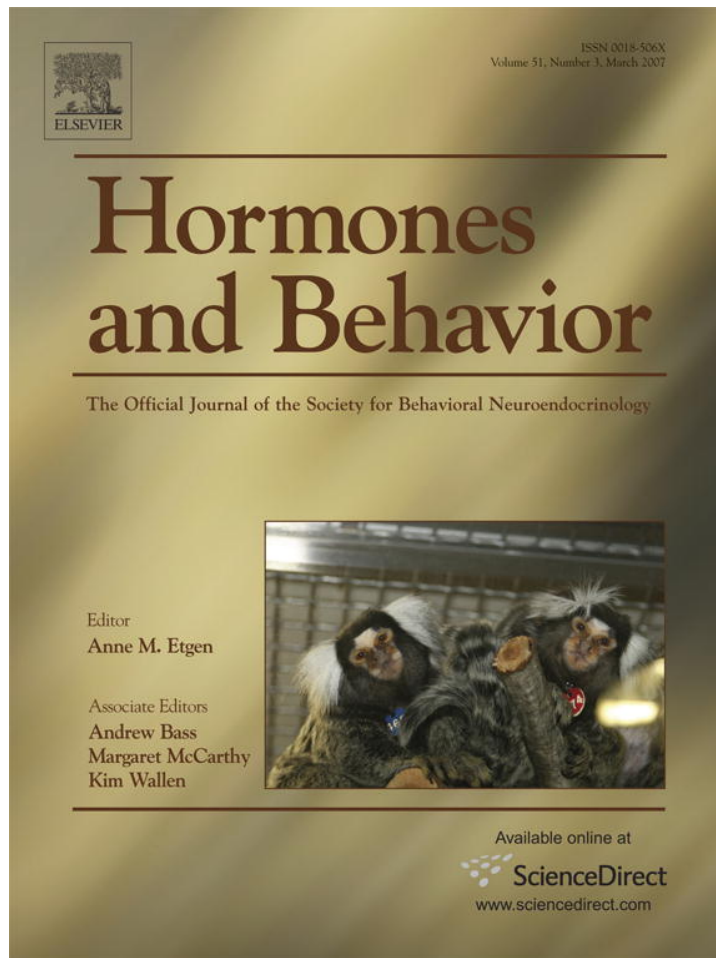


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Non-invasive measurement of small peptides in the common marmoset (*Callithrix jacchus*): A radiolabeled clearance study and endogenous excretion under varying social conditions

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Abstract

A non-invasive assay for measurement of oxytocin (OT) and vasopressin (AVP) in primates would enable researchers to study the relationship between the endocrine system and behavior without disturbing potentially endangered animals in their natural habitats. In order to test whether or not OT specifically would be measurable in the urine of a primate, 10 μ Ci of tritium-labeled OT were injected into the peripheral blood supply of four adult male common marmosets (*Callithrix jacchus*), with continuous urinary collection over 48 h. When urine was processed by HPLC separation and beta counting for radioactive clearance, the label was present in all samples in the fraction where OT elutes. Large amounts of OT were also seen in a fraction other than that containing the OT standard, indicating that OT is measurable but that it also undergoes substantial metabolic breakdown. In a second experiment, we isolated six common marmosets for 48 h and then exposed them to social contact to evaluate the effect of changing social stimuli on endogenous urinary measurement of both OT and AVP. Both were measured after HPLC separation to isolate the intact molecule and also to control for cross-reactivity with metabolites in subsequent RIA. Cortisol was also measured to objectively evaluate the stress response. A priori assumptions were that urinary OT and AVP would be lower during a period of isolation and higher during periods of social contact. These assumptions were met, leading us to conclude that peripheral OT and AVP are measurable via urinary assay and that such an assay is a valid means of evaluating social condition in this species.

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Introduction

Two hormones of importance to the study of social behavior are oxytocin (OT) and arginine vasopressin (AVP). OT is best known for its physiological role in the smooth muscle contractions associated with parturition and lactation in mammals, whereas AVP's physiological role is classically understood to concern osmoregulation. These are largely due to changes in peripheral levels of these hormones, which are released via the neurohypophysis. During the last 20 years, however, a number of additional roles have been evaluated for centrally released OT and AVP, which are produced in parvocellular and supraoptic

neurons of the hypothalamus and are thought to affect only the central nervous system, from which the peripheral system is often considered functionally distinct (Gimpl and Fahrenholz, 2001). These peptides are of considerable interest to those who study nonhuman primate behavior in both the field and in captivity, due to their roles in the regulation of complex social behaviors.

Central OT, for example, is important in establishing the bond between mother and infant, partly due to regulation of a decrease in blood pressure and cortisol levels upon commencement of suckling in mammals (Insel, 1992; Nelson and Panksepp, 1998) and during breastfeeding in human women, with accompanying positive emotion (Uvnäs-Moberg, 1998). The social role of central OT, however, goes beyond maternal behaviors. It is also essential for normal interactions between adult conspecifics, playing a role in formation of the pair bond (Bales and Carter,

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2003; Young and Wang, 2004) and in recognition of familiar individuals (Lim et al., 2005; Winslow and Insel, 2004; van Wimersma Greidanus and Maigret, 1996). OT also functions to regulate the stress response (Kalin et al., 1985; Heinrichs et al., 2003; Amico et al., 2004a), sexual behaviors (Arletti et al., 1992; Krüger et al., 2003; Argiolas and Melis, 2004), aggressive behaviors (Winslow and Insel, 1991a; Harmon et al., 2002) and grooming, particularly allogrooming (Drago et al., 1986; Wikstrom et al., 2003; Amico et al., 2004b). It is now understood that high levels of central OT are often associated with positive social stimuli, and low levels with negative stimuli in some mammals, apparently through OT's mediation of the activity of the hypothalamic–pituitary–adrenal (HPA) axis in a species- and sex-specific manner (Carter, 1998).

