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Title Page

Binding Characteristics of Two Oxytocin Variants and Vasopressin at Oxytocin Receptors from Four Primate Species with Different Social Behavior Patterns

Jack H. Taylor, Nancy A. Schulte, Jeffrey A. French, and Myron L. Toews

Callitrichid Research Center, Department of Psychology, University of Nebraska at Omaha (JHT, JAF)

Department of Biology, University of Nebraska at Omaha (JAF)

Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center (NAS, MLT)

Running Title Page

BINDING CHARACTERISTICS OF FOUR PRIMATE OXYTOCIN RECEPTORS

Corresponding Author

Myron Toews

mtoews@unmc.edu

Phone: 402-559-7197

FAX: 402-559-7495

Durham Research Center, 3042

985800 Nebraska Medical Center

Omaha, NE 68198-5800

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Abbreviations: **AVP** - Arginine-8-vasopressin; **CHO** – Immortalized Chinese hamster ovary cells; **HGH-BSA** - High glucose HEPES-buffered Dulbecco's Modified Eagle's Medium containing 0.1% bovine serum albumin; **Leu^8-OT** - Leucine-8-oxytocin; **NWM** – New World monkey; **OT** - Oxytocin; **OTR** – Oxytocin receptor; **Pro^8-OT** - Proline-8-oxytocin;

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ABSTRACT

A clade of New World monkeys (NWM) exhibits considerable diversity in both oxytocin ligand (OT) and receptor (OTR) structure. Most notable is the variant Pro^8-OT, with proline instead of leucine at the eighth position (Pro^8-OT), resulting in a rigid bend in the peptide backbone. A higher proportion of species that express Pro^8-OT also engage in biparental care and social monogamy. When marmosets (genus *Callithrix*), a biparental and monogamous Pro^8-OT NWM species, are administered the ancestral Leu^8-OT, there is no change in social behavior compared to saline treatment. However, when Pro^8-OT is administered, marmosets' socio-sexual and prosocial behaviors are altered. The studies here tested the hypothesis that OTR binding affinities and OT-induced intracellular Ca^{2+} potencies would favor the native oxytocin ligand in OTRs from four primate species, each representing a unique combination of ancestral lineage, breeding system, and native OT ligand: human (Leu^8-OT, monogamous, apes), macaque (Leu^8-OT, nonmonogamous, Old World monkey), marmoset (Pro^8-OT, monogamous, NWM) and Titi monkey (Leu^8-OT, monogamous, NWM). OTRs were expressed in CHO cells and tested for intact-cell binding affinities for Pro^8-OT, Leu^8-OT, and arginine vasopressin (AVP), as well as intracellular Ca^{2+} signaling after stimulation with Pro^8-OT, Leu^8-OT and AVP. Contrary to our hypothesis, Pro^8-OT bound at modestly higher affinities and stimulated calcium signaling at modestly higher potencies compared to Leu^8-OT in all four primate OTRs. Thus differences downstream from ligand-receptor binding event are more likely to explain the different behavioral responses to these two ligands.

Introduction

Oxytocin (OT) is a nonapeptide neurohormone that is critical for mammalian parturition, lactation, and parental behavior (Ellendorff *et al.*, 1982; Fuchs *et al.*, 1982; McNeilly *et al.*, 1983; Chan *et al.*, 1996; Lee *et al.*, 2009). OT binds and activates its canonical G protein-coupled receptor, the oxytocin receptor (OTR). Synthetic OT is used widely in clinical settings for inducing and accelerating labor. Because of its ability to modulate a wide variety of social behaviors (Lee *et al.*, 2009), it is currently being evaluated for its clinical use in disorders with a social component, such as autism spectrum disorder and schizophrenia (Bakermans-Kranenburg and van IJzendoorn, 2013; Feifel *et al.*, 2016; DeMayo *et al.*, 2017; Parker *et al.*, 2017). Considerable effort has been invested in engineering oxytocin analogs and formulations for potential therapeutic use and to extend understanding of OT actions, particularly CNS and behavioral effects (Manning *et al.*, 2012; Busnelli *et al.*, 2013; Muttenthaler *et al.*, 2017). Though these efforts with novel synthetic analogs have been reasonably successful, naturally occurring variants of the OT peptide could provide an alternate route to novel agents and therapies (Gruber *et al.*, 2012).

The nonapeptide family of hormone ligands is ancient and is present in nearly all animal lineages (Beets *et al.*, 2013; Lockard *et al.*, 2016). OT-like ligands generally vary at the third, fourth, or eighth amino acid position (Gruber *et al.*, 2012), and the amino acid at the eighth position strongly affects the activity of the peptide on its target organs (Sawyer and Manning, 1973; Manning *et al.*, 2012; Muttenthaler *et al.*, 2017). OT and the closely related nonapeptide arginine vasopressin (AVP) differ at amino acid positions three and eight and have vastly different roles in mammalian physiology, even though the affinity of OT for OTRs is only two-fold greater than the affinity of AVP for OTRs (Manning *et al.*, 2012). Despite having only a two-fold lower binding affinity for OTRs, AVP is over thirty-fold less potent than OT for generating OTR responses (Manning *et al.*, 2012). Variations among species in their nonapeptide receptors

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correlate with variations in their respective OT-like ligands, indicating ligand-receptor co-evolution (Koehbach *et al.*, 2013). The presence of OT-like peptides across diverse animal taxa suggests the universal importance of their functions, and their co-evolution with their ligands suggests a tightly aligned signaling system for these functions.

