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LC-MS/MS measurement of endogenous oxytocin in the posterior pituitary and CSF of macaques: a pilot study.

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Abstract

Oxytocin (OT) is a neuropeptide released into systemic circulation via the posterior pituitary (peripheral) and into the central nervous system via widespread OTergic pathways (central). Central OT plays a significant role in variety of functions from social and executive cognition to immune regulation. Many ongoing studies explore its therapeutic potential for variety of neuropsychiatric disorders. Measures of peripheral OT levels are most frequently used as an indicator of its concentration in the central nervous system in humans and animal models. In this study, LC-MS/MS was used to measure OT in pituitary samples collected from adult male macaque monkeys in order to explore the correlation between individual levels of OT in the CSF (central) and pituitary (peripheral). We quantified individual differences in the levels of OT in the pituitaries (44–151 ng/mg) and CSF (41–66 pg/ml) of these monkeys. A positive correlation between these two measures was identified. These preliminary results allow for future analyses to determine whether LC-MS/MS measures of peripheral OT can be used as markers of OT levels in the brain of nonhuman primates that serve as valuable models for many human neuropsychiatric disorders.

Keywords

Oxytocin; pituitary; macaque monkey; LC-MS/MS

Introduction

OT is a neuropeptide synthesized by parvocellular and magnocellular neurons of the hypothalamus [1]. OT is released into systemic circulation via the posterior pituitary (peripheral OT) and into the central nervous system via widespread OTergic pathways (central OT) [2]. OT has well-known physiological functions during labor and lactation

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Conflicts of interest

Authors have no conflicts of interest to disclose.

in humans and other mammals; however, recent studies have revealed that it plays anti-inflammatory, metabolic and cardioprotective roles [3–6]. Central OT acts as a neuromodulator and plays a role in social cognition, executive functions, maternal behavior, brain development, pain perception, etc. [7–11]. Evidence of the OT effects prompted investigation of its therapeutic potential in various neuropsychiatric disorders such as autism, schizophrenia, mood disorders and addiction [12]. Majority of these studies were conducted in rodents that widely used as models for studying learning, stress and social behaviors. Substantial differences between rodents and primates in the mechanisms that regulate these processes require utilizing nonhuman primates for bridging rodent to human research [13, 14].

Accurate measurement of both central and peripheral OT is required in order to determine whether the endogenous OT system is altered in these neuropsychiatric disorders. Measures of peripheral OT obtained in the plasma are most frequently used as an indicator of central OT levels in humans and animal studies. However, OT is a pulsatile peptide with a short half-life in plasma on the order of minutes [2, 15, 16]. Plasma levels of endogenous OT might be undetectable in subjects with a neuropsychiatric disorder that are associated with its deficit. Therefore, postmortem measures of endogenous OT in the posterior pituitary might greatly benefit our understanding of OT functioning and role in neuropsychiatric disorders.

In humans and other species in preclinical studies, levels of OT in the plasma and cerebral spinal fluid (CSF) have been measured with radioimmunoassay (RIA) [17, 18] and enzyme-linked immunoassays (ELISA) [19, 20]. These methods are well-established and commonly used to measure central and peripheral levels of OT. However, they frequently provide inconsistent results [21] due to inherent sensitivity and specificity issues found in OT immunoassays; pre-assay sample preparation such as extraction can also affect results [21, 22]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides an alternative to antibody-based assays due to its high selectivity, specificity, and sensitivity for detecting endogenous peptides in the plasma and CSF. Previously, LC-MS/MS was used to measure endogenous OT levels in rat and human plasma and CSF samples [23]. Recently, we reported using LC-MS/MS to measure endogenous OT in CSF of nonhuman primates[24]. Considering that nonhuman primates serve as valuable models for many human neuropsychiatric, neurodevelopmental and neurodegenerative disorders [25, 26], accurate measurement of OT in this species is especially important. There are no reports of applying an LC-MS/MS method to measuring levels of OT in the posterior pituitary and its correlation with central OT in the CSF. Therefore, in this study we aimed to use LC-MS/MS to measure OT in pituitary samples collected from adult male macaque monkeys in order to explore the correlation between individual levels of CSF (central) OT and pituitary (peripheral) OT.

