Leu$^8$ and Pro$^8$ oxytocin agonism differs across human, macaque, and marmoset vasopressin 1a receptors

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Oxytocin (OXT) is an important neuromodulator of social behaviors via activation of both oxytocin receptors (OXTR) and vasopressin (AVP) 1a receptors (AVPR1a). Marmosets are neotropical primates with a modified OXT ligand (Pro$^8$-OXT), and this ligand shows significant coevolution with traits including social monogamy and litter size. Pro$^8$-OXT produces more potent and efficacious responses at primate OXTR and stronger behavioral effects than the consensus mammalian OXT ligand (Leu$^8$-OXT).

Here, we tested whether OXT/AVP ligands show differential levels of crosstalk at primate AVPR1a. We measured binding affinities and Ca$^{2+}$ signaling responses of AVP, Pro$^8$-OXT and Leu$^8$-OXT at human, macaque, and marmoset AVPR1a. We found that AVP binds with higher affinity than OXT across AVPR1a, and marmoset AVPR1a show a 10-fold lower OXT binding affinity compared to human and macaque AVPR1a. Both Leu$^8$-OXT and Pro$^8$-OXT produce a less efficacious response than AVP at human AVPR1a and higher efficacious response than AVP at marmoset AVPR1a. These data suggest that OXT might partially antagonize endogenous human AVPR1a signaling and enhance marmoset AVPR1a signaling. These findings aid in further understanding inconsistencies observed following systemic intranasal administration of OXT and provide important insights into taxon-specific differences in nonapeptide ligand/receptor coevolution and behavior.
Leu8 important functional consequences presumably through OXT activation of OXTR. Sequential for receptor functioning. One tantalizing explanation for this coevolution is that a change in the OXT ligands can modulate social behavior, but Pro8-OXT often produces stronger effects11,14. Specifically, in marmosets (Callithrix spp.), a mostly socially monogamous and biparental primate that endogenously express Pro8-OXT, Pro8-OXT is more effective at modulating mate and stranger-directed behaviors than the ancestral Leu8-OXT ligand. Interestingly, even in rats, a species where males are not parental and parental behavior is OXT-dependent, Pro8-OXT was more effective at inducing parental effort from males than Leu8-OXT23. Pharmacological evidence also supports the notion that Pro8-OXT is more efficacious and potent than Leu8-OXT at primate AVTR1. Pro8-OXT binds with higher affinity at primate AVTR1 (in both Pro8-OXT and Leu8-OXT expressing primates) and produces greater calcium signaling responses compared with Leu8-OXT24,25, but others have reported minimal signaling differences23. These data strongly suggest these evolutionary changes in the OXT ligand lead to important functional consequences presumably through OXT activation of OXTR.

OXT signaling primarily occurs through the activation of OXTR, particularly via Gq-mediated Ca2+ mobilization and downstream signaling23,26. OXT also alters physiological and behavioral function by activating receptors of the closely related 'sister' nonapeptide arginine vasopressin (AVP)27,28. OXT activation of vasopressin 1a receptors (AVPR1a) can produce full or partial Gq-mediated Ca2+ signaling responses and can alter receptor desensitization and internalization29. OXT activation of AVPR1a leads to diverse behavioral outcomes30, and the AVP system has been strongly implicated as an important neural regulator in neurodevelopmental disorders including Autism Spectrum Disorders (ASD)31,32. Given the broad biological functions of OXT and AVP receptor signaling, and the fact that these OXT and AVP nonapeptides only differ at AA positions 3 and 8, AA substitutions in OXT-like ligands may differentially impact the degree of OXT ligand agonism across primate AVPR1a as they do at OXTR.

Because there is considerable crosstalk between OXT and AVP and their canonical receptors, we examined whether binding affinities, signaling potencies, and signaling efficacies of OXT isoforms varied across primate AVPR1a, with each OXT isoform showing differential levels of agonism at species-specific AVPR1a compared to 'maximal' AVP-induced responses. If these evolutionary changes in the OXT ligand structure correspond to functional alterations in the pharmacological properties of both OXTR and AVPR1a signaling across primates, these findings, in turn, may provide important insights into the taxon-specific and receptor-specific patterns underlying the OXT- and/or AVP-dependent modulation of social behavior in primates.

