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Oxytocin Structure and Function in New World Monkeys: From Pharmacology to Behavior

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Abstract

Oxytocin (OT) is a hypothalamic nonapeptide that mediates a host of physiological and behavioral processes including reproductive physiology and social attachments. While the OT sequence structure is highly conserved among mammals, New World monkeys (NWMs) represent an unusual ‘hot spot’ in OT structure variability among mammals. At least six distinct OT ligand variants among NWMs exist, yet it is currently unclear whether these evolved structural changes result in meaningful functional consequences. NWMs offer a new area to explore how these modifications to OT and its canonical G-protein coupled OT receptor (OTR) may mediate specific cellular, physiological, and behavioral outcomes. In this review, we highlight relationships between OT ligand and OTR structural variability specifically examining coevolution between OT ligands, OTRs, and physiological and behavioral phenotypes across NWMs. We consider whether these evolved modifications to the OT structure alter pharmacological profiles at human and marmoset OTRs including changes to receptor binding, intracellular signaling, and receptor internalization. Finally, we evaluate whether exogenous manipulation using OT variants in marmoset monkeys differentially enhance or impair behavioral processes involved in social relationships between pairmates, opposite-sex strangers, and parents and their offspring. Overall, it appears that changes to OT ligands in NWMs result in important changes ranging from cellular signaling to broad measures of social behavior.

Keywords

oxytocin; oxytocin receptor; marmoset; social behavior; receptor pharmacology; cell signaling

Introduction

Oxytocin (OT) can fairly be characterized as an essential element or the *sine qua non* of eutherian, placental mammals. This nine amino acid peptide hormone, synthesized by neurons in the supraoptic (SON) and paraventricular (PVN) nuclei of the neurohypophyseal tract, regulates the two physiological characteristics that are ubiquitous for female placental mammals. First, peripherally-secreted OT acts upon cells within the myometrium and endometrium of the uterus to stimulate uterine smooth muscle contractions associated with parturition and live birth (Arrowsmith & Wray 2014). Second, OT acting on the

myoepithelial cells in the mammary gland stimulates milk ejection during lactation (Crowley 2015). Clearly, successful offspring birth and postnatal growth are critically dependent on these two OT-mediated facets of female reproductive physiology.

In addition to these important peripheral functions, OT release from oxytocinergic neurons, particularly from the PVN, also project to critical mesolimbic and forebrain regions, both through direct synaptic connections, and via volume transmission from neuronal dendrites and soma (Ludwig & Leng 2006). These projections are known to mediate a host of social processes. The canonical social relationship mediated by OT is the bond between mothers and their offspring (Rilling & Young 2014). These social bonds ensure continued spatial proximity of mother to offspring and offspring receipt of lactational sustenance, both of which are necessary for growth and survival. It is no surprise, then, that the same neural architecture that underlies maternal-offspring attachments also mediate other social processes. This neural architecture appears to be critical for close affiliative social relationships, and OT is an important element in the complex phenomenon associated with the rewarding properties of affiliative social interactions (Lieberwirth & Wang 2014; Numan & Young 2016). These social relationship traits include pair bonding among reproductive adults, “friendships” among nonreproductive partners and social group members, and more multifaceted sociocognitive traits such as trust, emotional intelligence, social memory, in-group social preferences, and altruism (Feldman 2017).

The hypothalamus also contains neurons that synthesize an additional nonapeptide, arginine vasopressin (AVP). AVP has considerable structural similarity to mammalian OT, differing only at positions 3 and 8 in the mature peptides. AVP exerts significant effects on peripheral function (particularly osmoregulation and vascular tone), but, like OT, AVP projections in the CNS are involved in the regulation of multiple behavioral systems including social attachments among adults, social communication and cognition, and aggression (Caldwell & Young 2006; Caldwell *et al.* 2008; Albers 2012; Goodson 2013; Kelly & Goodson 2014). In this review we focus primarily on OT, but given the structural similarity of AVP and its important role in the modulation of social behavior, we also explore details of AVP signaling at OT receptors (OTRs).

Structural Changes in Nonapeptides

A necessary condition for both the peripheral and central effects of OT and AVP is the close coupling of the ligand with the cognate receptor(s). Nonapeptide-receptor binding results in a host of changes in cell properties that underlie the modulation of both physiological and behavioral states. Even subtle changes in ligand structure can alter binding properties with receptors and can differentially activate signaling cascades (Koehbach *et al.* 2013). Successful docking of nonapeptides with the extracellular and/or transmembrane elements of the receptors normally activate G protein-mediated intracellular processes (Busnelli *et al.* 2012; Stoop 2012; Stoop 2014; Grinevich *et al.* 2016), and, from a behavioral perspective, consequently alter neuronal function in cells that express receptors (Busnelli & Chini 2017). As with any ligand-receptor signaling system, a variety of alterations or modifications to the signaling molecule can enhance or inhibit cellular responses following OTR binding (Gimpl & Fahrenholz 2001; Zingg & Laporte 2003; Wyttenbach *et al.* 2008; Busnelli *et al.* 2016).

An additional feature that makes the OT/AVP system intriguing is the ability of both nonapeptides to bind with some affinity with both OT and AVP receptors (Gruber *et al.* 2012; Manning *et al.* 2012). These ‘crosstalk’ interactions between OT and AVP can exert significant physiological changes (Chini *et al.* 1996; Meyer-Lindenberg *et al.* 2011) and alter behavioral outputs (Song *et al.* 2016; Caldwell 2017; Song & Albers 2017).

In the last few years, reports have emerged in the literature of nucleotide changes in the coding regions for the OT gene (OXT) that leads to altered OT peptide structure among some mammals, particularly in New World monkeys (NWMs) (Lee *et al.* 2011; Ren *et al.* 2015; Vargas-Pinilla *et al.* 2015). These findings serve as a springboard to reevaluate the assumption that the OT ligand is conserved across mammals, and to also explore the consequences of this variation for cellular and behavioral outcomes. This review will first highlight the broad phylogenetic and evolutionary lineage of nonapeptides, ultimately focusing on the recent evidence for changes in the OXT gene in NWMs. As of this writing, six structural OT variants (including the ‘consensus’ mammalian sequence: Cys–Tyr–Ile–Gln–Asn–Cys–Pro–**Leu**–Gly–NH₂; Leu⁸-OT) have been identified in NWMs. We then characterize correlated changes in the OTR associated with ligand variation in NWMs. We also evaluate species differences among primates in AVP receptor structure, focusing on the AVP V1a receptor gene (AVPR1a). Second, we review details of nonapeptide ligand-receptor interactions in NWMs, focusing on receptor pharmacology and intracellular signaling pathways. This section highlights the ways in which ligand variation in OT can alter G-protein signaling cascades and points to the multiple ways in which ligand-receptor interactions may ultimately alter neural function serving as potent modulators for behavioral outputs. In our third section, we provide a description of the subtle and context-dependent nature of ligand-specific modulation of social behavior via exogenous manipulation of OT/AVP systems in marmoset monkeys (*Callithrix* spp.).

New World Monkey OT Evolution and Diversity

For those less familiar with primate taxonomy, we first provide a brief introduction to the evolution and phylogeny of the primate lineage, with the most detailed treatment of the NWMs. Diverging from other mammalian forms approximately 90 million years ago (Mya), the Order Primates are currently classified into five major groupings, as derived from extensive genomic phylogenies (Perelman *et al.* 2011). The most ancient primates include Lemuriformes (lemurs and lorises) and Tarsiiformes (tarsiers). The monkey and ape-like primates (Simiiformes) include four major groupings including two groups of hominoid primates: Hylobatidae (gibbons and siamangs) and hominid primates (orangutans, gorillas, chimpanzees, bonobos, and humans). The African and Asian Old World primates (superfamily Cercopithecoidea; e.g., macaques, baboons, guenons) constitute the third grouping of Simiiformes, and the NWMs of South and Central America (parvorder Platyrrhini) fill out the primate order. NWMs are estimated to have diverged from Old World primates and hominoids ~43 Mya (Perelman, Johnson, Roos, *et al.* 2011). Within this taxonomic group there are 17 genera that are classified into three families (see Fig. 1). Atelidae are large-bodied (7 – 9 kg) and Pitheciidae are intermediate sized (1 – 3 kg), and generally have diets of fruits and leaves. Cebidae are small-bodied primates (0.12 – 2.5 kg)

with more variable diets among species, including invertebrate and vertebrate prey (Hawes & Peres 2014).

