

# Sex and species differences in plasma oxytocin using an enzyme immunoassay

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**Abstract:** The neuropeptide hormone oxytocin (OT) is released peripherally and centrally and has been implicated in both physiology and behavior, especially sociosexual behaviors. Knowledge of OT levels in blood or other sources would be useful but these are rarely reported. Radioimmunoassay following extraction is the most commonly used method for measuring OT but is not ideal for use in small mammals in which blood volumes and concentrations of OT are low. Here we report a chemical and biological validation for a commercially available enzyme immunoassay for OT in unextracted plasma. In addition, comparisons of OT were made across species to allow comparison of the monogamous prairie vole (*Microtus ochrogaster* (Wagner, 1842)) to the polygynous Sprague Dawley rat. These species were chosen because OT plays a role in the formation of social bonds and we predicted that the highly social prairie vole would have higher plasma OT than the less social rat. Results of this comparison confirmed our hypothesis. Further, OT was significantly higher in females than in males in both species. Our results indicate that this enzyme immunoassay can be used to assay plasma OT in rodents and that the predicted correlations exist between plasma OT and gender as well as species-typical social behavior.

**Résumé :** L'hormone oxytocine (OT), un neuropeptide qui est libéré tant en périphérie que dans le centre de l'organisme, est impliquée dans la physiologie et le comportement, particulièrement les comportements sociosexuels. La connaissance des concentrations d'OT dans le sang et d'autres sources serait utile, mais elles sont rarement indiquées. Le dosage radioimmunologique après extraction est la méthode la plus communément utilisée pour la mesure d'OT, mais elle n'est pas idéale chez les petits mammifères chez qui les volumes sanguins sont réduits et les concentrations d'OT faibles. Nous fournissons ici une validation chimique et biologique d'un essai immunoenzymatique disponible dans le commerce pour mesurer l'OT dans du plasma sans extraction. De plus, nous avons fait des comparaisons interspécifiques d'OT chez le campagnol des prairies (*Microtus ochrogaster* (Wagner, 1842)), une espèce monogame, et le rat Sprague Dawley polygyne. Nous avons choisi ces espèces parce qu'OT joue un rôle dans la formation des liens sociaux et nous avons prédit que le campagnol des prairies qui est un animal très sociable aurait des concentrations plasmatiques d'OT plus élevées que le rat qui l'est moins. Les résultats de la comparaison confirment notre hypothèse. De plus, les concentrations d'OT sont significativement plus élevées chez les femelles que chez les mâles des deux espèces. Nos résultats démontrent que l'essai immunoenzymatique peut servir à doser l'OT plasmatique chez les rongeurs et que les corrélations prédites existent bien entre les concentrations plasmatiques d'OT et le sexe, de même qu'entre ces concentrations et les comportements sociaux particuliers à chaque espèce.

[Traduit par la Rédaction]

## Introduction

The purposes of this study were to examine the efficacy of a commercially available enzyme immunoassay (EIA) for measuring plasma oxytocin (OT) concentrations and to compare circulating OT in a polygynous rodent, the domestic rat (*Rattus norvegicus* (Berkenhout, 1769)), with that in a monogamous rodent, the prairie vole (*Microtus ochrogaster* (Wagner, 1842)). OT plays a critical role in mammalian reproduction in terms of both physiology and sociosexual behavior. In addition to affecting physiological aspects of reproduction such as milk letdown and sperm transport, OT in-

fluences a variety of behavioral responses, including memory and learning (Engelmann et al. 1996) and positive social behaviors such as social recognition (Ferguson et al. 2001), pair bond formation (Williams et al. 1994; Insel and Hulihan 1995; Cho et al. 1999), sexual behavior (Caldwell et al. 1986), and maternal behavior (Pedersen et al. 1982; Pedersen 1997; Russell et al. 2001).

