT/AVP standard solution in aCSF and perfused at 0.2, 0.6, 0.9, 1.5, and $3 \mu\text{L}/\text{min}$. Each flow rate was used until at least $9 \mu\text{L}$ of dialysate was collected. For instance, $3 \mu\text{L}/\text{min}$ was done for 3 min, and 0.9 for 10 min and $0.2 \mu\text{L}/\text{min}$ at 45 min. Standard solution was

continuously stirred at room temperature during this procedure and samples were collected into vials in the same way as during *in vivo* experiments. Sample analysis occurred immediately following collection. For each flow rate, 3 separate samples were collected and analyzed. To determine the percent recovery, the ratio between peak area of standard solution (i.e. 100 pM) and the peak areas of the collected dialysate was taken and expressed as a percentage. This was performed for both OXT and AVP.

OXT and AVP detection with capillary LC-MS³

OXT and AVP were measured using a modified version of a method previously used for enkephalins *in vivo* (Li et al., 2009; Mabrouk et al., 2011). Capillary LC columns were packed in house. Prior to packing columns with reversed-phase material, porous frits were placed at the tip of a 30 cm long 50/360 i.d./o.d. capillary (Polymicro, Phoenix, AZ). Frits were made by mixing 300 μ l of sodium silicate and 100 μ l of potassium silicate (KasilTM, PQ Corporation, Malvern, PA) and 100 μ l of formamide. Solution was stirred, then capillaries were dipped into the silicate solution for 2 s, allowing ~ 1 cm of the capillary to be filled via capillary action. Capillary was then baked in an oven overnight at 100°C. End of capillary was cut leaving only 1 mm of frit at tip.

Capillaries were flushed with 80% MeOH and 20% H₂O using a high pressure reservoir operating at 1000 psi. Then, a solution of 5 μ m Alltima C18 reversed-phase particles (Alltech, Deerfield, IL) was prepared by mixing 20 mg of packing material in 5 ml of 80% acetone and 20% H₂O. A miniature stir bar was added to this 5 ml vial to keep solution stirred during packing. Solution was placed in the high pressure reservoir operating at 1000 psi. Columns were packed to 4 cm. Compared to previous works using this setup (Li et al., 2009, Mabrouk et al., 2011), increasing the column i.d. from 25 to 50 μ m i.d. made them less susceptible to clogs.

ESI emitter tips were also prepared in-house from a 3 cm length of 40 μ m i.d. fused silica capillary using a laser puller (P-2000, Sutter Instruments, Novato, CA). Columns and tips were joined using a 2 cm PTFE 1/16" $\times$.010" sleeve adapter. An air driven fluid pump (DHSF-151, Haskel Inc., Burbank, CA) was used for sample loading and desalting (4000 psi), and a micro HPLC pump (MicroPro, Eldex Laboratories, Napa, CA) for gradient separation and eluting (700 psi). A Valco (VICI, Houston, TX) 6 port valve was used to switch between these pumps during operation. Samples were injected using a WPS-3000TPL autosampler (Dionex, Sunnyvale, CA), in partial loop injection mode (5 μ L loop).

The system was operated by loading 5 μ L of sample over 8 min followed by 2 min rinse with H₂O to desalt the column at 2.5 μ L/min. Following loading and desalting, injector valve switched to the gradient pump to elute the peptides at 300 nL/min. Mobile phase A consisted of LC-MS grade water with 0.5% acetic acid and mobile phase B consisted of LC-MS grade methanol with 0.5% acetic acid. The gradient program began with an isocratic step of 5% B then a linear increase to 80% B over 4 min, followed by a linear increase to 100% B in 1 min. An isocratic step at 100% B for 2 min was followed by a linear decrease down to 5% B over 0.5 min. Finally, 5% B was maintained isocratically for 2 min to re-equilibrate the LC system before the next injection. All valve switching and runs were controlled automatically with Xcalibur software (Thermo Fisher Scientific).