Nonapeptide evolution

Hypothalamic nonapeptides have a long evolutionary history – these molecules are expressed in a number of invertebrate species, and appear to have emerged approximately 600 million years ago (Gruber *et al.* 2012; Beets *et al.* 2013). During the course of evolution, vasopressin-like molecules appear to be the ‘ancestral’ peptide in vertebrates (vasotocin), and the similarity of peptide structure and the close proximity of the coding regions for OXT and AVP genes on the same chromosome in primitive jawed fishes suggests a gene duplication event of very early evolutionary origin, first emerging in ancestral jawed fishes ca. 500 million years ago (Gruber *et al.* 2012; Beets *et al.* 2013; Koehbach *et al.* 2013). The vertebrate OT family (isotocin-mesotocin-oxytocin) is generally associated with reproductive function, while the AVP family (vasotocin-vasopressin) is presumed to have origins in fluid homeostasis (Acher *et al.* 1995). These two nonapeptide families have significant similarities beyond both possessing nine amino acids, including an N-terminal six-residue ring structure formed by a disulfide bridge between two Cys residues at positions 1 and 6, a flexible C-terminal tail structure with a Pro and Gly at positions 7 and 9, and Asn at position 5.

Given the importance of the peripheral actions of OT for reproduction in placental mammals, it is not surprising that an implicit assumption in the literature has been that the mature OT peptide is highly conserved among the nearly 4,000 species of eutherians (Acher *et al.* 1995; Caldwell *et al.* 2008; Donaldson & Young 2008; Insel 2010; Gruber 2014). Comparative genomics analyses have supported this proposition. Wallis (2012) analyzed variation in the 27 nucleotides that code for the mature OT peptide across placental mammals, and calculated dN/dS ratios (the number of nonsynonymous nucleotide substitutions relative to the number of synonymous substitutions), in which a ratio < 1.0 implies stabilizing selection for protein structure, while a ratio > 1.0 implies positive selection for diversity in nucleotide sequences and hence protein variability (Wallis 2012). The dN/dS ratio for eutherian OXT gene is remarkably low (0.009), reflecting a highly conserved protein structure that is consistent with the centrality of OT for critical reproductive and social processes in mammals. Wallis (2012) reported an even lower dN/dS ratio for nucleotides coding for AVP gene among mammals (0.005), again implying an extreme conservation in the AVP ligand structure.

In light of the evidence for conserved OT ligand structure in mammals, it comes as some surprise that there is, in fact, considerable variation in OT structures among the NWMs. The first data suggesting multiple ligand forms were published in 2011 (Lee *et al.* 2011), and indicated that four species of NWMs sequenced for OXT (*Callithrix*, *Aotus*, *Saimiri*, and *Cebus*) showed an in-frame nonsynonymous substitution (NS) in the OXT codon for the 8th amino acid (CCG→CTG). Wallis (2012) also reported a similar NS substitution in the tree shrew (genus *Tupaia*). This NS leads to an alteration in the amino acid at the 8th position from the consensus mammalian leucine (Leu) to a proline (Pro), yielding a unique nonapeptide variant, Pro⁸-OT. Lee *et al.* (2011) further showed that this substitution was

expressed in mRNA and that neurohypophysial extracts contained Pro⁸-OT, indicating that the altered OXT codon at position 8 in these species is translated into a mature Pro⁸-OT structured peptide.

In 2015, two laboratories independently conducted a broader comparative analysis of NWMs (Ren *et al.* 2015; Vargas-Pinilla *et al.* 2015), both confirming the presence of Pro⁸-OT in the species sampled in Lee *et al.* (2011), and revealing additional variability in OXT in this taxonomic group, ultimately characterizing six OT-like variants (Leu⁸-OT, Pro⁸-OT, Val³-Pro⁸-OT, Phe²-OT, Ala⁸-OT, and Thr⁸-OT). Accession of available sequence data from NCBI for primates revealed that all species outside NWMs possess the consensus mammalian Leu⁸-OT. Fig 1 characterizes the mapping of these OT variants on the consensus molecular phylogeny of NWM, and full details on the amino acid substitutions in NWMs can be found in French *et al.* (2016). All three Families of NWMs have at least two genera that have divergent OT ligands from Leu⁸-OT, and there appears to have been multiple NS nucleotide changes in Pitheciidae and Atelidae, since exemplar genera contain at least three variants of OT. All genera of Cebidae, a Family that separated from the other NWM ~ 23 Mya, have OXT coding regions for Pro⁸-OT, suggesting that the mutation that was associated with the evolutionary divergence of this Family. Within the Cebidae, at least one species in the genera *Saguinus* expresses an additional NS in the codon at position 3 (Valine for Isoleucine; Vargas-Pinilla *et al.* 2015) as well as Proline at position eight. This finding, along with the emergence of relatively recent (7.5 – 9 Mya) variants in the other Families, indicates that mutations in the coding regions of OXT in NWM have continued after the separation of these families, and that some mutations have occurred in relatively recent evolutionary time. There has been a report of a seventh OT variant in NWMs, the howler monkey, *Alouatta seniculus*. Campbell and Cortés-Ortiz (personal communication, Nov 2017) have sequenced a Val³-Leu⁸-OT variant in this taxon. The potential existence of other, additional OT variants awaits a more complete and systematic sampling of species within the NWMs.

The OT variants in NWMs represent both structural and physicochemical divergence from consensus Leu⁸-OT. The Pro⁸ substitution, found in all Cebids and one genera of Atelidae, represents the most dramatic alteration from other variants, since the Pro-Pro sequence in the tail portion of the OT peptide at positions 7 and 8 places constraints on the rotational structure of the peptide, and can significantly alter ligand-cell membrane interaction profiles (Geisler & Chmielewski 2009). In addition, Pro⁸-OT differs in its hydrophilic profile from Leu⁸-OT, with greater hydrophilicity in Pro⁸ (Ren *et al.* 2015; Parreiras-e-Silva *et al.* 2017). For all OT variants in NWMs, each change in amino acid residues represents at least one physicochemical change (polarity, charge, or hydrophobicity), and hence each can be characterized as a ‘radical’ residue substitution (Zhang 2000). Indeed, Stoop (2012) argued that the structural changes associated with the Pro⁸ substitution represents a more dramatic change in the molecular architecture of OT than the corresponding changes from mesotocin to consensus Leu⁸-OT (Isoleucine to Leucine). In the OTR, both the N-terminus, which shows considerable structural variability across NWMs (Ren *et al.* 2015), and the first extracellular loop of the OTR are thought to be important for ligand selectivity and binding (Zingg *et al.* 2003), with the latter thought to be primarily interacting with the ligand C-terminus tail (where the majority of substitutions are found at the 8th position) of the OT

peptide (Chini *et al.* 1996; Wesley *et al.* 2002). Thus, changes in peptide structure of this magnitude have the potential to dramatically alter either receptor binding properties or post-binding intracellular signaling.

In light of the widespread and potentially consequential variation in OT ligands among NWMs, it is notable that NWMs have identical predicted protein structure for AVP. Ren *et al.* (2014) characterized AVP coding sequences for 33 species of primates, representing all four major clades. In all cases, the amino acid sequences for AVP were identical to the majority of eutherian mammals (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH₂; interestingly there are other variants (e.g., Lys⁸) present in a few nonprimate species). Among the available hominoid, prosimian, and Old World monkey species, all had identical nucleotides in the coding region for AVP (Ren *et al.* 2014).