Because it is generally assumed that OT does not readily penetrate the blood-brain barrier, OT is usually given as an intracerebroventricular (i.c.v.) injection and therefore most documented behavioral effects of OT are associated with central administration. However, peripheral administration of

Received 4 March 2004. Accepted 12 July 2004. Published on the NRC Research Press Web site at <http://cjz.nrc.ca> on 5 October 2004.

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OT also has been shown to alter behavior. For example, treatment with peripheral OT altered nociceptive threshold (Uvnäs-Moberg et al. 1998) and motivational state (Liberzon et al. 1997) in rats. Peripheral OT also decreased infanticide and increased maternal behavior in female house mice (McCarthy 1990), increased mating in sexually naive female prairie voles (Cushing and Carter 1999), and accelerated pair bond formation in female prairie voles (Cushing et al. 2001). Thus, circulating OT may influence behavior through either direct or indirect actions on the central nervous system.

There is now ample evidence that OT plays a role in social behavior in species across diverse mammalian taxa including primates, ungulates, and rodents (Kendrick et al. 1987; Winslow and Insel 1991; Carter et al. 1995). However, the neural and behavioral effects of OT appear to vary, depending upon social organization. For example, there are significant differences in the distribution of OT receptors between monogamous and polygynous rodents and it has been argued that these patterns underlie differences in social behavior (Insel and Shapiro 1992; Insel and Young 2001). There are also major differences in the regulation of OT between species. In rats, many of the effects of OT are steroid-dependent (Caldwell et al. 1986; Jirikowski et al. 1988; Johnson et al. 1989; Johnson 1992). However, in the monogamous prairie vole, OT increases positive social behavior without estrogen priming (Witt et al. 1990, 1991; Cho et al. 1999) and OT may even affect the response to estrogen (Cushing and Carter 1999).

In addition to the apparent interspecific differences in the role of OT in sexual and social behavior, a gender difference in the response to OT also might exist. Females are generally more social than males, particularly in polygynous species where males are solitary except when mating. Given the links between OT and social behavior, it seems reasonable to expect differences in behavior to correlate with gender differences in OT. Experimental data also are suggestive of such gender differences. Neonatal manipulations of OT increase intrasexual aggression associated with mate guarding in females as adults (Bales and Carter 2003). Also, OT gene expression and receptor binding increase in females after parental experience (Wang et al. 2000). In neither case were effects observed in males.

Therefore, the purpose of this study was twofold. Our first objective was to validate a commercially available EIA for OT. If valid, this assay has the advantage of requiring sample volumes much smaller than those necessary for a radioimmunoassay (RIA). Our second objective was to compare plasma OT between species differing in degree and extent of social behavior. Given the apparent interspecific differences in the actions of OT and its role in positive social behaviors (Pedersen et al. 1982; Williams et al. 1994; Insel and Hulihan 1995; Ferguson et al. 2001), we hypothesized that plasma OT would be higher in a monogamous species than in a polygynous species. Laboratory rats and prairie voles were chosen to test this hypothesis because these two species are widely used in studies of the functional effects of OT and have divergent social structures: prairie voles are socially monogamous and show long-term pair bonds and biparental care (Thomas and Birney 1979; Getz et al. 1981; Dewsbury 1987), while rats are polygynous and show little affiliation outside of copulation (Blanchard et al. 2001).

## Materials and methods

### Experimental animals

Subjects were adult prairie voles and Sprague Dawley® rats of both sexes. Prairie voles were fourth- or fifth-generation lab-reared animals that originated from voles trapped near Urbana, Illinois. Voles were weaned at 21 days of age and then housed in same-sex sibling pairs. Animals were maintained under a 14 h light : 10 h dark (14 h L : 10 h D) cycle and provided with high-fiber rabbit chow and water ad libitum. At the time of blood collection, voles ranged in age from 2 months to 8.5 months. This represents a reasonable age range for voles, as they are sexually mature at 2 months and can live and reproduce under laboratory conditions for several years.