### Results

**125I-OVTA competition binding with OXT and AVP at primate AVPR1a.** Saturation binding assays were performed to explore species-level differences in binding affinities for the antagonist radioligand 125I-OVTA to AVPR1a (SI Fig. 1). Bmax values (i.e., maximal binding) for human, macaque, and marmoset ranged from ~6.21–12.7 fmol/well with marmosets AVPR1a CHO clone showing the highest Bmax values (Table 1). Overall, human, marmoset, and macaque AVPR1a had relatively similar Kd values, ranging from 331–1165 pM (Table 1). The affinities for AVPR1a were also similar to those for 125I-OVTA at human, macaque, and marmoset OXTR (161–481 pM) published previously (Taylor et al., 2018). These findings confirm that there are only relatively small differences in binding affinities for 125I-OVTA among AVPR1a from these three primate species.

The competition binding assays with 125I-OVTA, AVP exhibited higher binding affinity to all primate AVPR1a than did either the Leu8-OXT or Pro8-OXT ligands, and Leu8-OXT and Pro8-OXT did not significantly differ from each other in affinity (Kd value) at any of the primate AVPR1a (Fig. 1). At human AVPR1a there was a significant difference in competitive binding affinity among ligands [F(2,9) = 45.17, p < 0.001], AVP displayed a significantly higher affinity (0.6 nM) than both Leu8-OXT (15.8 nM, p < 0.001) and Pro8-OXT (8.7 nM, p < 0.001), but Leu8-OXT and Pro8-OXT did not differ from each other (p = 0.26). At macaque AVPR1a there was a significant difference in binding affinity among ligands [F(2,6) = 52.99, p < 0.001]. AVP displayed significantly higher affinity (1.2 nM) than both Leu8-OXT (30.0 nM, p < 0.001) and Pro8-OXT (23.8 nM, p < 0.001), but Leu8-OXT

| Species   | Kd (nM) | Bmax (fmol/well) | Leu8-OXT | Pro8-OXT | Rank Order Potency |
|-----------|---------|-----------------|-----------|-----------|--------------------|
| Human     | 0.63 ± 0.24 | 15.78 ± 0.33 | 8.71 ± 0.37 | 6.21 | 1165 (AVP > Leu8 = Pro8) |
| Macaque   | 1.21 ± 0.10 | 30.02 ± 0.16 | 23.77 ± 0.17 | 8.50 | 331 (AVP > Leu8 = Pro8) |
| Marmoset  | 0.86 ± 0.02 | 247 ± 0.06 | 175.9 ± 0.05 | 12.71 | 944 (AVP > Leu8 = Pro8) |

Table 1. Binding affinities for ligands at primate AVPR1a. IC50 presented as nM ± Std. Error. Efficacy is presented as % maximum AVP response ± Std. Error. Human (n = 3); Macaque (n = 3); Marmoset (n = 5). Leu8 = Leu8-OXT; Pro8 = Pro8-OXT.
and Pro8-OXT did not differ from each other \( (p = 0.77) \). Finally, at marmoset AVPR1a there was also a significant difference in binding affinity among ligands \( F(2,6) = 119.1, p < 0.001 \). AVP displayed a significantly higher affinity (0.9 nM) than both Leu8-OXT (247 nM, \( p < 0.001 \)) and Pro8-OXT (176 nM, \( p < 0.001 \)), but Leu8-OXT and Pro8-OXT did not differ from each other \( (p = 0.69) \). Interestingly, though AVP affinities were similar for all primate AVPR1a, both Leu8-OXT and Pro8-OXT had affinities ~10 fold lower at marmoset AVPR1a compared to macaque and human AVPR1a (Table 1).