Like central OT, central AVP also has independent but profound effects on social behavior in many mammalian species in that it regulates anxiety (Landgraf, 2005), vigilance (Winslow and Insel, 1991b), aggression (Veenema et al., 2006), formation of monogamous pair bonds (Lim et al., 2004), and male parenting in pair-bonded species (Bartz and Hollander, 2006), all of which differ with AVP receptor location in the brain (Hammock and Young, 2005). The related avian peptide vasotocin has also been shown to play a role in social behavior and group size in several monogamous species of birds (Goodson et al., 2006) as well as aggressive behaviors within different populations of the same species (Maney et al., 2005).

Before many of our questions concerning the underlying causes of social behavior and the variation observed therein can be answered, however, an assay method must be in place to address them adequately. While cerebrospinal fluid has been assayed for OT successfully (Winslow et al., 2003), non-invasive methods have produced conflicting results over the years (Stewart, 1953; Frandsen and Jensen, 1971; Boyd et al., 1972; Forsling et al., 1973; Zebidi et al., 1978; Robinson and Walker, 1979; Ervin et al., 1985; Khan-Dawood, 1985; Amico et al., 1987; White-Traut et al., 1998). The result is that in spite of decades of investigation and some success, there is currently no widely used, non-invasive method to measure OT or AVP. This limitation has slowed the progress of research as invasive procedures are costlier, more time-consuming, and largely inapplicable to animals under behavioral conditions in the field or in captivity. Furthermore, they can cause considerable stress in the subject, which may itself cause changes in levels of OT and AVP (Carter and Altemus, 1997). Finally, invasive methods may not be well suited to studies involving human volunteers.

One of the primary reasons for controversy within the field of non-invasive peptide measurement is due to questions concerning the relevance of peripheral OT to behavior. It is clear that peripheral OT and AVP levels do not always covary with one another in the same animal. For example, changes in central OT, such as those observed on a circadian schedule in humans and monkeys, have no observable effects on peripheral OT concentrations (Amico et al., 1983). Furthermore, while small quantities of small peptides have been shown to cross the blood–brain barrier (BBB), either intact (Mens et al., 1983) or via their metabolites (Burbach et al., 1983), it has not yet been shown if or how this affects behavior.

While permeability of large quantities of intact peripheral OT and AVP across the blood–brain barrier (BBB) is physiologically unlikely, it is unnecessary to posit that this takes place in order to show that peripheral levels of these peptides can have behavioral relevance. The PVN and SON of the hypothalamus are known to project to the neurohypophysis for OT and AVP release into the bloodstream; it is also known, however, that the PVN is a major source of AVP and OT to numerous other regions within the brain itself (Kozorovitskiy et al., 2006), and that the SON may also release peptides centrally (Landgraf and Neumann, 2004); stimulus to either nucleus, therefore, may cause release of peptides into both the CNS and the periphery (Wotjak et al., 1998). Several empirical studies underscore the point that peripheral measures of these peptides may reflect central functioning. Urinary OT concentrations – themselves a proxy for peripheral OT levels via filtration of blood products in the kidney – were shown to be significantly higher in human children following tactile contact with a biological parent, but not with a student volunteer, while tactile contact with neither an adoptive parent nor a volunteer raised these concentrations significantly in children reared in emotionally deprived environments (Wismer Fries et al., 2005). Urinary OT and AVP were also found to differ between OT gene deletion mice, mice infused with OT, and mice whose OT release was stimulated by salt consumption (Polito et al., 2006). Furthermore, peripherally administered OT and AVP apparently affect partner preference in female and male voles respectively, when administered in a pulsatile fashion, indicating a possible central effect (Cushing and Carter, 2000; Cushing et al., 2001). Peripheral OT was also measured in human volunteers during self-stimulation, with levels increasing with psychophysiological arousal (Carmichael et al., 1987; Carmichael et al., 1994). Lastly, in autistic children, levels of plasma OT were found to be significantly lower than those of unaffected children and that these levels did not increase with age as they did in the control group (Modahl et al., 1998).

All of these studies suggest that peripheral OT and AVP levels may be linked to social condition, making investigation via non-invasive means potentially illuminating. One goal of this study was to incorporate the most precise technologies currently available to establish whether or not it is possible to isolate OT specifically in the urine of a nonhuman primate, to confirm its identity through a radiolabeled clearance study, and to identify possible metabolites. Clearance of exogenously administered OT, however, may not be indicative of how endogenous peptides are excreted under various social conditions. Therefore, the present study also examines whether or not endogenous OT and AVP can be reliably measured in the urine of a social primate, and whether or not these hormones differ when animals are exposed to different social conditions. A priori, we predicted that urinary levels of OT/AVP would be higher during social contact than during isolation. To evaluate the relationship between these peptides and the stress response, cortisol assays were also included in the analysis. Together, these experiments would reveal whether or not it is possible to measure these peptides in urine and whether or not such measures could be considered socially meaningful.

Materials and methods

Experiment I

Animals and housing

Four adult male common marmosets (*Callithrix jacchus*) from the Wisconsin National Primate Research Center (Madison, WI) were selected for this study, as this species is pair-bonded with strong male investment in offspring and hence are suitable candidates for measurement of both OT and AVP. Mean weight was 0.339 ± 0.0046 kg and mean age was 1137 ± 983.3 days. Dietary and husbandry conditions for this colony have been described elsewhere (Saltzman et al., 1997). Lighting consisted of a 12:12-h light/dark cycle and humidity was kept at approximately 50%. All housing conditions and social manipulations were preapproved by the Animal Care and Used Committee at the University of Wisconsin.