Collectively, the findings on OT and AVP ligand variation in NWMs raise two questions regarding genetic variability among species in the genes coding for these two peptide hormones. First, in spite of the widespread structural stability of OT throughout mammalian evolution, why do NWM represent a ‘hot-spot’ for genetic variation among placental mammals? As we have seen, multiple NS substitutions in OXT among these species lead to structural and potentially functional differences in the OT molecule. One other taxon – the cartilaginous sharks, skates, and rays – have also evolved multiple oxytocin-like peptides (Acher *et al.* 1995). This group of marine fishes utilize urea rather than salts for fluid homeostasis, and it has been argued that a conserved structure in OT-like peptides is not necessary for these osmoregulatory functions, thus stabilizing selection has been relaxed (Gimpl & Fahrenholz 2001). No comparable hypothesis has yet to be formulated for OT ligand diversity in NWMs, and features like body size, geographic distribution, and feeding ecology (Hawes & Peres 2014) fail to account for differences in OT structure. The second outstanding question derives from the observation that despite considerable variation in the OXT gene in NWM, there is no evidence for positive selection and hence variability in AVP.

Ligand-Receptor Coevolution: Macro- and Microscale

To exert their effects, OT and AVP must interact with the appropriate G protein-coupled receptors to effect downstream changes in cell, and ultimately, organismal, function. Markov *et al.* (2008) have argued that the analyses of ligand families and the corresponding structure of their receptors are among the best tests of coevolutionary processes. This is certainly the case with OT/AVP ligands and their receptors, given the centrality of their functions to survivorship and reproductive success, the early appearance of these signaling systems in vertebrates, and the diversity in nonapeptide ligand structures across different scales of evolutionary time. A single receptor has been identified that binds OT with high affinity (OTR), and it has wide distribution in the central nervous system (Vaidyanathan & Hammock 2017). Three receptor subtypes have been characterized for AVP (V1aR, V1bR, and V2R) in mammals. V2R is primarily expressed in the kidney, but both V1aR and V1bR are expressed widely throughout the brain (Song & Albers 2017).

When using a broad phylogenetic filter, nonapeptide receptor structure based on amino acid sequences in invertebrates are statistically distinct from all vertebrate receptors, and among

vertebrates, AVP-like receptors are structurally distinct from all OT-like receptors. Distinct clusters also form on the basis of OTR structure among taxa that have isotocin, mesotocin, and oxytocin as the major OT ligand (Koehbach *et al.* 2013). It is clear that genetic variation in nonapeptide receptors maps on to large-scale evolutionary changes that are linked to differences in the signaling ligand structure.

We conducted a similar phylogenetic analysis of OTR variability within a more restricted taxon, the Order Primates, with specific attention to nucleotide variation in the OTR gene among the NWMs (French *et al.* 2016). Fig 2 portrays a phylogenetic analysis for Simiiformes primates (monkeys and hominoids, with a prosimian as the reference out-group). Taxa can be differentiated according to consensus primate phylogeny, with OTR structures that are clearly distinct among prosimians, NWMs, Old World monkeys, and hominoids. Among NWMs, the three major clades also tend to cluster together on the basis of OTR structure. It is also apparent that OTR structure among species is significantly predicted by OT ligand structure (Ren *et al.* 2015; Vargas-Panilla *et al.* 2015). OTR structure in one genus of NWM (*Ateles*) stands out from the consensus primate phylogeny, in which the OTR structure is more like those of the small-bodied Cebidae. Notably, *Ateles* is the only NWM outside of the Cebids to express Pro⁸-OT (Fig 2). In this case, then, the OT ligand variant is a better predictor of OTR structure than consensus phylogenies. Ren *et al.* (2015) confirmed this trend by a comparing pairwise evolutionary distances among OT and OTR structure, and provided evidence for significant coevolution for species-specific ligands and changes in OTR structure among primates.

There are tantalizing results that link changes in OT and OTR structure with social phenotypes among NWMs. Vargas-Panilla *et al.* (2015) found a significant association between the OT ligand and litter size, with species possessing Pro⁸-OT more commonly producing more than one offspring at birth and extensive paternal care (a common trait among Callitrichid primates). Ren *et al.* (2015) also demonstrated a significant association between species classified as socially monogamous and OTR variability among primates. This finding was also demonstrated between social monogamy and V1aR variability among primates (Ren *et al.* 2014). Notably, V1aR receptor structure in the socially-monogamous and biparental titi monkey (Bales *et al.* 2017) is highly similar to Cebid species that also share these traits, despite considerable phylogenetic distance between titi monkeys and the Cebids (Ren *et al.* 2014).

In the remainder of this review, we expand upon these statistical associations among behavioral traits (social monogamy and biparental care) and nonapeptide and receptor variability in two distinct ways. First, we examine ligand-receptor pharmacology and downstream intracellular signaling via *in vitro* receptor binding and intracellular signaling assays to ascertain whether there are consequences of nonapeptide ligand variation for these processes. Second, we summarize our *in vivo* behavioral pharmacology experiments with marmosets testing whether OT agonist and OTR antagonist manipulations alter social behavior in marmosets in ways that are consistent with the phylogenetically-derived statistical associations between nonapeptide systems and sociality.

Overview of OTR Signaling

OTRs are members of the G-protein coupled receptor (GPCR) Group-A family, which are among the largest and most diverse mammalian protein families. GPCRs consist of seven membrane-spanning helices, an extracellular N-terminus, and an intracellular C-terminus, which interacts with G-protein messenger complexes. GPCRs ultimately function to transduce extracellular stimuli (release of neurotransmitter or hormone) into intracellular signaling events (changes in cell function and regulation). These signaling events are mostly mediated by receptor intracellular coupling to a heterotrimeric G-protein complex (G_{α} , G_{β} , G_{γ}), which upon activation changes conformational states that lead to specific changes in downstream cell activity. Much attention has focused on the G_{α} subunit due to the G_{α} subunit consisting of a number of isoforms (G_q , $G_{i/o}$, G_s), any of which can be preferentially activated by specific ligands. GPCRs possess multiple active conformation states that preferentially couple to isoforms, and these responses lead to a variety of distinct intracellular responses including stimulating adenylyl cyclase and production of cAMP (G_s), mobilizing intracellular calcium ions (Ca^{2+}) from the endoplasmic reticulum and regulating potassium ion (K^+) channels ($G_{q/11}$), inhibiting production of cAMP and also regulating ion channels ($G_{i/o}$), and all isoforms are involved in regulating a number of protein kinases which have many diverse functions (Simon *et al.* 1991). Through preferential activation of specific G-proteins, GPCR responses can exert a high degree of specificity in eliciting functional cellular responses (Maudsley *et al.* 2005).

When activated by OT, OTRs preferentially couple to both $G_{q/11}$ and $G_{i/o}$ -proteins (Gimpl & Fahrenholz 2001; Busnelli *et al.* 2012). A number of factors can influence OTR activation including circulating concentrations of OT ligand near OTRs and the density of OTR expression. For example, at lower OT concentrations [1–10 nM], OTRs preferentially couples to G_q , while at higher OT concentrations [11–100 nM] OTRs couple to both G_q and $G_{i/o}$ (Busnelli *et al.* 2012). Overall, OTRs primarily couple to G_q proteins, but this OT concentration gradient governing preferential OTR coupling to G-proteins is also evident within specific G_i isoform subtypes (G_{i1} , G_{i2} , and G_{i3}), and the G_o subtypes (G_{oA} and G_{oB}). Specifically, OTR activation via OT induces G_{i3} coupling at lower concentrations and G_{i1} , and G_{oB} coupling only at higher concentrations (Busnelli *et al.* 2012). This functional selectivity of coupling to G_q or $G_{i/o}$ has differential cellular outcomes. It has been shown in human embryonic kidney cells (HEK293) stably expressing OTRs that G_q coupling stimulates cell growth while $G_{i/o}$ coupling inhibits cell growth (Rimoldi *et al.* 2003). Moreover, in olfactory neuron cell lines (GN11), OTR coupling to G_q was shown to decrease K^+ inward rectifying currents, while coupling to $G_{i/o}$ increased these currents (Gravati *et al.* 2010). These data demonstrate that the coupling of OTR to specific G-proteins produce differential cellular changes depending on a variety of cellular contexts, and that OTR selectivity to specific G-proteins is physiologically relevant.