**Figure 1.** Competition Binding of \( ^{125}I \)-OVTA with OXT and AVP at Primate AVPR1a. Binding competition curves for AVP, Pro8-OXT, and Leu8-OXT for each of the primate AVPR1a. Increasing concentrations of competitor ligand (OXT, AVP) were added to fixed concentration of \( ^{125}I \)-OVTA in intact CHO cells expressing one of the primate AVPR1a. All values are expressed as percentage of maximal (control) binding in the absence of OXT or AVP.

**OXT and AVP stimulation of Ca\(^{2+}\) signaling at primate AVPR1a.** Both Leu8-OXT and Pro8-OXT are able to activate Ca\(^{2+}\) mobilization in AVPR1a, though in all species the rank order of potencies (Ca\(^{2+}\) EC\(_{50}\) values) for OT were significantly lower than for AVP at AVPR1a, and Leu8-OXT and Pro8-OXT were equipotent at all three primate AVPR1a (Fig. 2 and Table 2). At human AVPR1a there was a significant difference in Ca\(^{2+}\) response potency among ligands \( F(2,15) = 49.99, p < 0.001 \). AVP was significantly more potent (1.6 nM) at producing a Ca\(^{2+}\) response than both Leu8-OXT (69.3 nM, \( p < 0.001 \)) and Pro8-OXT (36.2 nM, \( p < 0.001 \)), but Leu8-OXT and Pro8-OXT did not differ from each other \( (p = 0.72) \). At macaque AVPR1a, there was a significant difference in Ca\(^{2+}\) response potency among ligands \( F(2,6) = 140.0, p < 0.001 \). AVP was significantly more potent (0.3 nM) at producing a Ca\(^{2+}\) response than both Leu8-OXT (31.4 nM, \( p < 0.001 \)) and Pro8-OXT (17.0 nM, \( p < 0.001 \), with no difference in potency between Leu8-OXT and Pro8-OXT \( (p = 0.20) \). The same pattern was also observed at marmoset AVPR1a (AVP = 0.1 nM, Leu8-OXT = 3.8 nM, Pro8-OXT = 3.14 nM) \( F(2,6) = 61.91, p < 0.001 \).
Though there were no differences in OXT potency (dose needed to produce 50% maximal response, EC50) at AVPR1a, there were significant species and ligand differences in the efficacy (maximal magnitude response) of OXT agonism at human and marmoset AVPR1a. At human AVPR1a, OXT functioned as a partial agonist compared to the canonical ligand AVP ($F_{(2,15)} = 18.99$, $p < 0.001$). Both Leu$^8$-OXT and Pro$^8$-OXT produced an approximately 45% maximal Ca$^{2+}$ response compared to AVP ($p < 0.001$). Conversely, at marmoset AVPR1a, OXT functioned as a superagonist, generating a larger maximal response compared to the canonical ligand AVP.

**Figure 2.** OXT and AVP Stimulation of Ca$^{2+}$ Signaling at Primate AVPR1a. Intracellular Ca$^{2+}$ responses in CHO cells expressing each of the primate AVPR1a in response to stimulation with varying concentrations of AVP, Pro$^8$-OXT, or Leu$^8$-OXT. All values are expressed as the percentage of the maximal AVP ($10^{-5}$ M) Ca$^{2+}$ response for each primate species AVPR1a.
Specifically, the Leu 8-OXT maximal response was 118% of that for AVP (p < 0.001), and the Pro8-OXT maximal response was 137% of that for AVP (p < 0.001). Compared to Leu8-OXT, Pro8-OXT produced a greater maximal Ca2+ response at AVPR1a than Leu8-OXT (p < 0.05). For macaque AVPR1a, AVP, Leu8-OXT, and Pro8-OXT all produced equal maximal Ca2+ responses [F(2,6) = 0.23, p = 0.80].