Procedures

A total of five doses of tritium-labeled OT (Cys-*Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂, MW=1007.23) suspended in ethanol (PerkinElmer, Boston, MA) were dried and resuspended in sterile saline for injection. Each 100- μ l injection contained approximately 7,400,000 counts of radioactivity. One of these (hereafter called the *reference sample*) was processed directly for HPLC separation and fractionated at 1-min intervals collected via a fractionator for the total 10-min run. The remaining four doses were injected into each subject via the femoral vein. Subjects were then moved immediately into metabolism cages from which all excreta could be collected individually. Metabolism cages were equipped with a wire mesh for fecal collection and a narrow funnel base for urine collection. The funnel was fitted with a clean test tube surrounded by dry ice, resulting in snap freezing of each sample immediately after void. Time of void was recorded in all cases, with each void being collected separately. The sides of the funnel were swabbed with ethanol following each void to ensure independence of sampling. Each fecal bolus was also collected individually and frozen on dry ice until assay. Test tubes were then capped and stored at -80 °C until assay. Animals remained in the metabolism cages for approximately 48 h, after which urinary radioactivity was shown to be close to baseline. The subjects were fed according to their usual schedule during this time, with water provided *ad libitum*.

Experiment II

Subjects

Subjects for this portion included six common male marmosets (*C. jacchus*), also housed at the Wisconsin National Primate Research Center as above. Mean weight [mean \pm SD] for subjects was 0.37 ± 0.052 kg and age [mean \pm SD] was 1194.2 ± 512.6 days. Each male was housed with a nonpregnant female (time mated [mean \pm SD]= 265 ± 147 days) and was provided food and water as in Experiment I. One male was eliminated from statistical consideration due to lack of consistent urination, reducing the number of subjects to five.

Procedures

Subjects were removed from their standard housing area during the first morning of the study and placed into the metabolism cage as detailed in Experiment I. Males were then individually housed for 48 h outside of visual or tactile contact with any conspecifics, as solitary housing is considered stressful for this species (Cross et al., 2004). It was not possible to completely eliminate the possibility of auditory or olfactory contact with other animals due to relative proximity of the housing colony. Collection took place at 30 min, 60 min, 240 min, 480 min, and 2880 min during the isolation period, with all voids taking place within the preset time periods pooled. Subjects were then returned to the housing area but remained in the metabolism cage. The males then were exposed to social contact with the mate for 6.5 h. This period of contact was broken down into subsections. In the first, visual (but not tactile) contact was possible. Samples were collected at 0, 30, 120 and 180 min during the visual condition, with each void taking place during the circumscribed time period being pooled. The cage of the male and his mate were then pushed against one another to permit tactile contact. Samples were collected at 0, 30, 120 and 180 min. The male was then released back into the cage with his mate. The male remained in contact with the female in his home cage for 30 min, after which he

was transferred back to the metabolism cage for a final sample. The male was then released permanently back into his home cage.

Urinary OT and AVP assays

Urine samples for Experiment I were processed within the first 2 weeks following collection and urine samples for Experiment II were processed after freezing them at -80 °C for several months. A portion of the urine (500 μ l) was acidified by the addition of 0.05 M HCl (1:1) for peptide measurement and a portion was used directly for creatinine measurement. 1 ml of acidified urine was purified by using solid-phase extraction (Oasis, HOB, 1 cm³, Waters or Strata, 30 mg/ml, Phenomenex). The method consisted of preparing the column with 2 ml of methanol and 2 ml of distilled water, applying the 1-ml sample, washing with 2 ml of 1.5% acetic acid, and eluting with 2 ml of methanol. Samples were dried and resuspended in 20 μ l of acetonitrile/water/0.13% trifluoroacetic acid (TFA) (1:1). Samples were injected onto the column by an automated sample injector, i.e. the microliter pickup (508, Beckman-Coulter Instruments). Marmoset samples and standards for OT and AVP were separated by HPLC using Beckman dual HPLC pumps (model no. 126,) connected to a diode array analyzer for UV detection at all wavelengths (model no. 168). All instruments were connected to a computer, and the samples were run and data analyzed by detection software (Beckman, Nouveau, System Gold). The technique used a 3 μ , C18-A 50 μ , 4.6 mm column for separating the peptides (MetaChem, Phenomenex, CA), with mobile phase A, 0.15% TFA in water, and phase B: 0.13% TFA in 95% acetonitrile/5% water. The flow rate was 3.0 ml/min, and the gradient was 15% B at 0, at 3.5–7.0 min, 15–25% B. Retention times for synthetic AVP and OT were 2.7 and 5.6 min, respectively (Sigma Chemical Co.). AVP and OT were expressed per mg of creatinine to control for fluid variability. Some samples were not available due to lack of urination during the predetermined time period or because of small sample volumes. For those samples insufficient in volume for OT, AVP, cortisol and creatinine assays, creatinine was always run plus maximum number of other tests possible. All samples of sufficient volume were assayed for all four assays. After separation for Experiment I, each 1-min fraction was dried, resuspended in scintillation cocktail and counted for 10 min with a Beta counter for measurement of radioactivity.

Fecal samples were processed for Experiment I only. They were prepared by first weighing each pellet and then adding 5 ml acidified ethanol to each. Pellets were broken up to release peptides, thoroughly vortexed for 3 min, and then centrifuged. The eluant was decanted into a clean tube and was dried down completely, then reconstituted with 1 ml of a 20:80 solution of acidified ethanol and pure water for SPE. SPE tubes were pretreated with 1 ml methanol and then 1 ml pure water. The sample was rinsed with 1 ml 10% ACN with TFA before being eluted with 1.1 ml of 80:20 ACN with TFA to elute, after which the samples were run in 50 μ l of 50:50 ACN with 1% TFA and then through HPLC. Each 1-min fraction was collected via fractionator, after which each was dried, resuspended in scintillation cocktail, and then counted for 10 min with a Beta counter for the measurement of radioactivity.