Sprague Dawley rats were obtained directly from Taconic Farms (Germantown, New York) or were F<sub>1</sub> generation of this stock born in our colony. Lab-reared rats were weaned at 21 days of age. All rats were housed in same-sex groups of two or three individuals. They were maintained under a 14 h L : 10 h D cycle and provided with rat chow and water ad libitum. Rats were 2–4 months of age at the time of blood collection. All husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park, and animals were cared for in accordance with the guidelines put forth by the Canadian Council on Animal Care.

### Treatments and blood collection

All blood collection was performed 3–5 h after lights were turned on. Animals were anesthetized using a combination of ketamine and xylazine (67.7 mg/kg ketamine and 13.33 mg/kg xylazine, intraperitoneal injection); ketamine does not elevate plasma OT (Zierer 1991) and xylazine also was not expected to affect plasma OT. Following anesthetization, blood was collected from the periorbital sinus using heparinized microcapillary tubes, and all collections were completed within 9 min of injection with anesthetic. Samples were placed on ice and then centrifuged at 4 °C, 3500 r/min for 12 min. Plasma was aliquoted into microcentrifuge tubes and frozen immediately on dry ice. Plasma was stored at –80 °C and thawed only at the time of assay.

### Experiment 1: validation of OT EIA

Sexually naive female prairie voles were used for validation of the OT EIA. All females received a single subcutaneous injection of OT (5 µg OT/50 µL isotonic saline) and blood was collected prior to injection ( $n = 11$ ) or at one of three time points after injection: 5 ( $n = 6$ ), 15 ( $n = 6$ ), or 60 ( $n = 5$ ) min.

### Experiment 2: species and sex differences

Prairie voles and rats of both sexes were used for comparisons of plasma OT. Blood samples for determination of baseline OT concentrations were collected from reproductively naive male and female voles ( $n = 12$  and 11, respectively) and male and female rats ( $n = 11$  for each sex). To avoid excessive handling, stage of estrus was not determined. RIA data indicate that plasma OT levels in cycling female rats vary by a factor of approximately two (Windle and Forsling 1993). Female prairie voles do not have a spon-

taneous estrous cycle; ovarian hormone levels remain low and constant unless the female is exposed to a male (Carter et al. 1980, 1989).

## EIA

Plasma OT was measured using the EIA kit developed by Assay Designs, Inc. (Ann Arbor, Michigan; Catalog No. 901-024). Assay Designs reports cross-reactivity with similar neuropeptides found in mammalian sera at less than 0.001% and a minimum detection limit of 4.68 pg OT/mL. The antibody recognizes the oxidized, active form of OT. Manufacturer's instructions were followed without modification; plasma was not extracted. The assay was validated for parallelism by assay of a serial dilution of pooled vole plasma. Accuracy of the assay was assessed by spiking samples of pooled vole plasma with varying amounts of the standard (known concentration) provided with the kit. Precision was determined from the variability surrounding multiple measurements of high and low controls (interassay CV) and variability in multiple measurements of unknowns (intra-assay CV).

Based on the parallelism data collected from initial measurements of plasma pools, plasma samples for both voles and rats were diluted 1:4 prior to assay (requiring 65  $\mu$ L of plasma to assay samples in duplicate). Vole plasma collected after injection with OT was initially diluted 1:4, but in all cases measured OT concentrations far exceeded the highest standard used to generate the standard curve. Fortunately a second aliquot was available for each vole, allowing reassay at a higher dilution factor. A 1:20 dilution resulted in values within the range of the standards, but samples were assayed singly owing to small remaining sample size. Thus, intra-assay CVs are unavailable for females treated with OT.

## Statistics

Linear regression was performed to assess parallelism of the dilution series to the standard curve and to assess the accuracy of the assay (agreement between expected and observed values for recovery). To determine whether plasma OT increased following injection with OT, a single-factor analysis of variance (ANOVA) was done and post hoc comparisons were made with a Fisher's PLSD. For interspecific comparisons of baseline OT, a two-factor ANOVA with species and sex as factors was performed. The criterion for statistical significance was  $P < 0.05$ .