OXT partial antagonism of AVP Ca2+ mobilization at human and marmoset AVPR1a. Partial agonists should also exhibit partial antagonism. To confirm this, Leu8-OXT and Pro8-OXT were coadministered with 10 nM AVP (a concentration of AVP that alone produces approximately 80% of the maximal Ca2+ response) for both human and marmoset AVPR1a. For human AVPR1a, both Leu8-OXT and Pro8-OXT caused a concentration-dependent decrease in AVP-stimulated Ca2+ mobilization. Both Leu8-OXT and Pro8-OXT at concentrations < 1 μM with and without coadministration of 10 nM AVP produced only 50% of the maximal Ca2+ mobilization response to AVP, confirming that both OXT ligands act as both partial agonists and partial antagonists at human AVPR1a (Fig. 3). For marmoset AVPR1a, OXT did not function as a partial antagonist of AVP (p < 0.05). For macaque AVPR1a, AVP, Leu8-OXT, and Pro8-OXT all produced equal maximal Ca2+ responses [F(2,6) = 0.23, p = 0.80].

Interestingly, in both human and marmoset AVPR1a, Pro8-OXT coadministered with AVP always produced a greater maximal response than Leu8-OXT when coadministered with AVP. This occurred even at concentrations of OXT that did not produce Ca2+ responses on their own. OXT partial antagonism experiments were not performed in macaque AVPR1a because OXT did not partially agonize or superagonize Ca2+ responses at macaque AVPR1a.

Coupling efficiency at primate AVPR1a. We measured a simplified form of coupling efficiency as a ratio of the concentration of ligand needed to mobilize Ca2+ responses in primate AVPR1a (potency/EC50) relative to the ligand binding affinity (Kd) at AVPR1a (Table 3). This metric provides insight into whether OXT/AVP ligands produce equal signaling responses across different receptors in the presence or absence of unbound/spare receptors, i.e., a extra receptors than what is required to produce a maximal response. Only the marmoset AVPR1a showed high coupling efficiency, with EC50 values that were about 1.8 log units higher than their Kd values for

| AVPR1a | Ca2+ EC50 | Ca2+ Response Efficacy (% Max AVP) | Rank Order Potency | Rank Order Efficacy |
|--------|-----------|-----------------------------------|--------------------|--------------------|
| Human  | 1.64 ± 0.05 | AVP, Leu8-OXT, Pro6-OXT, Leu14-OXT, Pro6-OXT | AVP > Leu8 = Pro6 | AVP > Leu8 = Pro6 |
| Macaque| 0.31 ± 0.09 | AVP, Leu8-OXT, Pro6-OXT, Leu14-OXT, Pro6-OXT | AVP = Leu8 = Pro6 | AVP = Leu8 = Pro6 |
| Marmoset| 0.05 ± 0.05 | AVP, Leu8-OXT, Pro6-OXT, Leu14-OXT, Pro6-OXT | AVP < Leu8 < Pro6 | AVP < Leu8 < Pro6 |

Table 2. Ca2+ mobilization potencies and efficacies for OXT/AVP at primate AVPR1a. EC50 presented as nM ± Std. Error. Efficacy is presented as % maximum AVP response ± Std. Error. Human (n = 6); Macaque (n = 3); Marmoset (n = 3).

Figure 3. OXT Partial Antagonism of AVP Ca2+ Mobilization at Human and Marmoset AVPR1a. Intracellular Ca2+ responses in CHO cells expressing each of the primate AVPR1a in response to stimulation with varying concentrations of Pro6-OXT or Leu8-OXT in the presence or absence of 10−8 M AVP (10 nM). All values are expressed as the relative percentage of the AVP (10−8 M) Ca2+ response for each primate species AVPR1a.
both Leu⁸-OXT and Pro⁸-OXT, respectively. Conversely, human AVPR1α showed negative coupling efficiency values, suggesting that human AVPR1α require relatively more AVPR1α to produce Ca²⁺ responses from OXT ligands compared to marmoset AVPR1α. Macaque AVPR1α coupling efficiencies for AVP, Leu⁸-OXT, and Pro⁸-OXT showed minimal difference (less than one log unit) compared to those for human and marmoset AVPR1α.