Independent analyses via RIA were performed for urine from Experiment II after HPLC separation for OT and for AVP, using I¹²⁵ RIA for OT and for AVP (Peninsula Lab. Inc., Bachem, San Carlos, CA). All samples from Experiment II were run in a single assay, with intra-assay coefficients of variation for a pool equaling 5.75, $n=11$ for OT and 4.13, $n=8$ for AVP (RIA's run according to kit instructions). Each 200- μ l sample was processed through HPLC and the fraction containing OT or AVP were collected based on retention times of the peptides, dried and reconstituted in assay buffer. During HPLC runs a mobile phase blank was injected between each sample to ensure a clean column and injection. All samples were fractionated before RIA due to nonspecific cross reactivity found with other fractions, and only the 6th fraction was used as this is where the unlabeled OT standard eluted. Procedural recoveries for AVP were 64% and for OT were 76.5%. Serial dilutions of urine were parallel to the standards (for OT, $t=0.23$, $p>0.05$; for AVP, $t=0.23$, $p>0.05$).

Statistical methods

For Experiment I, straightforward measurement of radioactivity in each urine sample was performed. For Experiment II, repeated measures ANOVA were performed on logged values for OT, AVP and cortisol between the isolation and contact periods of the study, and also for each contact subcondition. The

approach used a derivation from first principles method in order to compare animals and conditions to one another directly.

Results

Experiment I

Excretion of the radiolabel in urine

The radiolabel was present in some fractions of all urine samples. The total beta counts per minute (cpm) excreted by each animal during the first day of the study were approximately 210,254, 421,947, 138,971, and 291,880 cpm (only 2.8%, 5.7%, 1.8%, and 3.9% of the total injected amount), respectively. The temporal course of radiolabel excretion was remarkably consistent between animals. Of the total radioactivity voided during the first day of the study, a substantial portion (mean=38%) was voided by subjects within the first 30 min (± 10 min). A second substantial release of radioactivity into the urine occurs between the end of the first hour and the end of the second (mean=29.3% of total excreted radioactivity), with levels sufficiently close to baseline to permit safe return to the main housing area after approximately 48 h (Fig. 1). Fecal samples were also collected, separated via HPLC and counted, but no radioactivity above background levels was present in any of the fecal samples, except in a single case in which urinary contamination of the sample was possible.

Intact OT and metabolic products of injected radiolabel

Whereas the reference sample (pure radiolabeled OT sent through HPLC) produced a peak in the 7th minute fraction, indicating that the radiolabeled molecule eluted on the fractionator between 7 and 8 min (unlabeled OT eluted at 5.4 min, thus eluting in the 6th minute fraction, likely due to slight differences in molecular polarity), the greatest quantity

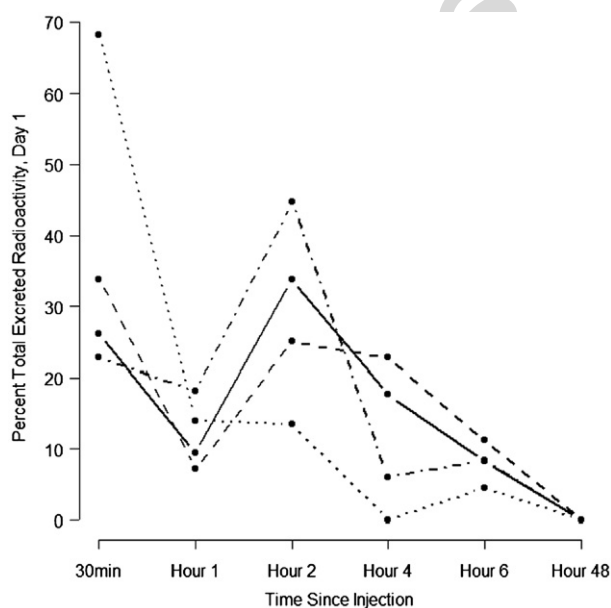


Fig. 1. Approximate timing of radiolabel excretion. Hatched lines represent individual subjects ($n=4$).

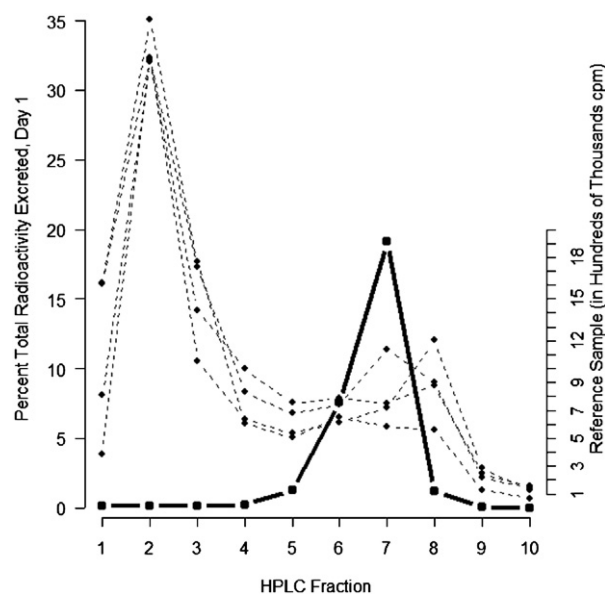


Fig. 2. HPLC fractions containing radioactivity in both the subjects (hatched lines) and the reference sample (solid line). When subjected to HPLC separation in 1-min fractions, pure radiolabeled OT elutes radiolabeled OT elutes in the fraction representing the elution from 6 to 7 min. Total beta counts in the reference sample, however, was 7,400,000; this quantity was diluted to 1:100 μ l as shown in order to permit processing via HPLC. The second fraction contains the vast majority of all excreted radiolabels during the first 24 h of the study, but a smaller quantity elutes at approximately the same time as the reference sample. This fraction was used for RIA in Experiment II to control for cross-reactivity.

of urinary radioactivity in test subjects elutes in the second minute fraction. This peak contained the majority of all excreted radioactivity during the first day across all animals (mean=33%, $\pm 1.44\%$). Importantly, however, all subjects also voided the radiolabel in a smaller quantity in the fraction matching the reference sample for intact OT. Quantities that remain intact are small but measurable via this technique (Fig. 2).