The column and emitter tip were coupled to a PV-550 nanospray ESI source (New Objective, Woburn, MA) interfaced to an LTQ XL linear ion trap (LIT) MS (Thermo Fisher Scientific, Waltham, MA). A +2.5 kV potential was applied to a liquid junction prior to the column for electrospray. The MS³ ion transition pathways set on the LIT for OXT (singly

charged) and AVP (doubly charged) were: 1007→723→706 and, 543→534→525, respectively and isolation width was set to 3 *m/z*.

Statistics

Dialysate concentrations were transformed to percent of baseline measurement to normalize pretreatment levels to 100 percent. All analyses were performed in Prism 5 (GraphPad, La Jolla, CA). The measurements were all continuous variables and the Kolmogorov-Smirnov test was used to assess normality of the residuals for each individual repeated measurement and this assumption was met. A two-tailed repeated measures analysis of variance (RM ANOVA) was performed on all microdialysis data followed by a post-hoc Tukey test to test the pairwise difference between every time point and baseline (i.e. 100%). P values < 0.05 were considered statistically significant.

Results

Detection and calibration

MS³ spectra revealed dominant peaks for AVP (*m/z* 525) and OXT (*m/z* 706) which were then used for quantification (fig. 1A, 1B). OXT and AVP in standards made up in aCSF were readily resolved and detected within 5 min as shown by the LC-MS trace in fig. 1C. For quantification, calibration curves were generated using 1, 10, 25, 50 and 100 pM OXT and AVP in aCSF to determine linearity and reproducibility of the method (fig. 1D). Each standard was injected 3 times. The average value of peak areas ± SEM was plotted against known concentration values. $R^2 = 0.9968$ and 0.9938 for OXT and AVP, respectively, thus establishing a high degree of linearity within this range (fig. 1D). Standard injections of 1 pM (5 amol) OXT and AVP were readily measured by the method indicating sufficient detection limits for *in vivo* analyses.

Microdialysis probe recovery

We have previously used microdialysis probes with polyacrylonitrile (PAN; molecular weight cut-off 40,000 Da) membrane (AN69, Hospal, Bologna, Italy) for opioid peptide measurements (Mabrouk et al., 2011). To determine if this membrane material was suitable for OXT and AVP measurements we performed *in vitro* recovery tests using different microdialysis flow rates (fig. 2). A 1 mm long probe (same dimensions as those used for *in vivo* experiments) was submerged into a standard solution containing 100 pM of both OXT and AVP at room temperature. Flow rates through the probe were incrementally adjusted from 3, 1.5, 0.9, 0.6 to 0.2 µL/min. Samples were collected until a total of 9 µL was available for an injection (amount needed for injection). Three fractions for each flow rate were collected then injected on LC-MS system. Data points are expressed as the percent recovery compared to 100 pM standard solution, i.e. the ratio was taken between collected fraction peak area and peak area of standard (100 pM). Recovery for OXT and AVP were around 5% at a flowrate of 3 µL/min and increased with decreasing flow rate, as expected, to as high as 40% at 0.2 µL/min (fig. 2). The 0.9 µL/min flowrate was selected for the current study since this achieves an easily managed sample volume of 9 µL in 10 min while maintaining reasonable relative recovery (~15 %). As is typical in microdialysis, it is possible to vary the flow rate to meet the needs of the experiment, e.g. lower flow rate may be desirable to achieve higher concentrations; although that would require methods of manipulating very small volumes if collected in the same 10 min window. These results demonstrate PAN membrane microdialysis probes are suitable for OXT and AVP sampling. Further, they show that high recoveries can be obtained at lower flow rates which are compatible with the microscale LC-MS method.