### Discussion

This study is the first to evaluate potential differences in Leu⁸-OXT and Pro⁸-OXT binding affinity, Ca²⁺ signaling potency and efficacy, OXT partial antagonism, and receptor coupling efficiency across a variety of primate AVPR1α. Previous studies have evaluated whether the documented coevolutionary changes in OXT ligands and OXTR in Platyrhini primates would produce demonstrable and unique properties for OXT-OXTR signaling in these species²⁵⁻²⁹. These studies found that changes to the OXT molecule, namely the Leu⁸ to Pro⁸ AA substitution in OXT, produced only modest changes in binding and signaling across primate OXTR. OXT exhibits a significant degree of ‘cross-talk’ with AVP receptors (primarily AVPR1α), and OXT and AVP exhibit potential overlap in behavioral outcomes via OXT and AVP signaling in the brain. It is therefore plausible that OXT modifications would lead to a functional selective advantage through differences in OXT interactions with AVPR1α. The data from this study support three key conclusions: (1) AVP binds with significantly higher affinity than OXT at human, marmoset, and macaque AVPR1α, and marmoset receptor AVPR1α show a 10-fold lower OXT binding affinity compared to human and marmoset AVPR1α. (2) There are no significant differences in binding affinity or Ca²⁺ signaling potency between Leu⁸-OXT and Pro⁸-OXT at primate AVPR1α. (3) Both OXT isoforms exhibit differential levels of agonism/antagonism across primate AVPR1α, acting as partial agonists and partial antagonists at human AVPR1α and as superagonists at marmoset AVPR1α.

The idea that differences in the OXT ligand structure would result in functional differences in primate AVPR1α binding and/or signaling properties was only partially supported. While Leu⁸-OXT and Pro⁸-OXT showed no differences in binding affinity or Ca²⁺ mobilization potencies at any of the primate AVPR1α, there was a significant difference in levels of OXT agonism both across primate AVPR1α and between AVT variants. Pro⁸-OXT produced a significantly higher maximal response compared to Leu⁸-OXT at marmoset AVPR1α. These pharmacological findings also partially align with previous work examining effects of Leu⁸-OXT and Pro⁸-OXT signaling at OXTR. Pro⁸-OXT exhibited modestly higher potencies than Leu⁸-OXT at primate OTRs²⁵, Pro⁸-OXT produced more efficacious Ca²⁺ responses at marmoset OXTR but not at human OXTR²⁴, and Pro⁸-OXT produced lower recruitment of β-arrestin and less receptor desensitization and internalization at both human OXTR and AVPR1α, where only human receptors were tested²⁹. Perhaps the most compelling finding from this study was that OXT exhibits differential agonism at human and marmoset AVPR1α. Similar to OXT signaling at OXTR, Pro⁸-OXT was more efficacious than Leu⁸-OXT at marmoset but not human AVPR1α. Previous pharmacological studies of marmoset OXTR did not explicitly test if different OXT ligands were partial agonists at marmoset and human OXTR²⁴, and that study did not make direct comparisons of OXT agonism to AVP agonism at OXTR. However, AVP appears to be a full agonist relative to both Leu⁸-OXT and Pro⁸-OXT at primate OXTR based on data reported across human, marmoset, macaque, and titi monkey OXTR²⁵.

The observation that Leu⁸-OXT and Pro⁸-OXT act as a partial agonists/antagonists at human AVPR1α is a novel finding. The first reported study evaluating the pharmacological profile of Pro⁸-OXT at human AVPR1α showed that Pro⁸-OXT is a full agonist at producing Ca²⁺ responses compared to AVP, while Pro⁸-OXT was only a partial agonist for β-arrestin recruitment at both human AVPR1α or OXTR²⁹. It is unclear what underlies the difference in Ca²⁺ responses from the human AVPR1α tested in this study and the human AVPR1α tested previously²⁹. Based on the clear partial agonism at human AVPR1α, we tested whether, as expected, OXT also functioned as a partial antagonist of the AVP Ca²⁺ response at both human and marmoset AVPR1α. Adding either OXT isoform along with AVP reduced the AVP Ca²⁺ response, but only for human AVPR1α. This confirms that OXT is a partial antagonist at human AVPR1α but not at marmoset AVPR1α. We further corroborated this finding by testing a marmoset AVPR1α clone with lower receptor expression (as indicated by saturation binding with ¹²⁵I-OVTA), and again both OXT ligands functioned as full agonists with slightly lower potency, with the Pro⁸-OXT response greater than for Leu⁸-OXT, eliminating concerns that species-differences in OXT agonism would lead to a functional selective advantage through differences in OXT interactions with AVPR1α. The data from this study support three key conclusions: (1) AVP binds with significantly higher affinity than OXT at human, marmoset, and macaque AVPR1α, and marmoset receptor AVPR1α show a 10-fold lower OXT binding affinity compared to human and macaque AVPR1α. (2) There are no significant differences in binding affinity or Ca²⁺ signaling potency between Leu⁸-OXT and Pro⁸-OXT at primate AVPR1α. (3) Both OXT isoforms exhibit differential levels of agonism/antagonism across primate AVPR1α, acting as partial agonists and partial antagonists at human AVPR1α and as superagonists at marmoset AVPR1α.