Despite variation across Animalia taxa, the OT ligand is highly conserved within eutherian mammals (Wallis, 2012). Recently, a nonsynonymous nucleotide substitution in the *OXT* gene coding for OT was discovered in four species of New World monkeys (NWM), resulting in a proline at amino acid position eight (Pro^8-OT) in place of the typical leucine (Leu^8-OT) (Lee *et al.*, 2011). Subsequent screening showed that the Pro^8-OT variant is present in at least twenty NWM species (Ren *et al.*, 2015; Vargas-Pinilla *et al.*, 2015). Additional OT variants were also identified, for a total of six different forms of OT in NWM, with at least one species from each NWM clade exhibiting an OT variant (Ren *et al.*, 2015; Vargas-Pinilla *et al.*, 2015). OTRs also vary in NWM, particularly in the N-terminus (Ren *et al.*, 2015; Vargas-Pinilla *et al.*, 2015), which is important for binding to the tail of the OT ligand (Postina *et al.*, 1996; Gimpl and Fahrenholz, 2001), and there is strong evidence for OT-OTR co-evolution (Koehbach *et al.*, 2013; Ren *et al.*, 2015; Vargas-Pinilla *et al.*, 2015). Moreover, *OXTR* variation is associated with social monogamy among primates (Ren *et al.*, 2015), and OT ligand variation at position eight is associated with litter size within the family *Cebidae* (Vargas-Pinilla *et al.*, 2015). Both native and non-native OT ligands modulate social behavior in NWM expressing Pro^8-OT (French *et al.*, 2016), but the native Pro^8-OT is more effective at modulating behavior than the ancestral Leu^8-OT in the marmoset, a monogamous and biparental NWM (Cavanaugh *et al.*, 2014; Mustoe *et al.*, 2015, 2018). Together these findings indicate that both OT ligand variation and the corresponding variations in OTRs among NWMs contribute to functional outcomes.

Based on the findings summarized above, we hypothesized that the binding affinities and signaling potencies of primate OT variants are different for different OTR variants, with each

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receptor variant preferring the ligand variant from the same species. The studies presented here test this hypothesis by measuring binding affinities and signaling potencies for Leu8, Pro8, and AVP at the OTRs from four primate species, each representing a unique combination of ancestral lineage, breeding system, and native OT ligand.

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Methods and Materials

OTR transfection and cell culture

Chinese hamster ovary (CHO; Female origin) cells were purchased from ATCC and cultured at 37° C with 5% CO_2 using Ham's F12 medium supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 100 µg/ml streptomycin. Human, marmoset, and macaque OTR plasmids (Table 1) were purchased from Genscript in a pcDNA3.1+ vector. The titi monkey plasmid was generated by amplifying and ligating the coding region of the titi *OXTR* from genomic titi monkey DNA (flanked with BamHI and XhoI restriction sites) and ligating it into a T-vector (pMD19). Competent *E. coli* were transformed using this vector, plated onto LB/ampicillin/IPTG/X-Gal plates, and incubated overnight at 37° C. White colonies were selected, then plasmid DNA was purified and sequenced. Sequence-confirmed plasmids were then digested with BamHI and XhoI and ligated into a pcDNA3.1+ vector. CHO cells were then transfected using Turbofect according to the manufacturer's instructions and kept under selective pressure using 400 µg/mL G418 antibiotic. Individual clonal lines were generated by plating batch-transfected cells at approximately 10 cells/mL (1 cell/100µL) into 96-well plates and then selecting wells for screening that originated from a single colony. Clonal lines were screened using an intact cell ^{125}I-ornithine vasotocin analog (^{125}I-OVTA) binding assay and selected for similar receptor expression across species, defined as total radioligand binding. All experiments were done in a single clone per species, except for the marmoset, in which two clones were used.

Intact cell saturation binding assays

CHO cells expressing primate OTRs were plated at 150,000 cells/mL (15,000 cells per well/100 µL) into 96-well plates and grown to 80-90% confluence. On the day of assay, growth medium was aspirated and cells were quickly washed once with 100 µL ice-cold high glucose

HEPES-buffered Dulbecco's Modified Eagle's Medium containing 0.1% bovine serum albumin (HGH-BSA) and then placed on ice. Then 50 µL of ice-cold ^{125}I-OVTA (PerkinElmer) in doubling concentrations from about 15 to 2000 pM were added in triplicate (technical replicates) to all wells and incubated for three hours on ice. At the end of the assay, an aliquot of the binding medium was collected to quantify free radioligand directly, eliminating any concerns about differential depletion of ligand due to differential receptor expression levels. Cells were then washed four times with 100 µL ice-cold HGH-BSA, solubilized with 100 µL 0.2 N NaOH, and counted on a gamma counter. Non-specific binding was defined as ^{125}I-OVTA binding occurring in the presence of excess competitor (10^{-4} M Leu^8-OT). Binding affinity (K_d) for ^{125}I-OVTA was determined using GraphPad Prism to fit the specific bound *vs.* free ligand data to a single-site binding equation. These assays were done at least three times on three different days using fresh aliquots of ^{125}I-OVTA and competitor, and K_d values were averaged across three biological replicates (five biological replicates for marmoset).