Matrix effects from in vivo samples

ESI-MS is known to be sensitive to matrix effects, such as high salt concentrations in samples that may alter analyte signal. Dialysate samples and standards may generate different signal intensities if such effects are not controlled. To counter matrix effects, we calibrated the system using standards dissolved in aCSF and employed a high pressure wash step to remove contaminants from the column while retaining peptides on the reversed phase column. To determine if calibration obtained from *in vitro* standards could be applied to *in vivo* measurements we performed a standard addition experiment. Both OXT and AVP could be readily detectable in PVN dialysates at basal levels (fig. 3A). A PVN dialysate sample gave a peak area of ~ 400 counts $\times$ s which corresponded to 10 pM obtained by an *in vitro* calibration (fig. 3B). The dialysate sample was then spiked with standard peptide to raise the concentration another 10 pM yielding a doubling of the peak area (fig. 3B). In order to further investigate the possibility of matrix effects over a range of concentrations, we compared signal intensities of OXT and AVP standards (1, 10 and 100 pM) made up in either aCSF or dialysate (fig. 3C, 3D). Dialysate was generated from the rat dorsolateral striatum, a brain region where OXT or AVP levels were not detectable. These results show that MS sensitivity is comparable in either dialysate or standards made in aCSF. Thus matrix effects have been overcome by the LC methods used.

In vivo microdialysis

We implanted microdialysis probes in the PVN which is a nucleus of the hypothalamus known to contain OXT and AVP. Basal dialysate concentrations of OXT and AVP were 5.4 ± 1.3 pM ($n = 12$) and 11.1 ± 2.9 pM ($n = 12$ rats), respectively. High K^+ (75 mM) stimulation caused a prompt increase in both OXT (530% maximal) and AVP (800% maximal) for the 20 min high K^+ was present in the aCSF (fig. 4A). When high K^+ was removed from the aCSF, peptide levels returned to baseline within the temporal resolution of the method (fig. 4A).

To further test and demonstrate the method, we determined if OXT and AVP could be stimulated osmotically as previously reported in the SON of the hypothalamus (for review see Ludwig and Leng, 2006). Systemic hypertonic saline injection caused a rapid increase in OXT levels immediately after injection (maximal 380%; fig. 4B). AVP levels initially remained stable post saline injection but then increased after 90 min and persisted (maximal 340% at 110 min) until the end of the experiment (fig. 4B). These microdialysis data are reported in absolute concentrations in supplementary figure S1.

An important feature of using LC-MSⁿ is the potential to detect multiple peptides and add peptides of interest to the sample assay. Since DYN mRNA is found in the PVN and kappa opioid receptors affect AVP release (Hallbeck, 2000; Tsushima et al., 1993) we made a preliminary attempt to determine if DYN₍₁₋₈₎ could also be measured in this assay. *In vitro* studies revealed that MS² of the doubly charged DYN₍₁₋₈₎ (m/z transition is 491 $\rightarrow$ 435) would give the most sensitive MS detection. Such studies also revealed that DYN₍₁₋₈₎ was readily separated chromatographically from OXT and AVP by the gradient method used. Separation and detection were indeed sufficient to resolve and detect all 3 peptides simultaneously from PVN microdialysates (fig. 5).

Discussion

A novel capillary LC-MS method was developed for simultaneously determining OXT and AVP in small samples with detection limits suitable for dialysate measurements. In the PVN, high K^+ aCSF stimulated the release of both peptides and an i.p. injection of hyperosmotic saline briefly stimulated OXT while causing a delayed yet long lasting

increase in AVP levels. In addition to the analytical method itself, we have optimized the microdialysis procedure for making such measurements.

PAN membrane has been shown to be superior for enkephalin recovery compared to cellulose, cuprophane or polycarbonate (Maidment et al., 1989). This is likely due to the high molecular weight cutoff of the material (40,000 Da) as well as other less characterized properties such as porosity or hydrophobicity. The current study demonstrates that this material (and overall concentric probe construction) is also suitable for the recovery of OXT and AVP. In other OXT or AVP microdialysis reports, a “U-shaped” probe is generally employed (Neumann et al., 1993; Ebner et al., 2000; Ludwig et al., 2005). Those experiments also frequently use perfusion flow rates of 3 $\mu\text{L}/\text{min}$ and above which reduces peptide relative recovery. We therefore lowered flow rates to elevate relative recovery for improved MS signal and detection.