Table 3. Coupling efficiencies for ligands at primate AVPR1α. Calculated as a Potency/Affinity Ratio \([-\log(Ca^{2+}\text{EC50}/K_i)]\).

|        | AVP      | Leu⁸-OXT | Pro⁸-OXT |
|--------|----------|----------|----------|
| Human  | −0.42    | −0.64    | −0.62    |
| Macaque| 0.59     | −0.02    | 0.15     |
| Marmoset| 1.28     | 1.81     | 1.75     |

The conclusion that OXT functions as a partial agonist and a partial antagonist for AVP activation of Ca²⁺ signaling responses in human AVPR1α has important implications. Though evidence for endogenously released OXT producing functionally important responses at AVP receptors is limited, some studies have shown that stimulating endogenous OXT release can induce social behavioral responses in rodents via AVPR1α. However, a
majority of studies that examine the effects of OXT on behavior use exogenous intranasal OXT administration, causing systemic distribution and leading to supraphysiological increases in circulating OXT throughout the periphery. OXT is known to exert dose-dependent behavioral effects\(^{35,36}\); thus further studies are warranted to evaluate whether high doses of OXT, in addition to activating OXTR, might also partially antagonize endogenous human AVPR1a signaling, which could aid in further understanding of the inconsistencies observed in behavioral responses following systemic administration of OXT\(^{37}\) and the reported ‘inverted U-shaped’ relationship between OXT dose and behavior\(^{38,39}\).

Moreover, differential OXT agonism at AVPR1a could have important implications for understanding the therapeutic potential of nonapeptide treatments in alleviating symptoms associated with neurodevelopmental disorders such as autism spectrum disorders (ASD). For instance, animal models of nonapeptide signaling may not generalize in a simple way to human clinical trials. The impact of intranasal OXT on behaviorally relevant clinical outcomes has shown mixed support in the literature\(^{40,41}\), but recent evidence has shown that peripheral use of both a highly selective AVPR1a antagonist and intranasal AVP administration has markedly improved behavioral outcomes for individuals with ASD\(^{43,44}\). These findings are important given that similar OXT treatment strategies for ASD have shown mixed efficacy\(^{42,43}\), and high doses of OXT could even mitigate potential therapeutic benefits of AVPR1a activation in ASD and surely other behavioral contexts as well. Whether the partial agonism/antagonism at human AVPR1a explains these anomalies merits further study.