Intact cell competition binding assays

CHO cells expressing primate OTRs were plated at 150,000 cells/mL (15,000 cells per well/100ul) into 96-well plates and grown to 80-90% confluence. On the day of assay, growth medium was aspirated and cells were quickly washed once with 100 µL ice-cold HGH-BSA and then placed on ice. Then 50 µL of roughly 50,000 CPM ice-cold ^{125}I-OVTA were added in triplicate (technical replicates) to all wells in the presence or absence of 10^{-11} to 10^{-5} M Pro^8-OT (CYIQNCPPG-NH2; Anaspec), Leu^8-OT (CYIQNCPLG-NH2; Anaspec) or AVP (CYFQNCPRG-NH2; Anaspec) and incubated for three hours on ice. At the end of the assay, an aliquot of the binding medium was collected to quantify free radioligand directly. Cells were then washed four times with 100 µL ice-cold HGH-BSA, solubilized with 100 µL 0.2 N NaOH, and counted on a gamma counter. Binding affinities (IC_{50}) were determined by plotting bound ^{125}I-OVTA *vs.* competitor concentration. IC_{50} values were then calculated using the Cheng-Prusoff equation

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and each receptor's affinity for ^{125}I-OVTA to produce K_i values. These assays were done at least three times on three different days using fresh aliquots of ^{125}I-OVTA and Leu^8-OT, Pro^8-OT, and AVP for three biological replicates per clone.

Ca^{2+} mobilization assays

CHO cells expressing primate OTRs were plated into 96-well plates and grown to 80-90% confluence. On the day of assay, growth medium was aspirated and cells were incubated at 37˚ C with 100 µL Fluo-4 Direct dye mixed in Fluo-4 Direct Ca^{2+} Assay Buffer with 5 mM probenecid for one hour. At the end of one hour, baseline fluorescence was measured at 37˚ C followed by stimulated fluorescence in the presence or absence of 10^{-11} to 10^{-6} M Pro^8-OT, Leu^8-OT, or AVP (3 x technical replicates). Peak fluorescence minus baseline fluorescence was then plotted as a function of ligand concentration to determine EC_{50} values. These assays were done at least three times on three different days using fresh aliquots of Leu^8-OT, Pro^8-OT, and AVP for three biological replicates per clone.

Data Analysis

Binding affinities (K_d) for ^{125}I-OVTA at each primate OTR were calculated by subtracting nonspecific binding and then plotting bound ^{125}I-OVTA *vs.* free ^{125}I-OVTA.

Because concentrations of ^{125}I-OVTA were not identical from experiment to experiment, technical replicates within each experiment (n=3) were normalized and binding affinities (K_i) were calculated using the Cheng-Prusoff equation and the measured binding affinity for ^{125}I-OVTA. Technical replicates were then averaged and used as biological replicates (n=3 per clone) to determine and compare K_i values for each ligand within species. A Bonferroni-corrected cutoff (p= .05 ÷ 3 = .0167) was used to determine statistically significant differences in K_i values.

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Within species differences in Ca^{2+} mobilization potency (EC_{50}) were determined by normalizing and averaging each technical replicate (n=3) and then using the biological replicates (n=3) to assess ligand comparisons (Pro^8-OT vs. Leu^8-OT, Pro^8-OT vs. AVP, Leu^8-OT vs. AVP). A Bonferroni-corrected cutoff (p= .05 ÷ 3 = .0167) was used to determine statistically significant differences in K_i's.

All data were analyzed using the nonlinear least-squares curve-fitting capabilities of GraphPad Prism.

Results

Saturation binding assays.

Saturation assays were performed on 96-well plates with 50 µL binding medium per well. Representative saturation curves for all receptors are shown in Figure 1. All of the binding and signaling assays for the human receptor were conducted with a single clone with a B_{max} value of 17 ± 6 fmol/well (n=3). All assays for the macaque receptor were with a clone with a B_{max} value of 12 ± 5 fmol/well (n=3). All assays for the titi receptor were with a clone with a B_{max} value of 44 ± 12 fmol/well (n=3). For the marmoset receptor, some assays were performed with a significantly higher expressing clone, R9, with a B_{max} value of 91 ± 20 fmol/well (n=2); additional experiments were performed with a clone with lower expression, R10, with a B_{max} value of 33 ± 5 fmol/well (n=4).

Saturation binding analyses revealed only relatively small differences in binding affinities for the radioligand ^{125}I-OVTA among the four species, ranging from 161 to 481 pM (Table 2). The human and macaque OTRs exhibited very similar affinities that were somewhat higher than those for titi and marmoset, with marmoset exhibiting the lowest affinity.