Experiment II

Urinary levels of OT, AVP and cortisol during contact were found to be higher on average than those collected during isolation across all animals. The difference between the contact period as a whole and the isolation portion was not significant for OT ($T=1.60$, p value=0.11, $SE=0.34$, $df=41$) and showed only nonsignificant trends for AVP ($T=1.80$, p value=0.07, $SE=0.37$, $df=41$), although for cortisol differences were significant ($T=2.21$, p value=0.03, $SE=0.12$, $df=41$) (Fig. 3). The visual subportion of contact, however, showed differences from isolation for all three hormones. OT showed only a nonsignificant trend towards increase upon onset of visual contact ($T=1.60$, p value=0.10, $SE=0.43$, $df=39$), but AVP changed significantly ($T=2.03$, p value=0.04, $SE=0.46$, $df=39$) as did cortisol levels ($T=3.32$, p value=0.002, $SE=0.14$, $df=39$) (Fig. 4). Neither tactile contact nor being reunited with the mate showed significant differences from the isolation condition for any hormone, although individual animals released very large quantities of OT or AVP in single

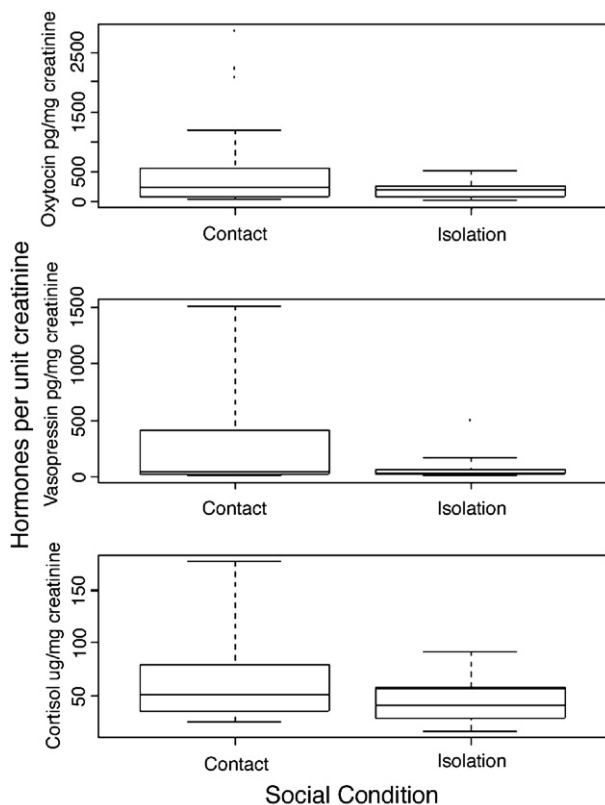


Fig. 3. Differences between contact and isolation for OT ($T=1.60$, p value=0.11, $SE=0.34$, $df=41$) AVP ($T=1.80$, p value=0.07, $SE=0.37$, $df=41$), and cortisol ($T=2.21$, p value=0.03, $SE=0.12$, $df=41$).

urine samples during other subconditions of the contact portion of the study. When these “bolus” releases are eliminated from consideration, the visual contact period produced significant increases relative to baseline for all hormones (OT: $T=1.86$, p value=0.05, $df=35$; AVP: $T=2.34$, p value=0.02, $df=34$; cortisol: $T=3.38$, p value=0.002, $df=35$). Variability due to subject effect was small for both OT and AVP ($SD=0.18$ vs. 1.18) and AVP ($SD=0.1$ vs. 1.30).

Discussion

The work presented here suggests that exogenous OT is released into the urine of *C. jacchus*, and that quantities of this peptide are measurable via the techniques described. It also demonstrates that endogenous OT and AVP are measurable in the urine and that these levels may alter with changing social condition, although more work is required in order to interpret the results, particularly for OT. Past efforts to measure OT in urine have showed variable success, perhaps due to very low levels of this peptide in excreta, breakdown of the intact molecule into metabolites, and also because of failure to employ the initial step of HPLC separation before RIA of samples. Without this step, cross-reactivity is high using this RIA assay system and the signal may be obscured. The very low levels of recovered radioactivity as shown in Experiment I indicate that most of the injected molecule was sequestered elsewhere in the body of the subjects. Rats that underwent sacrifice following a

similar study sequestered most radioactivity in the kidney (73%), pituitary gland (21%), adrenal gland (14.5%), and liver (17%) 1 h after jugular injection of radiolabeled OT (Aroskar et al., 1964).

The absence of fecal radioactivity indicates one or more of a number of possibilities. It may be the case that the tritium radiolabel disassociates or breaks down into water before it reaches the feces. Alternatively, it may take longer for the radiolabel to appear in the feces than the duration of the study period (48 h). Lastly, it is possible that OT is simply not measurable in fecal material.