OTR coupling to G_q is most widespread because endogenous concentrations of OT are usually in the 1–5 nM range, but it is possible that endogenous OT concentrations vary across the brain. OT concentrations in the brain can be modulated by a number of neuronal features including the number of OT fiber projections across various regions, local axonal release from these fibers, and distance from passive diffusion of OT from dendrites of OT

fibers (Grinevich *et al.* 2016). Moreover, OT concentrations can change in response to external stimuli. Some regions of the brain show up to 4-fold increases in basal OT concentrations in response to social stimuli (Zoicas *et al.* 2014), particularly in areas where levels of OTR expression are high (e.g., lateral septum, nucleus accumbens) (Ross *et al.* 2009), and this is an important mechanism by which OT modulates social behavior. Thus, the relationship between OT release in response to external stimuli and the heterogeneous distribution of OTRs across the brain are all key factors that can moderate specific cellular processes in response of OTR G-protein coupling.

The heterogeneous distribution in the brain of OTRs and G-proteins are an important mechanism by which OT system can finely tune cellular and behavioral responses. The distribution of OTRs in the brain varies across species that display differential patterns of social behavior (e.g., pair-living, biparental care, cooperation; (Freeman & Young 2016), and OTR distribution also differs between males and females of the same species (Dumais & Veenema 2016). Data on OTR expression in nonhuman primates is currently available for marmosets (Schorscher-Petcu *et al.* 2009), titi monkeys (Freeman *et al.* 2014), and macaques (Freeman *et al.* 2014). Marmosets show high OTR expression in the nucleus accumbens but not in the hippocampus. Conversely, titi monkeys show no OTR expression in the nucleus accumbens, but high OTR expression in the hippocampus (Freeman & Young 2016). Overall, OTR expression levels across broader taxa (i.e., primates and rodents) demonstrate two key phenomena. First, OTR expression is high for primates and rodents in areas involved in visual and olfactory processing respectively such as nucleus basalis of Meynert and superior colliculus in primates and olfactory bulb, lateral septum, and hippocampus in rodents (Freeman & Young 2016). Second, OTR expression is more variable in limbic structures like the nucleus accumbens and the bed nucleus of the stria terminalis (Freeman & Young 2016). The overlap between OT fibers and OTRs within the brain is also region-specific. In rodents, many regions that are innervated by OT neurons show prevalent OTR expression (Knobloch & Grinevich 2014; Knobloch *et al.* 2012), but this isn't always the case. Regions including the preoptic area, olfactory bulbs, ventral pallidum, and the ventromedial hypothalamic nucleus have high OTR expression but do not receive direct OT projections (Grinevich *et al.* 2016). Overall, this variable OTR expression in limbic structures may underlie species- and experience-dependent OTR regulation of social behavior, while high OTR expression in sensory processing areas is likely a conserved phenomenon.

Our current knowledge regarding OTR signaling points to an important intersection between variability in OTR induced cellular responses and variability in OTR expression across the brain. This intersection is critical toward advancing our understanding of variability in OT-mediated physiological and behavioral responses to social stimuli across and within species. Like OTRs, many neurotransmitter receptor distributions in the brain are also variable both across and within species. Importantly, unlike the OT system, in nearly all neurotransmitter systems, the structure of the ligand is strictly conserved; yet we have shown the OT peptide has undergone structural changes many times in the recent evolutionary history of NWMs, despite its pivotal physiological functions. How changes in OT/OTR structure-function relationships facilitate or respond to unique environmental conditions or stimuli will provide new insights into OTR signaling at multiple levels of biological organization.

Do Changes in OT Structure Correspond to Changes in OTR Signaling?

Here, we highlight how evolutionary changes in the structure of OT ligands can subtly tune OTR function through a variety of pharmacological properties (Table 1). Pharmacological data are just now emerging from work both in our lab and others demonstrating that the specific OT variants Leu⁸-OT, Pro⁸-OT, and Val³-Pro⁸-OT, and the related nonapeptide AVP, show distinct functional fingerprints upon activating cells stably expressing OTRs. We will focus on how these variants affect four *functional cellular outcomes* including binding affinity, intracellular Ca²⁺ mobilization, G-protein functional selectivity, and receptor internalization, and highlight how these outcomes elucidate physiological mechanisms that underlie the OT regulation of social behavior (Table 1).

1) OT Binding Affinity at OTRs

The physiological responses induced by OT release in the brain generally require OT interaction with OTRs. Importantly, the likelihood of OT induced OTR activation can vary based on a variety of biochemical and biophysical properties associated with the ligand-receptor interaction. Changes in the structure of the OT ligand can potentially enhance or diminish the ligand's ability to bind to the receptor and induce a cellular response. We currently know that both OT and AVP can bind to OTRs (Manning *et al.* 2012), but OT has a much higher affinity than AVP at least partially due to the differences in AVP structure and AVP's higher polarity. Conversely, AVP and OT both bind to vasopressin receptors with AVP showing higher affinity (Manning *et al.* 2012).

Data are currently available for NWM OT ligand variants and binding affinity at human (hOTR) and marmoset OTRs (mOTR). Pro⁸-OT has a stronger binding affinity than Leu⁸-OT at the mOTR, where Pro⁸-OT is the native ligand, and Pro⁸-OT also has stronger affinity than Leu⁸-OT at the hOTR, where Pro⁸-OT is not the native ligand. [Taylor *et al.* unpubl data]. Additionally Pro⁸-OT and Leu⁸-OT both have stronger binding affinities than AVP at each of these OTRs, which is consistent with previous findings of binding affinities between Leu⁸-OT and AVP at the hOTR and rodent OTRs (Manning *et al.* 2012). Of the OTRs tested thus far, Pro⁸-OT shows a higher OTR binding affinity than Leu⁸-OT regardless of whether it is the species' native ligand. Further work should explore whether this finding translates more broadly to other NWM OTRs and whether these differences persist at V1a and V1b receptors via OT crosstalk.

It is currently unknown whether Val³-Pro⁸-OT has a stronger or weaker affinity at hOTR and mOTRs. The structural changes of Val³-Pro⁸-OT may offer interesting insights into how conformational changes in the ligand can affect binding affinity at hOTRs and mOTRs given that the Val³-Pro⁸-OT variant has substitutions at both residue positions that differentiate mammalian OT from AVP. Thus it seems plausible that Val³-Pro⁸-OT may show a distinct binding affinity profile compared to the other ligands and future work should explore whether these changes result in enhanced or inhibited affinities to human and marmoset OTRs and AVP receptors.

2) OT Induced Ca^{2+} Mobilization

G_q -mediated Ca^{2+} cellular responses are an important second messenger system that is involved in numerous processes of signal transmission. Alterations in the efficacy or potency of Ca^{2+} mobilization in the cell and corresponding disruption of ion channel regulation via Ca^{2+} or K^+ -dependent ion channels can have significant effects on physiological function. While OT ligand changes can alter OTR binding affinities, OT ligand changes can also alter corresponding OTR/ G -protein coupling by enhancing or inhibiting the magnitude and selectivity of OTR/ G_q -coupling and downstream Ca^{2+} mobilization. While binding affinity provides insight into the sensitivity of OTRs to respond to OT, the magnitude and selectivity of the intracellular processes must be examined to evaluate meaningful physiological changes associated with OT signaling.