Competition binding assays with OT variants and AVP

In competition binding assays, Pro^8-OT exhibited a higher binding affinity than Leu^8-OT for all four species, with a 1.5-fold differences macaque, a 2-fold difference for marmoset, a 3-fold difference for human, and over 6-fold difference for titi. Only for titi and human was the difference in binding affinity statistically significant, (F's(1, 42) > 12.1, p < .016). For the human OTR, the difference was due to greater affinity for Pro^8-OT, while for the titi OTR, the difference was due to lower affinity for Leu^8-OT rather than higher affinity for Pro^8-OT compared to the other species (Figure 2; Table 2). For both OT variants, the absolute binding affinities were 3- to 5-fold higher for human, macaque, and marmoset than for titi.

Binding affinities for AVP were assessed alongside the two OT variants for all of the receptors. Compared to Pro^8-OT and Leu^8-OT, respectively, binding affinity for AVP was 20- and 8-fold lower for human, 11- and 6-fold lower for macaque, 13- and 6-fold lower for marmoset, but 13- and only 2-fold lower for titi. In fact, the affinity of the titi OTR for Leu^8-OT was not significantly higher than that for AVP (F's(1, 42) =2.07, p = .157). However, the rank order of potencies was the same for all species, with affinities for Pro8 > Leu8 > AVP.

Ca^{2+} signaling assays

In Ca^{2+} mobilization assays, the rank order of potencies was the same for all species and with the same pattern as for binding, Pro8 > Leu8 > AVP; however, the magnitude of the differences was smaller for signaling than for binding. Pro^8 and Leu^8-OT were roughly equipotent for all species, with only 1.5-fold greater potency of Pro8-OT *vs*. Leu^8-OT for human, macaque, marmoset, and titi (Figure 3; Table 3). Pro^8-OT consistently exhibited a slightly lower maximal response than Leu8 for all species except marmoset.

Ca^{2+} mobilization potencies for AVP were assessed alongside the two OT variants for all of the receptors. Compared to Pro^8-OT and Leu^8-OT, respectively, potency for AVP was 12- and 7-fold lower for human, 6- and 4-fold lower for macaque, 5- and 2-fold lower for marmoset, and 8- and 5-fold lower in titi. The absolute potencies for Pro^8-OT and Leu^8-OT for human and marmoset were similar, but the potency of AVP for the marmoset receptor was nearly 2-fold higher than it was for the human receptor. Potencies for each ligand across species were higher for human and marmoset OTR compared to macaque and titi monkey.

Ca^{2+} mobilization potencies relative to binding affinities were also computed as a metric of coupling efficiency (Table 4). In general, efficiencies within species were similar, with signaling EC_{50} values exhibiting potencies over two log units higher than the binding affinities for all three ligands. The macaque OTR was the least efficient, signaling at potencies less than two

log units higher than the binding affinity for all three ligands. Notably, in all species except titi monkey, AVP was equally or more efficient at mobilizing Ca^{2+} than Pro^{8}-OT or Leu^{8}-OT, per unit of binding affinity.

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Discussion

The studies here tested the hypothesis that the coevolution between Pro^8-OT and OTRs in NWM (Ren *et al.*, 2015; Vargas-Pinilla *et al.*, 2015) would confer greater selectivity in binding and signaling for Pro^8-OT over the ancestral Leu^8-OT at receptors from Pro^8-OT-expressing species, and conversely higher selectivity for Leu^8-OT at receptors from species expressing Leu^8-OT. The binding and signaling data in this study show that this hypothesis is at best only partially supported. For the marmoset OTR, the species-native Pro^8-OT bound with only modestly higher affinity and induced Ca^{2+} mobilization with higher potency than Leu^8-OT. In humans and titi monkeys, the species-non-native ligand Pro^8-OT also bound with higher affinity than the species-native ligand Leu^8-OT. For receptors from all three Leu^8-OT-expressing species, the two ligands were equipotent at mobilizing Ca^{2+}. The higher binding affinity for Pro^8-OT for all of the species, including those whose native hormone is Leu^8-OT, was unexpected and not consistent with our hypothesis of binding affinities for each species correlating with their native ligand. One explanation for the observed preference for Pro^8-OT over Leu^8-OT in all species may be that the flexible (Kotelchuck *et al.*, 1972; Brewster *et al.*, 1973) tail of Leu^8-OT can orient into a conformation that is similar to the more rigid structure of Pro^8-OT (Lee *et al.*, 2011) for only a smaller percentage of ligand-receptor interactions than Pro^8-OT, and that the optimal conformation for Leu^8-OT is one that is similar to the structure of Pro^8-OT. The lack of significant preferences for the endogenous ligand in terms of signaling potencies was similarly unexpected. Thus differences in other factors, perhaps downstream of the initial receptor binding and activation steps, are now the more likely explanations for the differential behavioral responses to OT in Leu^8-OT *vs.*Pro^8-OT-expressing species.