It is also noteworthy that differences in OXTR and AVPR1a function are important for our broader understanding of the coevolution of nonapeptide signaling system in Platyrhini primates. While OT acted as a partial agonist at human AVPR1a, OXT (both Leu\(^8\)-OXT and Pro\(^8\)-OXT) instead acted as a superagonist at marmoset AVPR1a. This is especially important from an evolutionary context because the Callitrichid clade has evolved widespread Pro\(^8\)-OXT expression of the OXT ligand, and the Pro\(^8\) ligand produces stronger behavioral effects\(^{44,45}\), potency and efficacy effects at marmoset OXTR\(^{24,25}\), and efficacy effects at marmoset AVPR1a (this study). It is unclear whether the higher agonism and coupling efficiency of OXT at marmoset AVPR1a is an important or conserved mechanism underlying the potential coevolution between OXTR and AVPR1a variability with socially monogamous phenotypes in primates\(^{15,46}\). More pharmacological and behavioral work utilizing Pro\(^8\)-OXT and other OXT ligands is needed across a broader sampling of primates. Such examples include Leu\(^8\)-OXT expressing titi monkeys that are viewed as socially monogamous and biparental and Pro\(^8\)-OXT expressing primates such as capuchins or squirrel monkeys that are highly social but non-monogamous/biparental. These data combined with the important data published on OXTR and AVPR1a central expression profiles in marmoset, titi, macaque, and humans would serve as a powerful tool to begin utilizing and targeting diverse non-human primate models of nonapeptide regulation of social behavioral phenotypes.

Clearly, there are many contributing factors to the ways in which OXT and AVP regulate physiological and behavioral outcomes across species, the relative roles of OXTR and AVPR1a activation, and how well these and other in vitro findings translate directly to neural transmission and ultimately behavioral modulation. These relationships are difficult to ascertain, especially in light of currently limited access to primate neural tissue and primate gene-editing techniques. An important first step is to evaluate whether the pharmacological and physiological findings and principles already established for nonapeptide biology in rodents are divergent or conserved across diverse nonhuman primate species. Our findings will serve as a roadmap to target specific pharmacological and physiological properties that may underlie species- or individual-level differences in behavioral and social phenotypes. Behavioral studies have been at the forefront of this effort and have elucidated many key findings about how OXT regulates social behavior in nonhuman primates\(^{14,47-49}\), but many of these studies have yet to identify specific neural mechanisms underlying these behavioral effects. Overall, the findings from this study provide important molecular insights into species-level differences in nonapeptide ligand/receptor coevolution and ‘cross-talk’ between OXT and AVP.

**Methods**

**Primate AVPR1a transfection and cell culture.** Chinese hamster ovary (CHO; Female origin) cells were purchased from American Type Culture Collection (Manassas, VA) 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 AVPR1a plasmids were purchased from Genscript (Piscataway, NJ) in a pcDNA3.1+ vector based on confirmed genetic sequences. CHO cells were transfected using Turbofect according to the manufacturer’s instructions and were kept under selective pressure using 400 μg/mL G418 antibiotic. Individual clonal lines were generated from batch-transfected cells by plating approximately 10 cells/mL (1 cell/100 μL) into 96-well plates. Clonal lines that originated from a single colony 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 specific radioligand binding. CHO cells showed no endogenous OXTR and AVPR1a binding or signaling activity in response to OXT and/or AVP ligands (SI Fig. 3).

**Intact cell saturation binding assays.** CHO cells expressing primate AVPR1a were plated at 150,000 cells/mL (15,000 cells per well/100 μL) into 96-well plates and incubated at 37 °C for 48 hours to achieve 80–90% confluence. The day of assay, growth medium was aspirated and cells were quickly washed once with 190 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. 50 μL of ice-cold HGH-BSA containing \(^{125}\)I-OVTA (PerkinElmer) in doubling concentrations (~15 to 2000 pM) was added in triplicate (technical replicates) and incubated for 3 hours on ice. 3 hours is the minimum incubation time on ice for \(^{125}\)I-OVTA and \(^{125}\)I-OVTA to reach equilibrium (SI Fig. 4) in CHO cells transfected with human and marmoset AVPR1a. Cells were washed four times with 100 μL ice-cold HGH-BSA, solubilized with 100 μL 0.2 N NaOH, and radioactivity quantified with a gamma counter. We also counted aliquots of the used binding medium (i.e., free \(^{125}\)I-OVTA) to quantify free radioligand
with 100 CPM 125I-OVTA were added in triplicate (technical replicates) to all wells in the presence or absence of 10 M Pro8-OXT (CYIQNCPPG-NH2; Anaspec), Leu8-OXT (CYIQNCPLG-NH2; Anaspec) or AVP (CYFQNCPGR-NH2; Anaspec), and incubated for three hours on ice. Cells were washed four times with 100 µL ice-cold HGH-BSA, solubilized with 100 μL 0.2 N NaOH, and radioactivity was quantified with a gamma counter.