Methods

Animals.

Pituitary (n=7) and CSF (n=7) samples were obtained from the Monkey Alcohol Tissue Research Resource (www.matrr.com). Both types of tissue were collected from alcohol-

naive adult male macaque monkeys (*Macaca fascicularis*) during standard necropsy procedure as previously described [27]. Prior to necropsy all animals were housed indoors in rooms with controlled temperature (20–22°C), humidity (65%), and a 12-h light cycle. Each subject was housed in a metal cage (0.8 m × 0.8 m × 0.9 m), fed a diet of nutritionally complete chow and fresh fruit, and had unlimited access to water. All procedures were conducted according to the Guide for the Care and Use of Laboratory Animals and approved by the ONPRC Animal Care and Use Committee.

Chemicals.

We purchased an unlabeled standard for OT from Sigma-Aldrich (St. Louis, MO); OT-d10 was purchased for use as an internal standard through a custom order from Cayman Chemical (Ann Arbor, MI). All other reagents were commercially available, and we purchased LC or MS grade chemicals when available.

CSF sample acquisition and preparation for LC-MS/MS analysis.

CSF samples (average volume 750 µl) were collected from the cisterna magna and stored at –80°C until used in the analysis [21]. LC-MS/MS analysis of OT in CSF was performed as previously described [24]. Using 15 ml polystyrene tubes, 1.0 ml of each standard, quality control (QC), or CSF sample was combined with 30 µl of internal standard mixture (34 ng/ml OT-d10 in 50:50 ACN:water, v:v) and incubated for 20 min on ice. As volumes of CSF samples ranged from 400–1000 µl, samples less than 1000 µl were brought to a final volume of 1.0 ml using charcoal stripped serum (Golden West Biologicals, Temecula, CA, USA) prior to addition of internal standard. To each tube, we added 2 ml of ice-cold ACN, vortexed thoroughly and centrifuged at 2500 × g for 10 min at 4°C. The supernatants were transferred to new 13 mm × 100 mm polypropylene tubes and evaporated in a 37°C water bath under forced air to a volume of less than 1 ml. We then added 5.5 ml of 0.2 M ammonium acetate (pH 3.0) in water, capped the tubes and mixed by inversion before centrifugation at 2500 × g for 10 min at 4°C.

Solid-phase extraction (SPE) columns (Strata × Drug B, 60 mg/6ml, Phenomenex, Torrance, CA) were conditioned prior to use by adding 2 ml of methanol, followed by 2 ml of water and 2 ml of 0.2M ammonium acetate (pH 3.0) in water; each was allowed to flow through by gravity before the next was added. The supernatants from the previous step were then added to SPE columns (one supernatant per column) by pouring. Columns were washed with 2 ml ammonium acetate (pH 3.0) in water followed by 2 ml water and 2 ml ACN and dried for 5 min in a vacuum manifold (5 in Hg; Millipore, Burlington, MA). OT was eluted with 2 × 1 ml 0.2% ammonium hydroxide in methanol into 12 mm × 75 mm polypropylene tubes and evaporated to dryness under forced air in a 37°C water bath. The extract in each tube was reconstituted in 100 µl 0.1% formic acid in 5:95 ACN:water (v:v), vortexed thoroughly and transferred to 96-well filter plates (Millipore GV Multiscreen 0.22 µm Durapore). After a 15-minute incubation at 4°C, plates were filtered into microtiter plates (Shimadzu, Kyoto, Japan) using a positive pressure manifold (8 min at 5 psi, followed by 15 seconds at 15 psi; Biotage, Uppsala, Sweden) and analyzed by LC-MS/MS.