The ability of AVP to bind and activate primate OTRs was also tested, because AVP binds and activates OTRs, and AVP is known to affect social behavior in primates, including titi monkeys and marmosets (Caldwell *et al.*, 2008; Jarcho *et al.*, 2011; Taylor and French, 2015;

Taylor *et al.*, 2017). In all species except titi monkey, AVP bound with much lower affinity to the OTR than Pro^8-OT or Leu^8-OT, and in all species AVP had lower potency for mobilizing Ca^{2+} than Pro^8-OT or Leu8-OT. These results show that the coevolution of Pro^8-OT and the OTR in marmosets has not altered selectivity for AVP *vs.* the two OT variants.

This study is the first description of NWM OT ligand variant binding in nonhuman primates, and the Ca^{2+} mobilization data inform the recent research investigating OTR signaling and NWM ligand variants in rodent models. Parreiras-e-Silva and colleagues (Parreiras-E-Silva *et al.*, 2017) found no differences in Ca^{2+} signaling at the human OTR between Pro^8-OT, Leu^8-OT, or the additional NWM OT variant Val^{3}Pro^8-OT. Our data also partially replicate those done by our collaborators (Pierce *et al.,* 2016) that found no difference in Ca^{2+} signaling between Pro^8-OT and Leu^8-OT at the human OTR but that Pro^8-OT was more efficacious than Leu^8-OT at inducing Ca^{2+} mobilization at the marmoset OTR. Taken together, these data indicate that the substitution of proline in place of leucine does not inhibit the G protein-coupled activity in species that express the ancestral Leu^8-OT. Perhaps more importantly, the Pro^8 substitution confers equal or greater potency for Ca^{2+} mobilization in a species in which Pro^8-OT is the native ligand.

These binding and signaling data provide a functional link between the genetic surveys of the OT system in NWM and the growing body of work comparing the behavioral effects of intranasal treatment with Pro^8-OT and Leu^8-OT in marmosets. Pro^8-OT, but not Leu^8-OT, enhances a variety of pair-mate- directed social approach behavior (Cavanaugh *et al.*, 2014, 2018). Moreover, Pro^8-OT increases the amount of social behavior that an OT-treated marmoset receives from their mate and reduces sociosexual behavior directed toward individuals other than the pair-mate (Cavanaugh *et al.*, 2014; Mustoe *et al.*, 2015). Leu^8-OT does affect some social behavior in marmosets, but Leu^8-OT never enhances a social behavior that Pro^8-OT does not also enhance (Mustoe *et al.*, 2018). The binding and signaling data

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support these behavioral findings. Pro^8-OT not only bound to the marmoset OTR with greater affinity but was also modestly more potent at stimulating Ca^{2+} mobilization. Though it is unlikely that this difference in signaling between Pro^8-OT and Leu^8-OT is the only contributing factor to the behavioral differences between treatment with Pro^8-OT and Leu^8-OT in marmosets, it is likely at least one contributing factor.

These binding and signaling data also shed new light on the clade-wise surveys of the *OXT*, *OXTR*, and *AVPR1A* genes in NWM. First and foremost, our data help to explain the finding that social monogamy and the OTR coevolved in NWM (Ren *et al.*, 2015; Vargas-Pinilla *et al.*, 2015), with Pro^8-OT binding and Ca^{2+}signaling both enhanced in the socially monogamous species that expresses Pro^8-OT natively. Moreover, Ca^{2+} signaling was not reduced by the substitution of proline for leucine at the eighth position in OTRs from Leu^8-OT species. This suggests that the OTR is permissive for this substitution, providing a potential mechanism for the coevolution of Pro^8-OT and OTRs in NWM (Ren *et al.*, 2015; Vargas-Pinilla *et al.*, 2015). The single nucleotide substitution that produced Pro^8-OT may have had modest consequences for neurotransmission, and thus the OTR may have evolved to accommodate this substitution. There is also a relationship between social monogamy and variation in the AVP V1a receptor gene (*AVPR1A*). Interestingly, in one of the only Leu^8-OT NWM that exhibits social monogamy, the titi monkey, Leu^8-OT and AVP bound the OTR with similar affinity. These genetic and signaling data suggest that interrogation of the NWM AVP receptors (V1aR, V1bR, V2R) may provide new insights into nonapeptide signaling in primates, and these studies are currently in progress.