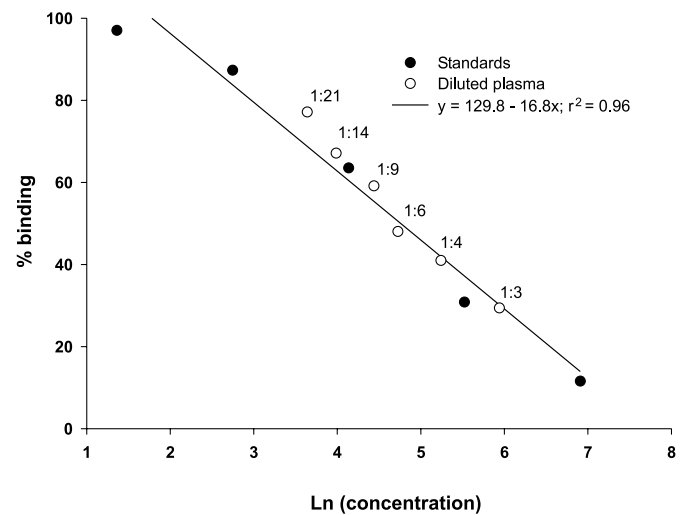
## Results

### Validation of EIA

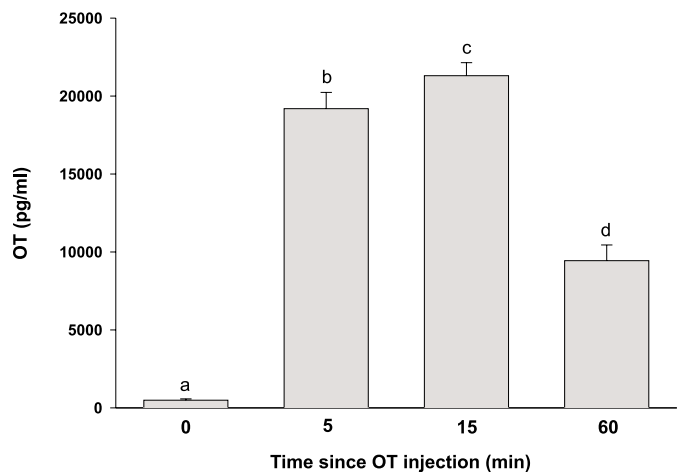
A dilution series of pooled vole plasma resulted in a displacement curve parallel to the standard curve ( $y = 129.88 - 16.79x$ ;  $r^2 = 0.96$ ; Fig. 1). Tests of accuracy resulted in a high correlation between expected and observed values ( $y = -2.20 + 0.79x$ ;  $r^2 = 1.00$ ) and average recovery was 71%. Intra-assay CV averaged 2.68%, interassay CV averaged 14.45%, and standard curves were similar across assays.

Injection with OT resulted in a significant increase in plasma OT ( $F_{[3,24]} = 242.69$ ,  $P < 0.0001$ ; Fig. 2), with all pairwise comparisons significant. Values were maximal 15 min after injection. Within 60 min, plasma OT was significantly lower ( $P < 0.0001$ ) but still elevated over baseline

levels ( $P < 0.0001$ ). Rough estimates of exogenous OT remaining in the plasma at each time point can be obtained by comparing the means for each group to that of the control group (time = 0) in Fig. 2. Clearly, the exogenous OT had not been entirely broken down at  $t = 60$  min, as the mean plasma OT at 60 min was 9440 pg/mL, while the mean plasma OT at  $t = 0$  min (no OT injection, all plasma OT is endogenous) was 366 pg/mL.



levels ( $P < 0.0001$ ). Fisher's PLSD: all pairwise comparisons significant at  $P < 0.05$ ). Values are means  $\pm$  SE. Groups not sharing the same letter are significantly different.