Pituitary tissue sample acquisition and preparation.

Pituitary samples were collected using standardized necropsy and then frozen at -80°C until used in the analysis [27]. All samples (approximately 80 mg per sample) were split in half, individually weighed, and placed into 12 mm $\times$ 75 mm glass tubes containing 1.5 ml ice-cold 1M acetic acid and 30 μl of OT-d10 internal standard. Tissues were homogenized on ice and the resulting homogenates were poured into individual 15 ml polystyrene tubes. The original glass tube was rinsed with 1 ml acetonitrile and this rinse was added to the appropriate 15 ml polystyrene tube containing the homogenate. After all homogenizations were complete, 3 ml acetonitrile were added to each tube and extraction proceeded as described for CSF samples.

Preparation of calibration curve and quality control samples.

OT purchased from Sigma was prepared in 50:50 acetonitrile:water and 1 ml aliquots of 100 $\mu\text{g}/\text{ml}$ were stored at -80°C . Calibration curves were prepared by thawing an aliquot and diluting further in 50:50 ACN to working concentrations for preparing the standard curve. We added 40 μl unlabeled OT in 50:50 acetonitrile:water (v/v) to each tube of 1 ml charcoal-stripped human serum. The final 11-point calibration curve ranged from 10 pg/ml to 80 ng/ml and included calibrators at 10 pg/ml, 25 pg/ml, 50 pg/ml, 100 pg/ml, 240 pg/ml, 720 pg/ml, 2 ng/ml, 6 ng/ml, 20 ng/ml, and 80 ng/ml. Calibrators were prepared immediately before sample preparation. Quality control (QC) samples were prepared by spiking unlabeled OT standard into normal human serum (Table 1) and stored at -80°C . These QCs were assayed in triplicate at the front of each assay and in duplicate after every 30 samples.

LC-MS/MS instrument parameters.

Microtiter plates were prepared as described above and loaded onto a SIL-30ACMP autosampler set at 4°C (Shimadzu). 40 μl of each standard, QC or sample were injected onto an ACE Excel 2 C18-PFP 50 mm $\times$ 2.1 mm column (Advanced Chromatography Technologies, LTD, Aberdeen, Scotland) at 50°C using reversed-phase chromatography. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The LC time gradient was created using two Nexera LC-30AD pumps (Shimadzu) running at 0.5 ml/min. Chromatography for CSF samples began at 10% B and ended at 16% B at 3.6 min. 1 min at 60% B was then required to wash the column, and the pumps re-equilibrated at 10% B for an additional 1.7 minutes (total run time approximately 6 min). A divert valve sent the LC effluent to waste outside of the 1.55–3.55 minute window during which OT eluted. Carryover on the column when analyzing pituitary samples was eliminated using a vigorous wash and re-equilibration program. After the 10%–16% B gradient ended at 3.6 min, the pumps proceeded to 95% B (3.8min) and held for 1 min. From 4.8–6.2 min, the pumps decreased to 5% B then increased to 95% B twice before returning to 10% B at 6.4 min to start 1.7 min of re-equilibration (total run time approximately 8 min). The divert valve settings for pituitary samples were also different, sending effluent to waste outside of the 1.25–3.55 min window during which oxytocin eluted, rather than the 1.55–3.55 min window used for CSF samples.

Heated electrospray injection in positive mode with scheduled multiple reaction monitoring (MRM) on a Shimadzu LCMS-8050 was used for detection of OT. The interface temperature was 270°C and heat block temperature was 170°C. Gas was supplied by a Peak Genius 1051 nitrogen and air generator (Peak Scientific, Inchinnan, UK). Nitrogen gas was used for nebulizing (1 L/min) and drying (8.2 L/min) while air was used for heating (7.8 L/min). A capillary B needle was used in the interface. Needle protrusion was set to 1.25 mm and probe distance was set to 1.0 mm; both were determined empirically. Argon (Airgas, Radnor, PA) was used for the collision-induced dissociation at 330 kPa. The MS/MS conditions for OT were optimized using the automated MRM optimization procedure in LabSolutions software (Shimadzu). The MRM transitions and other MS parameters used for the analysis can be found in Table 1S.