Alongside the work of Parreiras-e-Silva and colleagues (Parreiras-E-Silva *et al.*, 2017), this paper constitutes a “first look” at the characteristics of the marmoset and titi monkey OTRs when bound to the Pro^8-OT variant. As such we only explored two facets of GPCR function: ligand binding and Ca^{2+} signaling. Other characteristics of these receptors, such as differential

coupling to specific Gα subunits or bias for G proteins vs. β-arrestin, are beyond the scope of this project, but are nonetheless interesting future directions. The OTR is capable of coupling to a variety of Gα subunits resulting in a variety of cellular outcomes (see Gimpl and Fahrenholz, 2001; Mustoe *et al.*, 2018 for detailed reviews), and there is a need and a value to the production and characterization of ligands that are functionally selective at the OTR as tools to target specific signaling cascades. Indeed, even relatively small modifications to the OT ligand can alter the functional selectivity at the OTR, causing it to couple to different Gα subunits (Busnelli *et al.*, 2012), and it is already known that Pro^8-OT is less efficacious than Leu^8-OT at promoting β-arrestin recruitment and internalization at the human receptor (Parreiras-E-Silva *et al.*, 2017). It is possible that Pro^8-OT and Leu^8-OT may differentially promote coupling to specific Gα subunits or bias signaling via G proteins vs. β-arrestin in marmosets and titi monkeys as well, and these experiments may provide more insight into the evolution of this system in NWM. Another interesting possibility is that the OTRs may form dimers with various other GPCRs, and the Leu^8-OT and Pro^8-OT variants might exhibit selectivity for binding or activating one of these dimers *vs.* another. Such dimer selectivity would not be detected in these assays with only the OTR expressed. Thus multiple possible explanations for the ligand variation and its correlations with GPCR signaling and behavior remain to be explored.

A final potentially important outcome of these studies is that the higher binding affinity of Pro^8-OT *vs.* Leu^8-OT at OTRs from all species, including human, should presumably make Pro^8-OT a better ligand for future binding studies, in either a radio-labelled or fluorescently tagged form. The 3-fold higher binding affinity would allow the use of 3-fold lower concentrations of the ligand to achieve the same fractional receptor occupancy, thus decreasing the amount of ligand required and the corresponding cost and usage of the ligand. The tighter binding of the Pro^8-OT variant to the OTR may be useful in other contexts as well.

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Authorship Contributions

Participated in research design. JHT, NAS, JAF, and MLT

Conducted experiments: JHT and NAS

Performed Data Analysis: JHT, NAS, and MLT

Wrote or contributed to the writing of the manuscript: JHT, NAS, JAF, and MLT

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Footnotes

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Legends for Figures

Figure 1. Representative saturation assays for ^{125}I-OVTA binding to oxytocin receptors from each of the four species. Cells on 96-well plates were incubated on ice in 50 µL binding medium with the indicated concentrations of ^{125}I-OVTA for 3 hours, and specific binding was then quantified. Data are from a single experiment with all four receptors tested side-by-side in triplicate. Values for this experiment are in good agreement with the average values in Results and Table 1: Human, B_{max} = 9.7 fmol/well, K_d = 0.12 nM; Macaque, B_{max} = 6.9 fmol/well, K_d = 0.144 nM; Marmoset (R10), B_{max} = 34 fmol/well, K_d = 0.30 nM; and Titi, B_{max} = 30 fmol/well, K_d = 0.24 nM.

Figure 2. Competition curves for Pro^8-OT and Leu^8-OT for each primate species OTR. Increasing concentrations of competitor ligand (Pro^8-OT, Leu^8-OT, AVP) were added to a constant concentration of ^{125}I-OVTA in intact CHO cells expressing one of four primate OTRs. All values are expressed as the percentage of the maximal binding in the absence of OT or AVP.

Figure 3. Intracellular Ca^{2+} increases for each primate species OTR. Increasing concentrations of Pro^8,-OT, Leu^8-OT, or AVP were used to stimulate intracellular Ca^{2+} mobilization in CHO cells expressing one of four primate oxytocin receptors. All values are expressed as the percentage of the maximal response to 10^{-6} M Leu^8-OT for each species.

Tables

Table 1. Representative primate species OTRs

	Human	Macaque	Marmoset	Titi Monkey
Lineage (Family)	Old World (*Hominidae*)	Old World (*Cercopithidae*)	New World (*Callitrichidae*)	New World (*Pithecidae*)
Breeding System	Monogamous	Polygamous	Monogamous	Monogamous
Native OT Ligand	Leu^8-OT	Leu^8-OT	Pro^8-OT	Leu^8-OT

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Table 2. Binding affinities for ligands at various primate OTRs

OTR	^{125}I-OVTA K_d (± SEM), nM	K_i (± Log SEM), nM		
		Pro^8-OT	Leu^8-OT	AVP
Human (n=3)	0.161 (0.019)	22.78 (0.10)*	71.84 (0.10)	541.1 (0.07)* #
Macaque (n=3)	0.199 (0.036)	43.36 (0.07)	74.99 (0.08)	474.8 (0.10)* #
Marmoset (n=5; n=6)	0.481 (0.041)	81.31 (0.11)	170.2 (0.10)	1093 (0.13)* #
Titi Monkey (n=3)	0.289 (0.031)	146.9 (0.11)*	894.5 (0.12)	1924 (0.16) #

Note. * Indicates a significant within-species difference compared to Leu^8-OT using a Bonferroni-corrected cutoff of $p < .0167$. # Indicates a significant within-species difference compared to Pro^8-OT using a Bonferroni-corrected cutoff of $p < .0167$.