Studies have begun to evaluate whether changes in the OT ligand produce changes in the magnitude/efficacy (i.e., maximal calcium mobilization response) and potency (i.e., OT concentration required to produce 50% of the maximal Ca^{2+} response) of the Ca^{2+} mobilization response upon activating OTRs. In HEK293T cell lines stably expressing hOTRs, Parreira-e-Silva *et al.* (2017) tested the efficacy and potency of Pro^8 -, $\text{Val}^3\text{-Pro}^8$ -, and Leu^8 -OT at inducing Ca^{2+} mobilization responses. They found that all three ligands produced similar efficacy and potencies suggesting that all the OT ligands function as full agonists at hOTRs via the classic OTR activated G_q pathway. These findings are similar to that found by collaborative work in our lab exploring whether Leu^8 -OT and Pro^8 -OT produced differences in Ca^{2+} mobilization in CHO cell lines stably expressing hOTRs (Pierce *et al.* unpubl data). However, at mOTRs, there are some notable differences in Ca^{2+} mobilization responses, where Pro^8 -OT produced a larger maximal intracellular Ca^{2+} mobilization response compared to Leu^8 -OT, but the potency of this effect did not differ between OT ligands (Pierce *et al.* unpubl data) and matched measures of potencies at hOTRs (Busnelli *et al.* 2012; Parreira-e-Silva *et al.* 2017). These findings show that both Pro^8 -OT and Leu^8 -OT activation elicits similar patterns of Ca^{2+} mobilization triggered by OTR/ G_q -coupling at hOTRs, while Pro^8 -OT activation induces a stronger Ca^{2+} response compared to Leu^8 -OT at mOTRs. Overall, Pro^8 -OT has a higher binding affinity and induces larger G_q -mediated Ca^{2+} mobilization responses compared to Leu^8 -OT in mOTRs. Given these changes are limited to mOTRs, these pharmacological responses to Pro^8 -OT could have important physiological consequences for underlying OT regulation of marmoset behavior.

3) Functional Selectivity of OT Ligands

OT and many synthetic OT analogues can activate specific G-proteins in a manner that suggests OTRs display levels of functional selectivity. For example, carbetocin, atosiban, D-Nal-OVT show distinct functional selectivity for specific forms OTR/ G -protein coupling, which have been used as important research tools and have important therapeutic ramifications for targeted OT signaling (Busnelli *et al.* 2012; Busnelli *et al.* 2013). OT can produce a variety of outcomes related to sociality, as well as changes in other processes including pain modulation, appetite regulation, and reproduction. The variability in OT induced functional selectivity can serve as a source of diversity in how OT signaling events at OTRs enhance or inhibit many behavioral and physiological responses. Thus,

demonstrating that OT variants show functional selectivity of OTR/G-protein coupling can identify potential mechanisms underlying coevolved behavioral processes in NWMs.

While OTRs predominately couple to G_q , OTRs also couple to $G_{i/o}$ proteins, which results in reduced cellular cAMP production and also regulates Ca^{2+} and K^+ dependent ion channels, potentially mediating cell growth, pro-inflammatory effects, and neuronal excitability of OT (Rimoldi *et al.* 2003; Zhou *et al.* 2007; Gravati *et al.* 2010; Busnelli *et al.* 2012;). The regulation of these ion channels is important for signal transmission and altering cellular membrane potentials that are critical components of neural transmission. This functional selectivity of activating $G_{i/o}$ or G_q more or less favorably is referred to as ‘biased agonism’ (Kenakin 2007; Busnelli *et al.* 2012), and can have important cellular consequences based on the diversity of G-protein mediated responses.

OTR coupling to $G_{i/o}$ can be measured in a number of ways. Coupling can be measured directly via measuring bioluminescence resonance energy transfer (BRET) between OTRs and G subunits (Busnelli *et al.* 2012; Parreira-e-Silva *et al.* 2017), or indirectly by measuring changes in membrane potential (e.g., cell hyperpolarization) following pharmacologic disruption of $G_{i/o}$ coupling via pertussis toxin PTX (Pierce *et al.* unpubl data). Parreira-e-Silva *et al.* (2017) showed that OT ligand induced OTR coupling varies by the specific G_i subunit. G_{i1} and G_{i3} subunits show similar levels of hOTR coupling following OT activation regardless of the OT ligand. On the other hand G_{i2} showed stronger coupling to hOTRs, but only when activated by Leu⁸-OT, suggesting that Leu⁸-OT shows a lower degree of functional selectivity at inducing G-coupling (Parreira-e-Silva *et al.* 2017). However, OT ligand induced G_i coupling was significantly lower than G_q coupling for all ligands, suggesting that OTRs mainly couple to G_q regardless of OTR activation by Leu⁸, Pro⁸, or Val³-Pro⁸-OT. Whether these results are similar in mOTRs have yet to be evaluated using BRET. However, recent work from our lab collaborators shows that when disrupting $G_{i/o}$ coupling via PTX, mOTRs showed a significant reduction in efficacy of Leu⁸-OT-mediated hyperpolarization, while PTX treatment did not affect Pro⁸-OT-induced hyperpolarization. Similarly at hOTRs, PTX treatment reduced the efficacy of Leu⁸-OT-mediated but did not significantly affect Pro⁸-OT-mediated hyperpolarization (Pierce *et al.* unpubl data). Taken together, these data suggest that hyperpolarization responses following Pro⁸-OT activation of OTRs are almost completely G_q -mediated, and future work should explore potential downstream functional consequences of via functional selectivity of Leu⁸-OT and Pro⁸-OT at OTRs.

Differences in the ways that Pro⁸-OT and Leu⁸-OT induce $G_{i/o}$ coupling may have important consequences on regulating Ca^{2+} and K^+ dependent ion channels. Leu⁸-OT and Pro⁸-OT produced concentration-dependent decreases in hyperpolarization at both hOTRs and mOTRs, and Leu⁸-OT displayed greater potency than Pro⁸-OT with regard to changes in membrane potential (Pierce *et al.* unpubl data). Given that G_q and $G_{i/o}$ coupling are important for regulating ion channels, future work should examine the degree to which these OT ligands directly regulate Ca^{2+} and K^+ -dependent inward rectifying and intermediate conductance (IK) channels (Gravati *et al.* 2010) as a potential source for functional selectivity of OT activation via OTR/G-protein coupling.

4) OTR Desensitization and Internalization

Receptor desensitization and internalization are important processes that can greatly affect receptor sensitivity and responsiveness to long-term ligand stimulation. These processes are regulated by a number of G-protein receptor kinases (GRK) and arrestins (e.g., β -arrestin1 and 2), which prevent G-protein coupling with the receptor and signal receptor endocytosis. An important consideration for OTR function is the extent to which OT ligands show stronger or weaker efficacy or biases at activating GRKs and β -arrestin recruitment leading to receptor desensitization and internalization.

Thus far, one study has evaluated and demonstrated that OT ligands produce differential effects on OTR desensitization and internalization. Parreira-e-Silva *et al.* (2017) showed that Pro⁸-OT activation of OTRs resulted in a marked reduction in potency for both β -arrestin1 and β -arrestin2 recruitment compared to Leu⁸-OT in hOTRs, and Val³-Pro⁸-OT showed an even greater reduction in potency compared to Leu⁸- and Pro⁸-OT. Pro⁸-OT showed a significant reduction in β -arrestin1 recruitment efficacy, producing only 72% of the maximal response induced by Leu⁸-OT at β -arrestin1, but Pro⁸-OT showed no significant difference in β -arrestin2 recruitment efficacy compared to Leu⁸-OT at hOTRs. Val³-Pro⁸-OT was associated with a significant reduction in recruitment efficacy at both β -arrestin1 and 2 producing only 62% and 70% of the Leu⁸-OT response, respectively. Overall these results reveal that both Pro⁸-OT and Val³-Pro⁸-OT induce significantly diminished OTR desensitization compared to Leu⁸-OT at hOTRs, suggesting potential biases in OTR recruitment of β -arrestins. Notably, Val³-Pro⁸-OT, shares a similar pharmacological profile with AVP in regard to hOTR β -arrestin recruitment at OTRs.