Half-maximal inhibitory concentrations (IC50) were determined by plotting bound 125I-OVTA vs. competitor concentration. IC50 values were then corrected using the Cheng-Prusoff equation with each receptor’s Kd for 125I-OVTA to produce Kc values for the competing ligands. Assays were done at least three times on three different days using fresh aliquots of 125I-OVTA and Leu8-OXT, Pro8-OXT, and AVP with at least three biological replicates per clone.

Ca2+ mobilization assays. CHO cells expressing primate AVPR1a were plated at 150,000 cells/mL (15,000 cells per well/100 µL) into 96-well plates and incubated at 37 °C for 48 hours/grown to 80–90% confluence. On the day of assay, growth medium was aspirated and cells were incubated at 37 °C with 100 µL Fluoro-4 Direct dye mixed in Fluoro-4 Direct Ca2+ Assay Buffer with 5 mM probenecid for ~45 minutes. Using a FlexStation 2 (Molecular Devices), baseline fluorescence was measured at 37 °C followed by stimulated fluorescence in the presence or absence of 10−12 to 10−6 M Pro8-OXT, Leu8-OXT, or AVP (3 × technical replicates). Peak fluorescence minus baseline fluorescence was plotted as a function of ligand concentration to determine EC50 values. Assays were done at least three times on three different days using fresh aliquots of Leu8-OXT, Pro8-OXT, and AVP for three biological replicates per clone. We determined the degree of OXT Ca2+ agonism/antagonism at AVPR1a by repeating the same procedures for Leu8-OXT alone, Pro8-OXT alone, and OXT ligands coadministered with 10−6 M AVP (10 nM) (3 × technical and biological replicates) with concentrations of OXT from 10−10 to 10−6 M compared to coadministration of OXT concentrations from 10−10 to 10−4 M together with 10−8 AVP.

Data analyses. Binding affinities for 125I-OVTA at each primate AVPR1a were calculated by subtracting non-specific binding and then plotting bound 125I-OVTA vs. free 125I-OVTA. Because concentrations of 125I-OVTA were not identical from experiment to experiment, technical replicates within each experiment (n = 3) were normalized and then corrected using the Cheng-Prusoff equation. Technical replicates were averaged and used as biological replicates (n = 3 per clone) to determine and compare Kc values for each ligand within species. Differences in Ca2+ mobilization potency (EC50) and maximal response to OXT were determined by normalizing OXT-induced (Log M) Ca2+ responses as a percentage of maximal (100%) AVP-induced Ca2+ response. We averaged across technical replicates (n = 3) within each biological replicate and then averaged across the biological replicates (n = 3), normalized the data, and tested for significant differences of best-fit LogEC50 using one-way ANOVA analyses. Post hoc analyses to assess ligand comparisons (Pro8-OXT vs. Leu8-OXT, Pro8-OXT vs. AVP, Leu8-OXT vs. AVP) were performed using Tukey’s posthoc test with a Bonferroni-corrected cutoff to determine statistically significant differences in best-fit LogEC50. All best-fit data (Kc, EC50, and Ca2+ maximal responses) were analyzed using the nonlinear least squares curve-fitting capabilities of GraphPad Prism.

Data availability
Raw data and clonal cell lines (CHO clonal cell lines expressing either human, macaque, or marmoset AVPR1a) are available upon request.

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Designed the project: A.M., J.T., M.T., N.S. and J.F. Constructed the cell lines: N.S., J.T. and A.M. Performed the experiments: A.M., N.S. and J.T. Analyzed the data: A.M., N.S. and J.T. Drafted the initial version of the manuscript: A.M. Revisited the manuscript: A.M. and M.T. Reviewed the final version(s) of the manuscript: A.M., N.S., J.T., M.T. and J.F. Secured funding: J.F., J.T. and A.M.

Competing interests
The authors declare no competing interests.

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