Data analysis.

The LC-MS/MS data were analyzed using LabSolutions version 5.72 (Shimadzu). Target reference ion ratios were set according to the reference ion ratio of the highest standard. Default ion allowance for peak identification was 30% relative to this target ratio. Linear regression with 1/C weighting was used for analysis of calibration curves.

Results and Discussion

We used an LC-MS/MS method described in our previous report to measure OT in the CSF and pituitary of macaques [24]. Instrument parameters used for the analysis can be found in Table 1S (supplementary materials). Assay metrics, including accuracy, precision, limits of quantitation and detection, extraction efficiency, and matrix effects can be found in Tables 1–2.

Figure 1A shows separation of OT and the internal standard OT-d10. Excellent linearity was observed for OT within the calibration range ($R > 0.99$). The limits of quantitation for OT can be found in Table 2 and were determined by the lowest and highest concentration calibrators that were successfully integrated with accuracy between 80% and 120% [28]. This resulted in a lower limit of quantitation (LLOQ) of 10 pg/ml and upper limit of quantitation (ULOQ) of 80 ng/ml. Accuracies and precisions were acceptable across three QCs ranging from 30–400 pg/ml (Table 1). In total, 4 transitions were monitored for OT and OT-d10; the 1007.40>723.40 transition was used for quantitation of OT. Analysis required approximately 3.5 min and an additional 2.5 min of equilibration for instrument stabilization between injections was needed for a total run time of approximately 6 min/injection.

In lieu of a limit of detection (LOD) we determined the method detection limit (MDL) using a previously published method [29]. The LOD and LOQ are traditionally reported as the concentration at which the signal:noise ratio for a target analyte is 1:3 (LOD) or 1:10 (LOQ). In modern MS/MS instrumentation many sources of noise have been eliminated, making signal:noise comparisons often meaningless to LOD and LOQ determinations [30]. The MDL is defined as the minimum concentration of an analyte that can be measured and reported with 99% confidence that the analyte concentration is greater than zero, using data collected from analysis of the target analyte near the LOQ to evaluate uncertainty in the MS/MS system [29]. For the current method we determined the MDL for OT by performing

replicate injections of the lowest concentration calibrator on different days and calculated the MDL using a formula described for this purpose [29]. The MDL for OT was 3 pg/ml (Table 2).

We selected chromatographic conditions that provided good peak shapes for OT and OT-d10 and optimal separation of the compounds from any interfering peaks resulting from the pituitary or CSF matrices. There were no significant interfering peaks within the analysis window for OT as observed through testing of blank matrices during the method development process. These requirements were met by using a C18-PFP column in conjunction with the described mobile phases paired with the LC gradient program described. Figure 1B shows a representative analysis of OT standard at the LLOQ of 10 pg/ml; Figure 1C depicts a serum blank showing no major interferences at the retention time of OT.