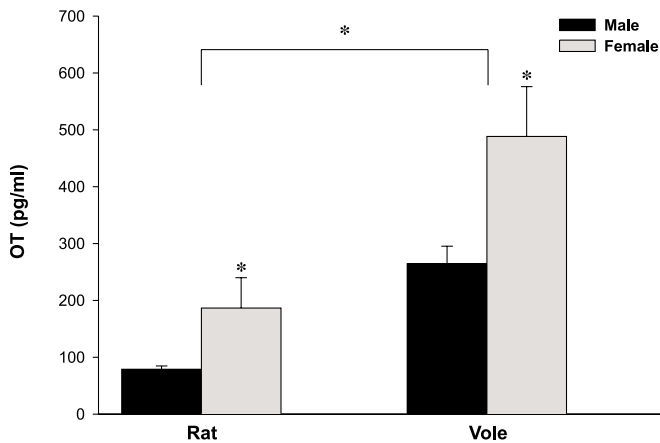


levels ( $P < 0.0001$ ). Rough estimates of exogenous OT remaining in the plasma at each time point can be obtained by comparing the means for each group to that of the control group (time = 0) in Fig. 2. Clearly, the exogenous OT had not been entirely broken down at  $t = 60$  min, as the mean plasma OT at 60 min was 9440 pg/mL, while the mean plasma OT at  $t = 0$  min (no OT injection, all plasma OT is endogenous) was 366 pg/mL.

### Species and sex differences

There were significant differences in plasma OT by species and by sex, with voles having significantly higher OT than rats ( $F_{[1,41]} = 21.24$ ;  $P < 0.0001$ ) and females having higher OT than males ( $F_{[1,41]} = 9.82$ ;  $P < 0.001$ ; Fig. 3). The average OT concentration was  $488.3 \pm 87.6$  pg/mL in female

**Fig. 3.** Baseline plasma oxytocin concentrations in Sprague Dawley rats (*Rattus norvegicus*) and prairie voles of both sexes ( $P_{\text{species}} < 0.0001$ ,  $P_{\text{sex}} = 0.01$ ; asterisks indicate significant differences). Values are means  $\pm$  SE.



voles ( $n = 11$ ),  $264.4 \pm 31.0$  pg/mL in male voles ( $n = 12$ ),  $186.5 \pm 53.4$  pg/mL in female rats ( $n = 11$ ), and  $78.9 \pm 5.8$  pg/mL in male rats ( $n = 11$ ).

## Discussion

Measured concentrations for the serial dilution of pooled plasma were parallel to the standard curve, indicating that extraction procedures are unnecessary and that a 1:4 dilution eliminates significant matrix effects. In situations where OT is elevated, a greater dilution of plasma may be necessary in order to have the assayed concentrations within the working portion of the standard curve. The OT assay was also valid in terms of quantitative recovery, as there was good agreement between expected and observed OT concentrations for spiked samples. These results and the reported minimum detection limit compare favorably with published EIA and RIA results for OT (e.g., Landgraf 1981; Chiodera et al. 1984; Vecsernyés et al. 1994; Prakash et al. 1998). Our results also indicate that the assay is biologically valid. As expected, exogenous administration of OT resulted in significant increases in plasma levels. Plasma OT was elevated within 5 min of injection, reached maximal concentration at 15 min, and began to decline within 60 min, presumably because of breakdown of the OT.

OT concentrations observed in this study were substantially higher than those reported using RIA and extracted plasma. Studies using RIA have reported values ranging from 2 to 41.5 pg/mL in rats (Lang et al. 1983; Windle and Forsling 1993; Uvnäs-Moberg et al. 1999). While it is difficult to quantitatively compare measurements among assays, especially among different assay systems, we assessed relative differences between species by comparison of split samples measured by both EIA and RIA (run courtesy of J. Amico, University of Pittsburgh). The RIA had a detection range of 1–20 pg/mL. Results from this RIA showed that OT in rat plasma from our laboratory fell within the standard curve. However, all prairie vole plasma assayed by RIA far exceeded the standard curve. Thus, results from both assays

supported the hypothesis that prairie voles have significantly higher baseline OT levels.