We found that LC-MS³ sensitivity for both AVP and OXT to be similar to what we have previously observed with enkephalins enabling basal levels to be readily detected at low picomolar concentrations (Haskins et al., 2001; Li et al., 2009; Mabrouk et al., 2011). The detection limit of the current method, expressed as mass, 5 amol (1 pM detection limit in a 5 μL sample), compares favorably with the ~100 amol (0.1 pg/100 μL sample) limits typically reported for RIAs for these peptides (Neumann et al., 1993; Ludwig et al., 1994; Ebner et al., 2000). In principle the low detection limit could be used to improve temporal resolution (by collecting fractions at smaller intervals) or spatial resolution (collection of smaller samples from smaller probes); however, such advances would require better sample handling capability. The capillary LC-MS³ method used here has several features that allow such low detection limits. The stationary phase is highly retentive to peptides allowing a relatively large volume sample to be loaded and concentrated within the small volume of the column (total column volume equals 31 nL compared to the injection volume of 5 μL). The use of a rinse step following injection washes away salt that normally causes detrimental ionization suppression of analytes by ESI-MS. Loading and rinsing at 2.5 $\mu\text{L}/\text{min}$ for 10 min is required to adequately reduce the salt content on the column for reliable results. Elution at a relatively low flow rate of 300 nL/min may also improve sensitivity of the ESI process (Emmett and Caprioli, 1994). The use of largely different flow rates in the same method requires two pumps, one set for fast loading and rinsing and the other for elution. Fortunately, the switching of these pumps is easily automated (Haskins et al., 2001; Li et al., 2009). We determined that the desalting step was sufficient to remove salts and contaminants from dialysates since standard peak areas for OXT and AVP were similar in both matrices (fig. 3B).

Some modifications were used for this group of experiments to improve throughput and reliability compared to previous reports on this system (Haskins et al., 2001; Li et al., 2009; Mabrouk et al., 2011). One main difference is the use of 50 μm i.d. compared to 25 μm i.d. LC columns. This modification has greatly improved the number of injections performed per column (30–50 compared to 10–20) and allows for faster loading and desalting (10 min compared to 15 min) since column backpressure is reduced. Also we have optimized and fully automated our capillary autosampler and MS method which has improved throughput compared to prior configurations.

Applying the online desalting technique and capillary LC-ESI-MS, we were able to detect low picomolar basal concentrations in dialysate with good reliability (6.7% and 10.3% RSD for OXT and AVP, respectively). We therefore further validated the method under physiological conditions. Both peptides were released by reverse dialyzing high K^+ (75 mM) in the aCSF to depolarize PVN cells. Interestingly, Hattori et al (1992) failed to show high K^+ stimulation of OXT release in the PVN in ranges of 50 mM to 150 mM. Although

sampling period of that study was 20 min compared to 10 min in the current study, this does not explain the lack of effect they observed. On the other hand, others have found K^+ stimulated release of magnocellular cells of the SON to release OXT and AVP (Ludwig et al., 2005). Therefore it is not surprising that the OXT and AVP containing cells of the PVN would also respond to high K^+ depolarization.

Previous works have shown that local hypersmotic saline application (via reverse dialysis) to the PVN can induce the release of OXT (Hattori et al., 1992) as well as AVP (Landgraf and Ludwig, 1991). Additionally, it has been shown that systemic administration of hypersmotic saline can induce release of both AVP and OXT in the SON (Ludwig et al., 1994). Therefore we aimed to use our newly developed method to determine if i.p. administration of hypersmotic saline induced simultaneous release of AVP and OXT in the PVN. We found that hypersmotic saline administration caused a rapid and short-lived increase in OXT in the PVN. This increase in OXT likely represents an acute response to the i.p. injection stressor. OXT but not AVP in the PVN is released during an acute stressor (shaker stress) in line with our findings (Nishioka et al. 1998). Interestingly, a previous study found intravenous administration of hypersmotic saline did not increase OXT in the PVN but did increase plasma OXT levels (Hattori, et al. 1992). Our study used i.p. injections rather than intravenous administration of hypersmotic saline. Thus, the stress of the i.p. injection compared to the non stressful intravenous injection likely explains the different findings between these studies.