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Table 3. Ca^{2+} mobilization potencies for ligands at various primate OTRs

	Ca^{2+} EC_{50} (± Log SEM), nM		
OTR (n=3)	Pro^8-OT	Leu^8-OT	AVP
Human	0.072 (0.12)	0.127 (0.10)	0.864 (0.07)* #
Macaque	1.341 (0.11)	2.025 (0.11)	7.981 (0.12)* #
Marmoset	0.115 (0.15)	0.176 (0.14)	0.459 (0.14) #
Titi Monkey	0.595 (0.09)	1.010 (0.09)	4.821 (0.09)* #

Note. * Indicates a significant within-species difference compared to Leu^8-OT using a Bonferroni-corrected cutoff of $p < .0167$. # Indicates a significant within-species difference compared to Pro^8-OT using a Bonferroni-corrected cutoff of $p < .0167$.

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Table 4. Coupling efficiencies for ligands at various primate OTRs

	Potency/Affinity ratio -Log(Ca^{2+} IC_{50}/K_i)		
OTR	Pro^8-OT	Leu^8-OT	AVP
Human	2.51	2.75	2.80
Macaque	1.51	1.57	1.77
Marmoset	2.85	3.00	3.38
Titi Monkey	2.39	2.95	2.60

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Figures

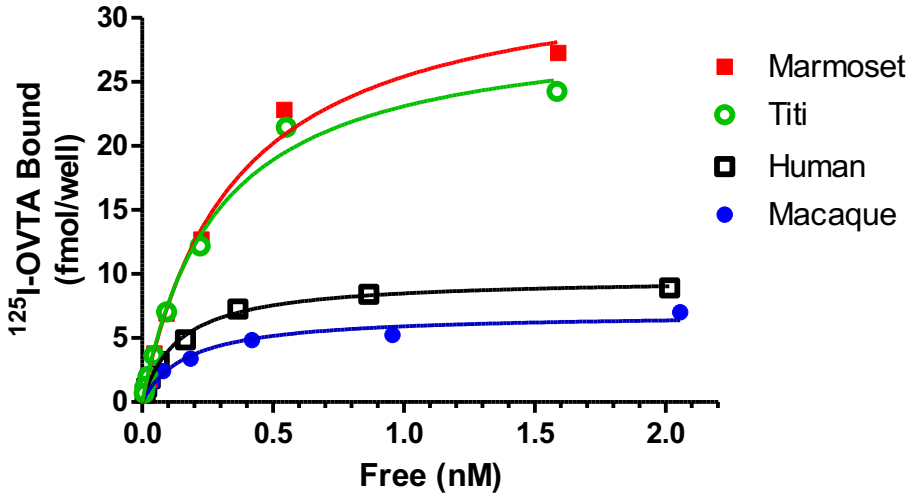

Figure 1.

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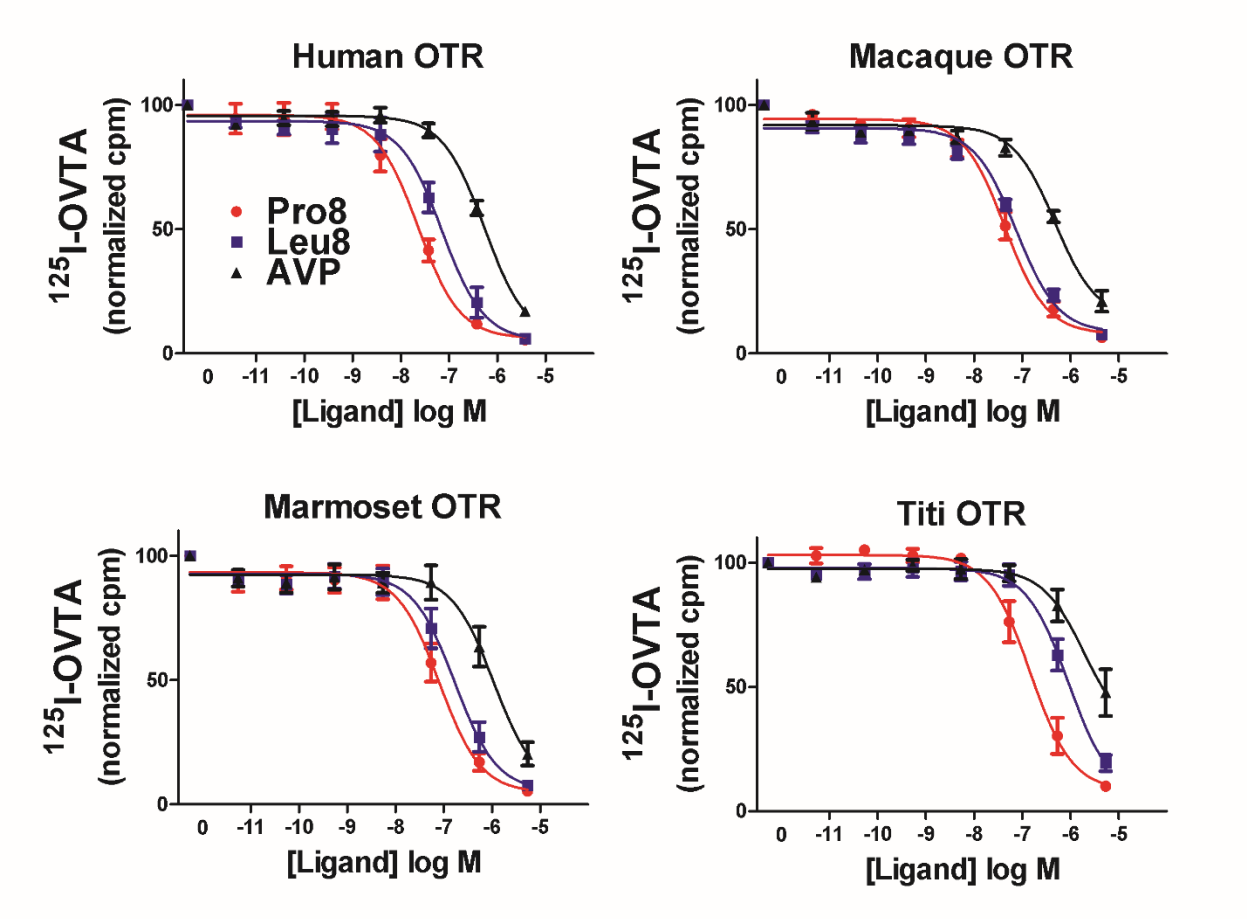