While urinary metabolites in the second fraction bear investigation in the common marmoset, it should be emphasized that they may not be identical to OT metabolites of another species, due to possible species differences in peptide cleavage. Therefore, this result is not necessarily applicable in the absence of future radiolabeled clearance work in other species, which was partly why the only the intact molecule (which should be identical in all mammals) was selected for analysis in Experiment II, in spite of its containing a considerably smaller

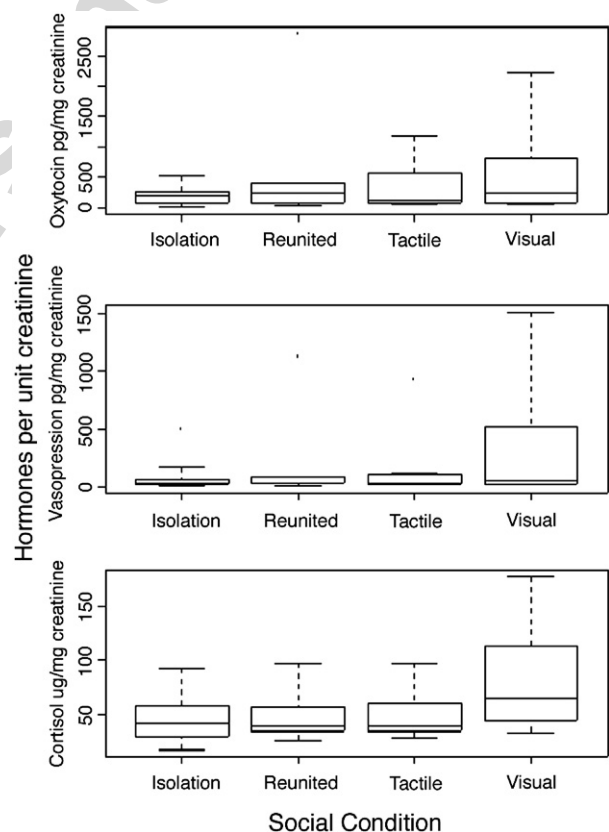


Fig. 4. Concentration of excreted OT ($T=1.60$, p value=0.10, $SE=0.43$, $df=39$) AVP ($T=2.03$, p value=0.04, $SE=0.46$, $df=39$) and cortisol levels ($T=3.32$, p value=0.002, $SE=0.14$, $df=39$) during the different contact subportions of the study. Values were logged and then the exponential taken to convert back into standard units. Visual contact stimulated the greatest increase of each hormone relative to the isolation condition. Note the extreme values for OT and AVP (dots above plot) during each of the other conditions. These outliers represent single bolus releases on the part of a single subject. The cause for these releases is currently unknown, but without these points, differences are significant for all three hormones during the visual portion (OT: p value=0.05, AVP: p value=0.02, cortisol: p value=0.002).

portion of the radioactivity. Furthermore, as only the amino acid tyrosine bore the radiolabel, it is possible that other metabolic byproducts exist that could not be measured via this technique. One possible avenue for future research could be to radiolabel each of OT's nine amino acids and to observe all metabolic products. Nonetheless, while this study demonstrates that much intact OT is broken down into metabolites, small quantities do remain intact in sufficient quantities to permit urinary assay via the methods described.

Using this knowledge, we were able to conclude that endogenous OT and AVP are also measurable in the urine of the common marmoset in Experiment II, and moreover, that these levels change with social condition. Contact overall did not differ significantly from isolation, but the visual subportion did, indicating that the type of social contact may be important to the release of these peptides in this species. Also, although levels of cortisol were high during isolation, all animals showed a substantial and immediate increase upon commencement of visual contact. Levels decreased relatively rapidly afterward. It is possible that seeing the mate without the option for tactile contact is a stressful experience in a pair-bonded species. Moreover, some animals did release single, large boluses of OT and/or AVP during contact other than visual contact for reasons that remain unclear. It is worth noting, however, that no very large (more than 3 SD from the mean) releases of OT or AVP took place during the isolation condition in any of the subjects (which was nearly seven times longer in duration), indicating that these releases may have been nonrandom – in fact each bolus release was measured in the first or second urine sample collected after the contact condition changed, indicating a socially relevant response. More investigation of these very large releases is clearly called for. Finally, rapid change in peptide levels following exposure to conspecifics also underscore the importance of collecting the *first* urinary sample after observing a behavior of interest, a conclusion that is also well-supported by Experiment I. Ideally, a regimen of urine collection paired with behavioral observation would yield the best results.

Some limitations are also evident in this study, most notably nonsignificant results for endogenous OT change in the urine during visual contact. This may have been due to low power and small sample size. While elimination of outliers led to significant differences in all three hormones, the cause of these releases during portions of the study other than visual contact is unknown and requires investigation.

Nonetheless, this publication is the first to describe an effective method for measuring OT and AVP in the urine of a social, nonhuman primate under behavioral conditions. This work sets the stage for future research into the role of OT and AVP in primates, both in captivity and in the field.