The differences between the concentrations reported in this study and previous studies may be due to one of several factors. First, comparatively high OT values could result from differences between the EIA and RIA procedures or the antibodies used. RIA requires extraction, and even correction for recovery may not completely compensate for loss or breakdown of OT. Results from other studies also suggest that there are inherent differences between EIAs and RIAs, and systematic comparisons of the two assay systems have shown that EIAs tend to yield higher values (Buntin et al. 1999; Shirtcliff et al. 2001). Measured by EIA, plasma OT in humans was recently reported to range from 200 pg/mL to more than 600 pg/mL (Zak et al. 2003), whereas previous studies using RIA reported average values of 3.6 pg/mL (Amico et al. 1981). Additionally, a recent study of OT in rhesus monkeys reported much higher values using this EIA than those reported using an RIA. Winslow et al. (2003) found average values for plasma OT to be approximately 225–280 pg/mL, whereas previous studies reported plasma OT in the same species to range from 8 to 33.4 pg/mL (Amico et al. 1990). RIA may also have more restrictive detection limits for OT; for example, Vecsernyés et al. (1994) reported basal OT for male rats to be 9.6 pg/mL, but the minimum detection limit for that assay was near 10 pg/mL. Thus, the RIA was used for measurements at the lower limits of detection, where OT concentrations could be under- or over-estimated. Finally, OT could have increased in response to the stress associated with handling. In male rats, OT increased within 5 min of physical restraint (Lang et al. 1983); however, increases in plasma OT do not occur in response to all types of stressors (Lang et al. 1983; Engelmann et al. 1999) and injectable anesthetic does not appear to affect OT levels (Zierer 1991). Moreover, all animals were anesthetized, so if there was any effect, it would have been similar across all groups.

In both species, there was a significant difference in plasma OT between sexes, with females having higher circulating concentrations of OT than males. A review of the literature regarding sex differences in plasma OT is, at best, ambiguous. Vecsernyés et al. (1994) reported no sex differences in rats, while Windle and Forsling (1993) reported significantly higher levels in male rats than in female rats. In contrast, our results provide evidence that females can have significantly higher plasma OT concentrations than males. Higher levels of OT in females are consistent with the notions that females are more spontaneously social than males and that this difference might reflect the effects of OT (Witt et al. 1992; Cho et al. 1999). Females tend to be more social than males, particularly in polygynous species such as rats, where males typically come into contact with conspecifics only to mate, whereas females not only spend time with their litter but are more tolerant of conspecifics. The sex differences in OT levels that we observed in the prairie vole are also consistent with other data on the role of neuropeptides in social behavior. In the prairie vole, there is evidence that endogenous OT plays a major role in the development of pair bonds in females, while arginine vasopressin (AVP) may be especially important in the social behavior of males (Insel et al. 1998; Cushing et al. 2001). Centrally adminis-

tered AVP resulted in a significant increase in social behavior in prairie voles (Cho et al. 1999; Young et al. 1999); in both rats and voles, males produce more AVP than females (De Vries et al. 1994; De Vries and Villalba 1997). A sex-related difference in mechanisms underlying social behavior is consistent with the results from the present study demonstrating lower OT in males of both species relative to conspecific females. Both the sex differences in plasma OT observed here and the modulation of male social behavior by AVP support the hypothesis that the mechanisms underlying social behavior may be sexually dimorphic.