Similarly to desensitization, Pro⁸-OT and Val³-Pro⁸-OT are both much weaker at inducing hOTR internalization (Parreira-e-Silva *et al.* 2017). Leu⁸-OT induced a robust and time-dependent loss of hOTRs from the plasma membrane (and thus a loss in OTR signaling), and Pro⁸-OT only moderately induced hOTR loss from the membrane (about half as potent). Val³-Pro⁸-OT induced *no* internalization of hOTRs at all. These findings show that Leu⁸-OT robustly desensitizes and internalizes hOTRs, Pro⁸-OT does so to a lesser extent, and Val³-Pro⁸-OT induces very little desensitization and no internalization. When activated by Pro⁸-OT or Val³-Pro⁸-OT, hOTRs are likely to remain more responsive to OT activation. These OT differences are quite remarkable, and further experiments examining desensitization, internalization, and the recruitment of other trafficking and recycling signalers may be a fruitful area for future investigations.

Summary of OT Ligands and OTR Pharmacology

OT ligands clearly produce a number of different functional consequences in OTR binding, signaling, and internalization. First, Pro⁸-OT appears to bind with higher affinity and, at least in mOTRs, induces stronger Ca²⁺ mobilization responses. Second, Pro⁸-OT appears to induce OTR coupling almost exclusively through G_q, while Leu⁸-OT shows less selective G-protein coupling. Certainly more work is needed to evaluate whether Pro⁸-OT shows true functional selectivity or 'biased agonism'. Finally, Pro⁸-OT and Val³-Pro⁸-OT activation of hOTRs show lower affinities for β -arrestin recruitment, which likely results in more responsive OTR functioning. Taken together, these pharmacological data suggest

mechanisms through which OT/OTR coevolution may lead to functional differences in physiological and behavioral outcomes. Much more work is needed to chart out the pharmacological cartography through which these ligands may directly modify signal transduction. Such approaches should include directly measuring neural function to evaluate whether the *in vitro* findings translate to native tissues including neuronal cultures and other neuronal cell contexts. Moreover, exploring pharmacological profiles in other primate species expressing Leu⁸-OT with similar behavioral repertoires (e.g., titi monkeys), or Pro⁸-OT expressing species with differing behavioral repertoires (e.g., capuchin or spider monkeys), and the functional consequences of OT crosstalk at V1a and V1b receptors (Bales *et al.* 2007; Gupta *et al.* 2008; Schorscher-Petcu *et al.* 2010; Song & Albers 2017) will advance our understanding of the physiological importance of these signaling responses in OTRs.

Finally, recent attention has shifted toward potential signaling impacts related to OTR homo- and heterodimerization with promising results. For instance, synthetic bivalent OT ligands have been shown to display super-potent properties (~1000 times greater potency) to activate OTR/Gq coupling compared to OT (Busnelli *et al.* 2016). This increased potency is believed to be the result of bivalent OT ligands specifically targeting OTR dimers. Additionally these bivalent OT ligands produce significant behavioral consequences exhibiting 100 times greater potency at promoting sociability in mice compared to endogenous OT and 40 times greater potency at promoting sociability in zebrafish compared to endogenous isotocin (Busnelli *et al.* 2016). OTRs also form heterodimers with other Class A GPCRs. One compelling example includes the formation of heterodimers between OTRs and Dopamine (DA) 2 receptors (D2Rs), which, in turn, exhibit reciprocal interactions (Romero-Fernandez *et al.* 2013; de la Mora *et al.* 2016). Specifically OT activation increases D2R affinity for DA and DA agonists, while DA activation of D2Rs enhances OT-induced signaling. With recently converging evidence for direct synergistic effects of OT neuronal release modulating DA neural activity and social behavior (Hung *et al.* 2017; Xiao *et al.* 2017), OTR/D2R heterodimers may represent an important physiological mechanism through which these behavioral outcomes can be modulated (De La Mora *et al.* 2016). Whether OT ligand variants enhance or inhibit the conformational changes necessary to facilitate or impair OTR homo- and heterodimerization is an important area for future investigation.

Do Changes in OT Structure Correspond to Changes in Social Behavior?

We have demonstrated there are considerable structural differences in OT ligands and OTRs in NWM. We have also demonstrated these OT ligands induce differences in the pharmacological properties of OTRs. The final arbiter of the importance of these structural and functional changes from the perspective of social behavior is the degree to which Pro⁸-OT ligand variants selectively modify behavioral and social processes in NWM. This taxonomic group represents a ‘hot-spot’ for OT ligand variation among placental mammals, and this group corresponds with a ‘hot-spot’ for many uncommon behavioral phenotypes among mammals including biparental care and social monogamy (Lukas & Clutton-Brock 2013; French *et al.* 2017). Currently, data examining the impact of exogenous manipulation with OT ligand variants on social behavior are available for marmoset monkeys (*Callithrix* spp.). The social and mating systems of marmosets have been characterized variously as

socially monogamous, polyandrous, polygynous, and ‘flexible’ (Díaz-Muñoz & Bales 2016). Marmosets express many of the specific individual behavioral traits that are commonly associated with social monogamy (French *et al.* 2017). For example, adult male and female marmosets are successfully housed in long-term pair-living contexts, engage in high levels of affiliative behavior, and maintain proximity with pairmates, all of which are hallmarks of species that are traditionally viewed as socially monogamous (Agmo *et al.* 2012; Smith *et al.* 2010; Cavanaugh *et al.* 2014). Marmosets also live in extended family groups and exhibit cooperative breeding with both parents and siblings providing caregiving to offspring (Digby 1995; Snowdon 1996). Finally, marmosets display prosocial preferences including ‘altruistic’ food provisioning (Burkart *et al.* 2007; Mustoe *et al.* 2015). In this section, we evaluate whether OT ligand variants (Pro⁸-OT and Leu⁸-OT) influence behavioral outcomes across several components of sociality (mate-directed behavior, stranger-directed behavior, and parental behavior). To this end, we can determine whether specific behavioral components show 1) *OT-specific effects* where treatment with Pro⁸-OT and/or Leu⁸-OT produce different behavioral outcomes, 2) *OT-generic effects* where both OT ligand treatments have similar effects on behavioral outcomes, or 3) *no OT effect* where behavioral measures are not altered by OT treatment.

In our work on nonapeptide modulation of social behavior in marmosets, we have utilized intranasal administration of nonapeptides (Pro⁸-OT, Leu⁸-OT, and/or AVP), and oral administration of a non-peptide OTR antagonist (OTA: L368,899; Boccia *et al.* 2007). Subsequent to these treatments, social behavior in marmosets was recorded in a number of behavioral paradigms. First, we routinely record spontaneous social interactions that occur in normal home environments. The most common experimental paradigm is the *partner preference test*, in which an OT-treated marmoset can preferentially investigate and socially interact with pairmates or strangers. In a *novel environment stressor*, OT-treated marmosets were removed from their home enclosure and either separated with or isolated from their pairmate. This task allows for the investigation of social buffering (Smith & French 1997; Rukstalis & French 2005; Hennessy *et al.* 2009), a phenomenon in which stressors are experienced as less severe (measured in animals physiologically or behaviorally) when a social partner is present compared to when a social partner is absent. In a *food-sharing task*, treated marmosets were given sole access to food and permitted to allocate to others (e.g. mate, stranger, and offspring). This allows for the investigation of prosociality, altruism, inequity aversion, and offspring provisioning. In cases when marmosets were removed from their home environment and separated from their mates, we observed behavior during *reunion with their mate* in their home enclosure. Finally, in an *infant interest test*, treated marmosets were exposed to simulated infant stimuli and control stimuli. Much like in the partner preference test, these stimuli are separated into different compartments, so marmosets can investigate stimuli independently.