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References

- Amico, J.A., Tenicela, R., Johnston, J., Robinson, A.G., 1983. A time-dependent peak of oxytocin exists in cerebrospinal fluid but not in plasma of humans. *J. Clin. Endocrinol. Metab.* 57, 947–951.
- Amico, J.A., Ulbrecht, J.S., Robinson, A.G., 1987. Clearance studies of OT in humans using radioimmunoassay measurements of the hormone in plasma and urine. *J. Clin. Endocrinol. Metab.* 64, 340–345.
- Amico, J.A., Mantella, R.C., Vollmer, R.R., Li, X., 2004a. Anxiety and stress responses in female OT deficient mice. *J. Neuroendocrinol.* 16, 319–324.
- Amico, J.A., Vollmer, R.R., Karam, J.R., Lee, P.R., Li, X., Koenig, J.I., McCarthy, M.M., 2004b. Centrally administered OT elicits exaggerated grooming in OT null mice. *Pharmacol. Biochem. Behav.* 78, 333–339.
- Argiolas, A., Melis, M.R., 2004. The role of OT and the paraventricular nucleus in the sexual behaviour of male mammals. *Physiol. Behav.* 83, 309–317.
- Arletti, R., Benelli, A., Bertolini, A., 1992. OT involvement in male and female sexual behavior. *Ann. N. Y. Acad. Sci.* 652, 180–193.
- Aroskar, J.P., Chan, W.Y., Stouffer, J.E., Schneider, C.H., Murti, V.V.S., Vigneaud, V., 1964. Renal excretion and tissue distribution of radioactivity after administration of tritium-labeled OT to rats. *Endocrinology* 74, 226–232.
- Bales, K.L., Carter, C.S., 2003. Developmental exposure to OT facilitates partner preferences in male prairie voles (*Microtus ochrogaster*). *Behav. Neurosci.* 117, 854–859.
- Bartz, J.A., Hollander, E., 2006. The neuroscience of affiliation: forging links between basic and clinical research on neuropeptides and social behavior. *Horm. Behav.* 50, 518–528.
- Boyd, N.R.H., Jackson, D.B., Hollingsworth, S., Forsling, M.L., Chard, T., 1972. The development of a radioimmunoassay for oxytocin: the extraction of oxytocin from urine and determination of the excretion rate for exogenous and endogenous oxytocin in human urine. *J. Endocrinol.* 52, 59–67.
- Burbach, J.P., Bohus, B., Kovacs, G.L., Van Nispen, J.W., Greven, H.M., De Wied, D., 1983. Oxytocin is a precursor of potent behaviourally active neuropeptides. *Eur. J. Pharmacol.* 94, 125–131.
- Carmichael, M.S., Humbert, R., Dixen, J., Palmisano, G., Greenleaf, W., Davidson, J.M., 1987. Plasma OT increases in the human sexual response. *J. Clin. Endocrinol. Metab.* 64, 27–31.
- Carmichael, M.S., Warburton, V.L., Dixen, J., Davidson, J.M., 1994. Relationships among cardiovascular, muscular, and oxytocin responses during human sexual activity. *Arch. Sex Behav.* 23, 59–79.
- Carter, C.S., 1998. Neuroendocrine perspectives on social attachment and love. *Psychoneuroendocrinology* 23, 779–818.
- Carter, C.S., Altemus, M., 1997. Integrative functions of lactational hormones in social behavior and stress management. *Ann. N. Y. Acad. Sci.* 807, 164–174.
- Cross, N., Pines, M.K., Rogers, L.J., 2004. Saliva sampling to assess cortisol levels in unrestrained common marmosets and the effect of behavioral stress. *Am. J. Primatol.* 62, 107–114.
- Cushing, B.S., Carter, C.S., 2000. Peripheral pulses of oxytocin increase partner preferences in female, but not male, prairie voles. *Horm. Behav.* 37, 49–56.
- Cushing, B.S., Martin, J.O., Young, L.J., Carter, C.S., 2001. The effects of peptides on partner preference formation are predicted by habitat in prairie voles. *Horm. Behav.* 39, 48–58.
- Drago, F., Pedersen, C.A., Caldwell, J.D., Prange Jr., A.J., 1986. Oxytocin potentially enhances novelty-induced grooming behavior in the rat. *Brain Res.* 368, 287–295.
- Ervin, M.G., Leake, R.D., Ross, M.G., Calvario, G.C., Fisher, D.A., 1985. Arginine vasotocin in ovine fetal blood, urine, and amniotic fluid. *J. Clin. Invest.* 75, 1696–1701.