Our findings of interspecific differences lend support to the hypothesis that OT may have a role in the establishment of affiliative social behaviors. Independently of sex, the highly social prairie vole had significantly higher plasma concentrations of OT than the typically less contact-prone rat. These results fit with both experimental work involving treatment with OT and observations of social behavior in both species. In both rats (Witt et al. 1992) and voles (Witt et al. 1991; Cho et al. 1999), treatment with exogenous OT is followed by increased social contact. Furthermore, pair bonds are typical of prairie voles (Thomas and Birney 1979; Getz et al. 1981; Carter et al. 1995), but not rats. It is not uncommon for free-living prairie voles to live communally, in a situation where social interactions are frequent, positive, and occur outside the immediate context of copulation (Getz et al. 1981). In contrast, interactions within mixed-sex rat colonies are often agonistic, particularly between members of the same sex, and interactions between males and females appear to reflect only sexual advances on the part of the male (Blanchard et al. 2001).

In addition to the degree of social behavior, there are other lines of evidence that predict higher plasma OT in prairie voles than in rats. Male and female rats typically come into contact when the female is sexually receptive, and not before. In contrast, female prairie voles require prolonged exposure to stimuli from males to become sexually receptive. Thus, social contact in rats follows sexual receptivity, while social contact in prairie voles precedes sexual receptivity. If OT is modulating these behavioral responses, then prairie voles should have higher levels of OT in order to display a greater willingness to engage in social interaction to initiate reproduction. A comparison of possible mechanisms for the regulation of social behavior in rats and voles supports this hypothesis. In rats, the ability of OT to stimulate sociosexual behavior is dependent upon estrogen priming, as increasing levels of estrogen up-regulate OT receptors (Jirikowski et al. 1988; Johnson et al. 1989; Johnson 1992). In contrast, in prairie voles not only does estrogen not up-regulate OT receptors (Witt et al. 1991), but OT actually increases sensitivity to estrogen (Cushing and Carter 1999). Thus, high circulating estrogen levels are not required for social behavior in prairie voles, and social contact in this species may occur while females are still reproductively immature (Carter et al. 1989). However, in rats, sexual receptivity often precedes social contact with a male, suggesting that one purpose of elevated estradiol on the night of estrus in the female rat is to up-regulate OT and (or) OT receptors (Caldwell et al. 1986; Johnson et al. 1989; Johnson 1992), thus coordinating social behavior of the female with her endocrine status.

In conclusion, our results indicate that the EIA is valid and can be used to reliably measure plasma OT concentrations. This assay has a number of advantages over available RIA kits: the shelf life of the reagents is longer than for an RIA using an  $^{125}\text{I}$  label; radioactive materials are unnecessary; and the EIA has a wider detection range. Procedures for other OT EIAs have been published (Prakash et al. 1998; Péqueux et al. 2001; Kawasaki et al. 2002), but a distinct advantage of this assay is that it is commercially available, making it more accessible and requiring less investment of time. Because vole and rat plasma assayed by EIA do not need to be extracted, relatively little plasma is required for this assay in comparison with other published assays for OT (Chiodera et al. 1984; Vecsernyés et al. 1994; Prakash et al. 1998). For example, in the present study we were able to get reliable data with as little as 65  $\mu\text{L}$  of plasma. This is particularly important when working with small mammals and can allow survival blood sampling. Caution is warranted, however, in applying this assay to other species; a validation should be done beforehand, as many species and assay systems do require extraction. Finally, higher plasma OT in prairie voles versus rats further supports the hypothesis that OT plays a role in social behavior (Carter 1998). Although the data suggest a correlation between basal plasma OT and species-typical social behavior, it is simply a correlation. To make more definitive conclusions, studies relating social behavior and plasma OT must be conducted in more detail and using a wider range of species.

## Acknowledgements

We thank Janet Amico for RIA comparisons and Pamela Epperson, Yukiyo Yamamoto, and Karyn Levine for their support and assistance. We also thank Dr. Nancy Schmidt and Karen Collins at Assay Designs, Inc. for their assistance. Funding was provided by National Institutes of Health: HD 38490 (C.S.C., B.S.C., M.A.O.), MH 01992 (B.S.C.), and HD 41293 (K.M.K.).

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