Figure 2.

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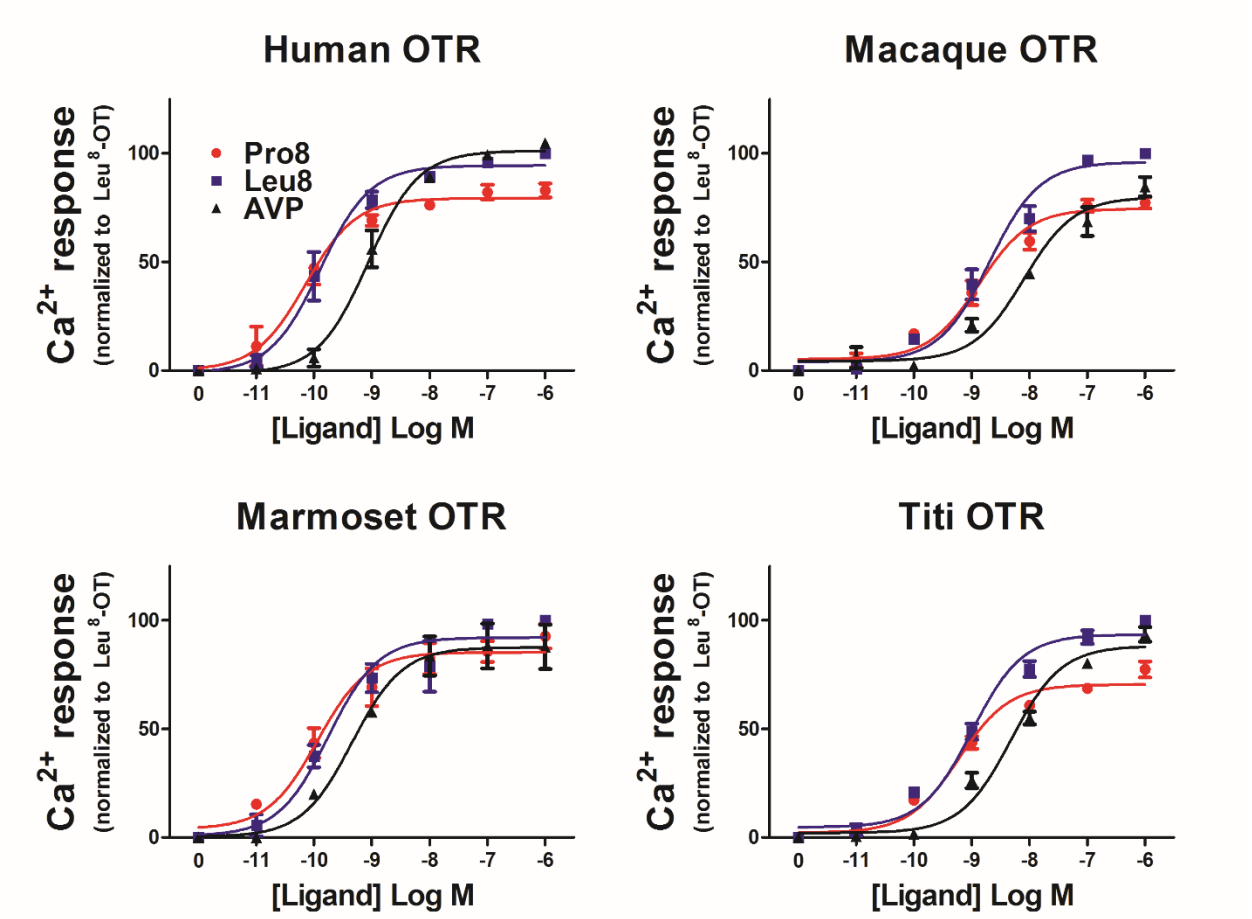

Figure 3.