We did find that hypersmotic saline increased AVP levels and this effect took approximately 1.5 h to become statistically significant compared to baseline. This result is in accord with previous work which showed that for AVP in the SON, a significant effect of systemic hypersmotic saline was reached only after 2 h (Ludwig et al., 1994). However the same authors also showed that OXT increased in this same pattern in the SON following systemic hypersmotic stimulation. The SON contains magnocellular cells while the PVN contains both magnocellular and parvocellular cells and these 2 nuclei likely have different functional roles (for review see Ludwig et al., 2006). Therefore it is not surprising that they responded differently to systemically administered hypertonic saline solution.

It will be important for future studies to address the function of other neurotransmitters and neuropeptides in the PVN and their impact on OXT and AVP release. As shown in fig. 6, we have started investigating the feasibility of measuring OXT and AVP as well as opioid peptides simultaneously in microdialysate samples. Further work will be required to fully validate the method for DYN, but these preliminary results strongly suggest that it will be possible to monitor opioid peptides and OXT/AVP simultaneously. They further point to the power of using LC-MSⁿ for assessing neurotransmitter interactions *in vivo*.

In summary, we have developed a sensitive and reliable assay to measure both OXT and AVP simultaneously with capillary LC-MSⁿ. This method is compatible with small sample volumes which has improved microdialysis probe recovery (lower flow rates) as well as the temporal resolution of release monitoring (10 min sampling). Furthermore, the technique is more straightforward than RIA since samples are analyzed within ~15 min after collection with no sample preparation. When coupled to microdialysis, LC-MSⁿ may prove to be a valuable alternative to RIA for investigators seeking to understand the roles of neuropeptides in diverse biological function and neurological pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mass spectra (MS³) for AVP (A) and OXT (B) with highest intensity granddaughter ion peaks at 525 and 706, respectively. Total (top) and reconstructed ion chromatograms (lower) for AVP and OXT from LC-MS analyses of a 50 pM standard made up in aCSF (C). RT is retention time in min. OXT and AVP standards were analyzed on LC-MS in triplicate at 1, 10, 25, 50, and 100 pM demonstrating linearity within this concentration range (D).

Figure 2.

In vitro recovery of OXT and AVP for a 100 pM standard solution as a function of flow rate using PAN membrane microdialysis probes. As expected, lower flow rates improve relative recovery. The 0.9 μl/min flowrate was chosen for in vivo experiments since it gave adequate recovery and fraction collection volumes.

Figure 3.

OXT and AVP were readily detectable in PVN dialysates (A). Standard addition experiment to compare MS signal from aCSF and dialysate matrices (B). RT is retention time in min. Peak area is compared for 10 pM standard OXT and AVP in aCSF (left) and a PVN dialysate sample (center). Spiking the dialysate with additional 10 pM peptide resulted in an additive signal (right). Standard curves (1, 10 and 100 pM) were run in both aCSF and dialysate which showed that both OXT (C) and AVP (D) signal intensities were similar regardless of sample matrix. The result shows that the system sensitivity is the same in either aCSF or dialysate samples.

Figure 4.

High K⁺ aCSF stimulated OXT ($F_{(6,5)} = 11.76$, $p < 0.0001$) and AVP ($F_{(6,5)} = 6.90$, $p = 0.002$) release (A). Black bar represents the time in which high K⁺ was in aCSF. Systemic injection of hypertonic saline (black arrow) caused a transient increase in OXT ($F_{(12,5)} = 7.33$, $p < 0.0001$) while causing a delayed and long lasting increase in AVP (B) ($F_{(12,5)} = 8.65$, $p < 0.0001$). RM ANOVA and a post-hoc Tukey test were performed to compare basal levels against post drug levels. All *in vivo* microdialysis data are expressed as percent of baseline $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to basal levels in the PVN.

Figure 5.
Total (top) and reconstructed ion chromatograms (lower) for DYN₍₁₋₈₎, AVP and OXT from LC-MS analyses of dialysate from the PVN. Chromatogram is representative of 3 separate PVN dialysate injections made using this method. RT is retention time in min.