- Forsling, M.L., Martin, M.J., Sturdy, J.C., Burton, A.M., 1973. Observations on the release and clearance of neurophysin and neurohypophysial hormones in the rat. *J. Endocrinol.* 57, 307–315.
- Frandsen, P., Jensen, S.E., 1971. Excretion of oxytocin and vasopressin in human urine. *Acta Endocrinol. (Copenh)* 66, 540–546.
- Gimpl, G., Fahrenholz, F., 2001. The oxytocin receptor system: structure, function and regulation. *Physiol. Rev.* 81, 629–683.
- Goodson, J.L., Evans, A.K., Wang, Y., 2006. Neuropeptide binding reflects convergent and divergent evolution in species-typical group sizes. *Horm. Behav.* 50, 223–236.
- Hammock, E.A., Young, L.J., 2005. Microsatellite instability generates diversity in brain and sociobehavioral traits. *Science* 308, 1630–1634.
- Harmon, A.C., Huhman, K.L., Moore, T.O., Albers, H.E., 2002. OT inhibits aggression in female Syrian hamsters. *J. Neuroendocrinol.* 14, 963–969.
- Heinrichs, M., Baumgartner, T., Kirschbaum, C., Ehlert, U., 2003. Social support and oxytocin interact to suppress cortisol and subjective responses to psychological stress. *Biol. Psychiatry* 54, 1389–1398.
- Insel, T.R., 1992. Oxytocin – a neuropeptide for affiliation: evidence from behavioral, receptor autoradiographic, and comparative studies. *Psychoneuroendocrinology* 17, 3–35.
- Kalin, N.H., Gibbs, D.M., Barksdale, C.M., Shelton, S.E., Carnes, M., 1985. Behavioural stress decreases plasma oxytocin concentrations in primates. *Life Sci.* 36, 1275–1280.
- Khan-Dawood, F.S., 1985. Methods of extraction and concentration of OT for immunoassay. In: Amico, J.A., Robinson, A.G. (Eds.), *Oxytocin: Clinical and Laboratory Studies*. International Congress Series, vol. 666. Excerpta Medica, Amsterdam, pp. 16–23.
- Kozorovitskiy, Y., Hughes, M., Lee, K., Gould, E., 2006. Fatherhood affects dendritic spines and vasopressin V1a receptors in the primate prefrontal cortex. *Nat. Neurosci.* 9, 1094–1095.
- Krüger, T.H.C., Haake, P., Chereath, D., Knapp, W., Janssen, O.E., Exton, M.S., Schedlowski, Hartmann, U., 2003. Specificity of the neuroendocrine response to orgasm during sexual arousal in men. *J. Endocrinol.* 177, 57–64.
- Landgraf, R., 2005. Neuropeptides in anxiety modulation. *Handb. Exp. Pharmacol.* 169, 335–369.
- Landgraf, R., Neumann, I.D., 2004. Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. *Front. Neuroendocrinol.* 25, 150–176.
- Lim, M.M., Wang, Z., Olazabal, D.E., Ren, X., Terwilliger, E.F., Young, L.J., 2004. Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene. *Nature* 429, 754–757.
- Lim, M.M., Bielsky, I.F., Young, L.J., 2005. Vasopressin and oxytocin in social recognition and bonding: potential animal models of autism. *Int. J. Dev. Neurosci.* 23, 235–243.
- Maney, D.L., Erwin, K.L., Goode, C.T., 2005. Neuroendocrine correlates of behavioral polymorphism in white-throated sparrows. *Horm. Behav.* 48, 196–206.
- Mens, W.B., Witter, A., van Wimersma Greidanus, T.B., 1983. Penetration of neurohypophysial hormones from plasma into cerebrospinal fluid (CSF): half-times of disappearance of these neuropeptides from CSF. *Brain Res.* 262, 143–149.
- Modahl, C., Green, L., Fein, D., Morris, M., Waterhouse, L., Feinstein, C., Levin, H., 1998. Plasma OT levels in autistic children. *Biol. Psychiatry* 43, 270–277.
- Nelson, E.E., Panksepp, J., 1998. Brain substrates of infant–mother attachment: contributions of opioids, OT, and norepinephrine. *Neurosci. Biobehav. Rev.* 22, 437–452.
- Polito, A.B., Goldstein, D.L., Sanchez, L., Cool, D.R., Morris, M., 2006. Urinary oxytocin as a non-invasive biomarker for neurohypophysial hormone secretion. *Peptides* 11, 2877–2884.
- Robinson, I.C.A.F., Walker, J.M., 1979. Extraction of small amounts of OT from biological fluids by means of agarose-bound neurophysin. *J. Endocrinol.* 80, 191–202.
- Saltzman, W., Severin, J.M., Schultz-Darken, N.J., Abbott, D.H., 1997. Behavioral and social correlates of escape from suppression of ovulation in female common marmosets housed with the natal family. *Am. J. Primat.* 41, 1–21.
- Stewart, W.C., 1953. An oxytocic material from normal human urine. *Gynaecologia* 136, 87–93.
- Uvnäs-Moberg, K., 1998. Oxytocin may mediate the benefits of positive social interaction and emotions. *Psychoneuroendocrinology* 23, 819–835.
- van Wimersma Greidanus, T.B., Maigret, C., 1996. The role of limbic vasopressin and OT in social recognition. *Brain Res.* 713, 153–159.
- Veenema, A.H., Blume, A., Niederle, D., Buwalda, B., Neumann, I.D., 2006. Effects of early life stress on adult male aggression and hypothalamic vasopressin and serotonin. *Eur. J. Neurosci.* 24, 1711–1720.
- White-Traut, R., Powlesland, J., Gelhar, D., Chatterton, R., Morris, M., 1998. Methodological issues in the measurement of oxytocin in human neonates. *J. Nurs. Meas.* 6, 155–174.
- Wikström, S., Gunnarsson, T., Nordin, C., 2003. Tactile stimulus and neurohormonal response: a pilot study. *Int. J. Neurosci.* 113, 787–793.
- Winslow, J.T., Insel, T.R., 1991a. Social status in pairs of male squirrel monkeys determines the behavioral response to central OT administration. *J. Neurosci.* 11, 2032–2038.
- Winslow, J.T., Insel, T.R., 1991b. Vasopressin modulates male squirrel monkeys' behavior during social separation. *Eur. J. Pharmacol.* 200, 95–101.
- Winslow, J.T., Insel, T.R., 2004. Neuroendocrine basis of social recognition. *Curr. Opin. Neurobiol.* 14, 248–253.
- Winslow, J.T., Noble, P.L., Lyons, C.K., Sterk, S.M., Insel, T.R., 2003. Rearing effects on cerebrospinal fluid concentration and social buffering in rhesus monkeys. *Neuropsychopharmacology* 28, 910–918.
- Wismer Fries, A.B., Ziegler, T.E., Kurian, J.R., Jacoris, S., Pollak, S.D., 2005. Early experience in humans is associated with changes in neuropeptides critical for regulating social behavior. *Proc. Natl. Acad. Sci. U. S. A.* 102, 17237–17240.
- Wotjak, C.T., Ganster, J., Kohl, G., Holsboer, F., Landgraf, R., Engelmann, M., 1998. Dissociated central and peripheral release of vasopressin, but not oxytocin, in response to repeated swim stress: new insights into the secretory capacities of peptidergic neurons. *Neuroscience* 85, 1209–1222.
- Young, L.J., Wang, Z., 2004. The neurobiology of pair bonding. *Nat. Neurosci.* 7, 1048–1054.
- Zebidi, A., Geelen, G., Allevard, A.M., Sempore, B., Jarsaillon, E., Meunier, C., Gharib, C., 1978. Radioimmunoassay of urinary OT in man. *C. R. Seances Soc. Biol. Fil.* 172, 1155–1161.