One can view how OT may influence social relationships through two distinct behavioral processes. The first mechanism involves OT enhancing strong social attachment relationships that are ‘endogenous’ to bonded marmoset pairs. The second mechanism is ‘exogenous’ to the pairs, and derives from OT promoting disinterest toward unfamiliar strangers of the opposite sex or by increasing aggressive exclusion of strangers by one or both members of the marmoset pair. We review the evidence for each of these mechanisms

for maintaining social relationships among partners (Anzenberger 1985), first by discussing the impact of OT manipulations on mate-directed behavior, followed by an evaluation of these manipulations on stranger-directed behavior.

Mate-Directed Behavior

If OT is critical for regulating mate-directed behaviors, and Pro⁸-OT produces enhanced mOTR signaling, then we would expect that Pro⁸-OT would show greater enhancement of mate-directed affiliative behavior than Leu⁸-OT. It is clear that OT is involved in mate-directed behavior in marmosets because evidence from OTA treated marmosets show a reduction in mate-directed social approach in a variety of contexts, including reduced proximity during partner preference tests (Smith *et al.* 2010; Cavanaugh *et al.* 2014), reduced proximity during and after a novel environment stressor (Cavanaugh *et al.* 2016; Cavanaugh *et al.* unpubl data), and reduced food sharing behavior in the home cage (Smith *et al.* 2010); though OTA doesn't always have this effect (Cavanaugh *et al.* 2014; Mustoe *et al.* 2015; Mustoe *et al.* 2016).

Studies examining marmoset partner-preference tests only showed modest effects of OT in the enhancement mate-directed behavior (Smith *et al.* 2010; Cavanaugh *et al.*, 2014). With regard to OT ligand specificity, Cavanaugh *et al.* (2014) found that Pro⁸-OT enhanced the amount of time spent in close proximity with their mate during a partner-preference test but in females only, while Leu⁸-OT had no effect for both males and females. However, the vast majority of findings in marmosets suggest that both OT ligands produce little to no effects on mate-directed behaviors including social approach, grooming, or huddling behaviors (all affiliative behaviors associated with pair-bond maintenance) (Cavanaugh *et al.* 2014; Cavanaugh *et al.* 2015); cooperative food sharing in a experimental prosocial food-sharing tasks (Mustoe *et al.* 2015; Mustoe *et al.* 2016); and affiliative behaviors (time in proximity, grooming, huddling) upon reunion following a long-term social separation (Cavanaugh *et al.* unpubl data). While it would seem most likely that Pro⁸-OT treatments would produce larger effects on mate-directed behavior than Leu⁸-OT in marmosets, the data suggest there are minimal OT-specific effects. Furthermore, OT in general (either ligand) does not appear to strongly enhance mate-directed behaviors in marmosets, but OT does affect marmoset social behavior in a variety of other important ways.

One way in which OT does produce behavioral changes between marmoset mates is inducing changes in how untreated marmosets interact with their OT-treated mates. Individuals who are treated with OT received increased affiliative behavior from their mates (Cavanaugh *et al.* 2015). In some cases, the effects of OT are similar for both ligands. For instance, females treated with either Pro⁸-OT or Leu⁸-OT received more grooming from their pairmates. Males treated with either Pro⁸-OT or Leu⁸-OT are approached by their mates more frequently (Cavanaugh *et al.* 2015), suggesting these are OT-general effects. In other cases, the effect of OT appears to be specific to Pro⁸-OT, where Pro⁸-OT treated individuals received increased duration of gaze from their mate (Cavanaugh *et al.* unpubl data). Overall, OT treatments may be subtly changing *some characteristic(s)* in marmosets that make them more likely to receive increased affiliation from their mate, and these OT changes appear to be both OT-specific and OT-general effects depending on the behavior.

Stranger-Directed Behavior

Our data reveal that OT manipulations produce more robust behavioral changes with regard to interactions with opposite-sex strangers compared to interactions with their pairmates. Marmosets gregariously interact with strangers when given the opportunity (Cavanaugh *et al.* 2014; Mustoe *et al.* 2015; Smith *et al.* 2010). Activation of the OT system significantly reduces these stranger-directed behaviors, and these effects are specific to Pro⁸-OT. Pro⁸-OT treated marmosets spent less time with opposite sex strangers in the partner preference test, and were slower to engage in sexual solicitation displays with strangers, but these measures were unaffected by Leu⁸-OT treatments (Cavanaugh *et al.* 2014). Similarly, untreated marmosets preferentially donated food rewards to strangers vs. pairmates in a prosocial food-sharing task (Mustoe *et al.* 2015). Marmosets treated with Pro⁸-OT showed a significant reduction in stranger-directed food sharing, but Leu⁸-OT treatment did not alter stranger-directed food sharing. In two experimental contexts, then, Pro⁸-OT reduces stranger-directed behavior while Leu⁸-OT had no effect, which supports the view that there is greater OT-specificity for stranger-directed behavior than mate-directed behavior.

Parental Behavior

Experimental treatment with OT alters several components of parental behavior in marmosets. Saito and Nakamura (2011) examined the rates fathers shared food with young offspring under two conditions: intracerebroventricular administration of Leu⁸-OT or saline treatment. Leu⁸-OT treatment lead to greater transfer of food from fathers to offspring, by decreasing the rates of food sharing refusals (Saito & Nakamura 2011). In contrast, our lab showed that Pro⁸-OT treated male marmosets were *less* likely to engage in alloparental care via sharing food with their *younger* siblings (Taylor *et al.* 2017). Importantly, many contextual and procedural features between these studies differed, making a direct comparison between the opposing Leu⁸- and Pro⁸-OT effects difficult. A recent study addresses whether OT variants differentially affect parental behavior utilizing a rodent model (a Leu⁸-OT species) (Parreiras-e-Silva *et al.* 2017). Administration of NWM OT ligand variants (Pro⁸-OT and Val³-Pro⁸-OT) to adult male and female rats (intranasally) produced differential effects on caregiving behavior toward pups. Female rats treated with Val³-Pro⁸-OT and Pro⁸-OT they were faster to retrieve separated pups compared to females treated with Leu⁸-OT or saline. Male rats, not normally parental, retrieved pups more rapidly when treated with any OT ligand (Pro⁸-OT, Leu⁸-OT or Val³Pro⁸-OT) compared to saline (Parreiras-e-Silva *et al.* 2017). This indicates that even in a Leu⁸-OT rodent species, Pro⁸-OT is as effective or more effective (in females at least) at enhancing parental behavior as Leu⁸-OT. As is the case with mate- and stranger-directed behavior, Pro⁸-OT tends to produce stronger behavioral effects.

Data comparing differences in parental behavior following either Pro⁸-OT or AVP treatments are also of interest due to the potential for OT and AVP cross-activation at OTRs. Taylor and French (2015) measured responses of adult male and female marmosets to acoustic and visual stimuli associated with infants. Both Pro⁸-OT and AVP treatments enhanced responsiveness to infant stimuli (reduced latency to approach and increased investigation of infant stimuli), but the effects were sex-specific. Pro⁸-OT enhanced

responsiveness to infant stimuli in males, and AVP enhanced responsiveness to infant stimuli in females. Pro⁸-OT and AVP do not produce the same effects on responses to infant and juvenile stimuli (Taylor & French 2015).