As shown in Figure 3A, the concentration of endogenous OT in the CSF samples of cynomolgus macaques ranged from 41 pg/ml to 66 pg/ml, similar to a previous report using LC-MS/MS analysis [31], enzyme immunoassay (for example, [32] where CSF levels of OT ranged between 36 and 134 pg/ml in adult monkey, but see [33]: 3 – 32 pg/ml) or RIA (for example, [34] with -CSF OT range between 20 and 80 pg/ml in adult rat; but see [33] 2.3 – 15.9 pg/ml in adult monkey). In the pituitary, the OT concentration ranged between 44 ng/mg to 151 ng/mg (Figure 3B). To our knowledge, this is the first report of OT content in the posterior pituitary of macaques. In humans, the only available study on pituitary OT content reported 10.2 ± 5.9 ng/gland measured in fetuses at 14–17 weeks' of gestation [35]. In rodents, the pituitary levels of OT were previously reported at the average range between 2.5 and 3.2 ng/ μ l using RIA [36]. Importantly, we observed positive correlation between pituitary and CSF concentrations of OT (Figure 3C). Many studies have evaluated peripheral (blood, salivary) OT concentrations as potential biomarkers of behavioral and cognitive functioning in humans [37–39]. In both human and monkey, quantifying OT content in peripheral samples is a common approach due to an easier acquisition process than invasive central (CSF or brain tissue) samples. The functional significance of the blood OT content as an indicator of central OT has been recently demonstrated in children [40]. The results obtained in this preliminary study indicate similar findings in nonhuman primates; however, considering the small sample size and given differences in pharmacokinetics and temporal dynamics in secretion of peripheral and central OT [41], plasma level of OT might be a rough indicator of the OT activity in the brain of monkeys. Additional studies in nonhuman primates are needed to confirm correlation between levels of OT in the CSF and blood.

Conclusion

We used a validated LC-MS/MS method to quantify endogenous levels of OT in pituitary samples of macaque monkeys. Using this method, we revealed that CSF and pituitary OT concentrations were positively correlated. These preliminary results allow for future analyses to determine whether LC-MS/MS measures of peripheral OT can be used as markers of OT levels in the brain of nonhuman primates. One of the main limitations of the study is that results were acquired in a small sample of seven monkeys and future studies with larger number of subjects are needed to replicate the observed preliminary results.

Given sex-dependent differences in OT functioning, it will also be important to document a relationship between levels of endogenous OT in the CSF and pituitary in female monkeys. Additionally, the reported results were acquired in adult monkeys only and future studies are needed to reveal whether the correlation between the central and peripheral OT levels is age-dependent in monkeys.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative chromatograms of OT analyzed by LC-MS/MS.

A. Analysis of OT (720 pg/ml) and internal standard OT-d10 (1 ng/ml) demonstrating relative retention times and MRM transitions used for quantification. B. OT at the lower limit of quantification (10 pg/ml). C. Extracted serum blank injection showing no significant interference peaks at the retention time for OT. CPS, counts per second; min, minutes; d, deuterium.

Figure 2. Representative MRM chromatograms of OT.

A. Analysis of OT (48 pg/ml) in CSF. B. Analysis of OT (0.05 µg/mg) in pituitary.

CPS, counts per second; min, minutes

Figure 3. Levels of endogenous OT in the CSF and pituitary of cynomolgus macaques measured with LC-MS/MS.

The average and individual OT levels measured in the CSF (A) and pituitary (B). The horizontal lines show average [OT] (n=7) and circles depict individual OT measures. C. Individual levels of OT measured in the pituitary (peripheral) positively correlated with the CSF levels of OT (Spearman correlation, $r=0.78$, $p<0.05$).

Table 1.

Accuracies and precisions (expressed as the coefficients of variation, CVs) for analysis of OT in normal human serum (n=2 assays).

Analyte	Spiked Value (pg/ml)	Mean Assayed Value (pg/ml)	Accuracy	Intra-assay CV	Inter-assay CV
OT QC 1	30	29	96.7%	1.4%	3.6%
OT QC 2	120	107	89.2%	1.2%	6.5%
OT QC 3	400	377	94.3%	4.9%	6.1%

QC, quality control. These data represent analysis of OT spiked into human serum (2 replicates of each QC in each of 2 assays)

Table 2.

LOQs, MDL, extraction efficiency, and matrix effect for analysis of OT.

Target	LLOQ (pg/ml)	ULOQ (ng/ml)	MDL (pg/ml)	Extraction Efficiency (%)	Matrix Effect (%)
OT	10	80	3	73	208

LOQ, limit of quantification; LLOQ, lower LOQ; ULOQ, upper LOQ; MDL, method detection limit