Summary of Pro⁸-OT and Leu⁸-OT Effects on Social Behavior

Treatment effects that are mixed and/or context dependent make summarizing the collection of results from these experiments challenging but useful. As such we have taken a “bird’s eye view” of the published work comparing Pro⁸-OT, Leu⁸-OT, OTA, and AVP, and a summary is shown in Table 2. Here we highlight a few patterns that are apparent. *First*, OTRs must be involved in augmenting social behavior, since blocking the OTR with an antagonist reduces measures of sociality across a wide variety of experimental contexts. *Second*, when Pro⁸-OT treatments produce an effect on behavior it is almost always in the expected direction; Pro⁸-OT enhances mate-directed or mate-received behavior and reduces stranger-directed behavior. Two notable exceptions are the findings that young male marmosets treated with Pro⁸-OT reduce food sharing directed toward siblings (Taylor *et al.* 2017), and adult marmosets treated with Pro⁸-OT increased time spent alone and reduced time spent with the mate (Cavanaugh *et al.* 2014). *Third*, Leu⁸-OT treatments produced mixed effects on whether social behavior was enhanced or impaired, and Leu⁸-OT never enhanced a social behavior that Pro⁸-OT did not also enhance. Differences between Pro⁸-OT and Leu⁸-OT across behavioral contexts may be explained by the observations that Leu⁸-OT functions as a partial agonist with lower efficacy than Pro⁸-OT for mOTRs, and that Leu⁸-OT leads to increased OTR internalization (Pierce *et al.* unpubl data; Parreiras-e-Silva *et al.* 2017), assuming full activation of OTRs are necessary to induce these behaviors. It is also possible that the lack of effects or the mixed results may be due to inconsistent and poor penetrance of OT into brain regions with receptors following intranasal delivery (Leng & Ludwig 2016) or potential differences in Pro⁸-OT penetrance. *Finally*, the behaviors that consistently appear most sensitive to OT treatments (stranger-directed behaviors) also show the greatest degree of Pro⁸-OT specificity.

Concluding Remarks

It is clear that OT is among one of the most pervasive signaling systems modulating physiology and social behavior in mammals, and changes to this signaling molecule are likely to result in meaningful and targeted consequences. Pro⁸-OT as a signaling molecule at OTRs shows increased binding affinity, produces increased intracellular Ca²⁺ mobilization, and induces reduced receptor internalization compared to the ancestral mammalian Leu⁸-OT molecule. Importantly, Pro⁸-OT also elicits specific behavioral changes in pair-bonded marmosets in some contexts, especially with regard to reducing social interest directed toward opposite-sex strangers. Together, these findings demonstrate that the unique OTR pharmacological properties associated with activation by the Pro⁸-OT ligand result in potentially important functional consequences (Fig 3). The extent to which these OT structural changes modify neuronal processes, enhance or diminish OT/OTR interactions with other neuronal systems, or possess targeted therapeutic potential has yet to be thoroughly explored. However, our observations that naturally occurring modifications in the OT/OTR system among NWMs lead to significant functional consequences provides an

intriguing ‘natural experiment’ to explore OT under a new lens. Our approach should be viewed as complementary to efforts that explore laboratory-induced changes in the OT molecule as a means to discover super potent and efficacious synthetic ligands as potential candidates for clinically relevant endpoints (e.g., Busnelli *et al.* 2016; Muttenthaler *et al.* 2017). Overall, these findings from OT pharmacology to OT-induced behavioral changes clearly demonstrate that OT/OTR structural modifications in NWM that arise from natural selection can orchestrate important changes in OT-mediated physiological and behavioral outcomes.

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Figure 1.

Consensus primate phylogeny constructed from data from 10kTrees (Arnold *et al.* 2010) for selected genera/species for which OT ligand is known. Colored phylogenetic roots indicate separate OT ligands (2D structures shown for each OT ligand); red circles represent pair-living and beige variable pair-living/non-pair-living, and stars indicate the presence of biparental care via provisioning and/or protecting behavior for the species. Behavioral phenotypes derived from Lukas and Clutton-Brock 2013; French *et al.* 2017; and/or University of Michigan Museum of Zoology (animaldiversity.org).

Figure 2. Phylogeny constructed from OTR sequence across primates demonstrating significant coevolution of OT ligand structure and OTR sequences across primates. OT ligands across genera are indicated by colors matched from Figure 1. Figure modified from Ren *et al.* 2015.

Figure 3.

A schematic summary of this review of the main findings based on OTR functional pharmacology outcomes such as receptor binding and G-protein signaling, and social behavior outcomes (in marmosets) following activation of Pro⁸-OT. Arrows indicate direction of effect compared to Leu⁸-OT induced activation of OTRs at h- and mOTRs. “?” represent findings that have yet to be determined or evaluated. The pharmacological outcomes associated with OT activation are known to be involved in neuronal membrane/cell excitability (Gong *et al.* 2015), long-term potentiation (Lin *et al.* 2012), and regulating many facets of social behavior (Blume *et al.* 2008; Jurek *et al.* 2012; Wang *et al.* 2015). For a full representation of the OT signaling network see Chatterjee *et al.* 2016.

Table 1

Description of potential functional pharmacological outcomes associated with OTR signaling

<p>1) OT Binding Affinity</p>	<p>Definition: The concentration of OT ligand needed to occupy 50% of OTRs (IC_{50}).</p> <p>Question: Do different structural OT ligands enhance or reduce binding affinity with OTRs?</p> <p>Outcome: OT ligand affinity to the receptor has important implications not only in how natural ligand variants interact with OTRs, but these OT ligands may exhibit similar properties with regard to their crosstalk with AVP receptors.</p>
<p>2) OT Induced Ca^{2+} Mobilization</p>	<p>Definition: The maximal response/efficacy of Ca^{2+} mobilization and the OT ligand potency (EC_{50}) of the concentration needed to induce 50% maximal response.</p> <p>Question: Are there differences in the magnitude of intracellular responses based on the structure of the OT ligand, and does this response vary in potency between OT ligands?</p> <p>Outcome: While certain ligands may share equal or better binding affinity to the receptor, differences in response magnitude/potency may suggest that certain OT ligands are 'better' at enhancing cellular function.</p>
<p>3) Functional Selectivity of OT Ligands</p>	<p>Definition: Increased favorability/bias for OTRs to couple to one G-protein over another.</p> <p>Question: Do specific OT ligands show different biases of activation of G_q over $G_{i/o}$ or vice versa?</p> <p>Outcome: OT ligands may preferentially activate certain physiological outcomes through activating specific cellular pathways over others (e.g., OT ligands may only activate G_q and fail to activate $G_{i/o}$ protein coupling).</p>
<p>4) OTR Desensitization and Internalization</p>	<p>Definition: Decrease in receptor responsiveness over time via increased affinity for/recruitment of β-arrestins leading to the transport and degradation of OTRs from the plasma membrane.</p> <p>Question: Do specific OT ligands enhance β-arrestin recruitment and loss of OTR from the plasma membrane?</p> <p>Outcome: Differences in how OT ligands facilitate faster or slower desensitization/internalization following acute or chronic OTR stimulation can elucidate additional intracellular mechanisms for differential OT ligand function.</p>

Table 2

Oxytocin ligand variants and social behavior in marmosets

Behavior	Details	Pro8-OT	Leu8-OT	AVP	OTA
Social Approach					
	Partner preference test (3 weeks cohabitation) ^a		-↑		↓
	Proximity (undisturbed) ^c	-	-		-
	Partner preference Test (8+ weeks cohabitation) ^b	↑F,↓M	-		-
	Proximity during novel environment stressor ^f	↑	-		↓
	Proximity during reunion ⁱ	-	-		↓
	Responsiveness to infant Stimuli ^e	↑M,-F		-M,↑F	-
Social Attention					
	Grooming(undisturbed) ^c	-	-		-
	Huddling(undisturbed) ^c	-	-		-
	Solicit grooming(undisturbed, reunion) ^{c,i}	-	-		-
	Gaze ^j	-	-		-
Social Attractiveness					
	Received Grooming (undisturbed) ^c	↑F,-M	↑F,-M		-
	Received Grooming (reunion) ⁱ	-	↓-		↓-
	Approached by mate ⁱ	-	-		-
	Received gaze ⁱ	↑	↓-		↓
Food Sharing					
	Mate-Directed prosocial food sharing task ^{d,g}	-	-		-
	Mate-Directed food sharing (undisturbed) ^a		-		↓
	Sibling directed food sharing ^h	↓M			
	Offspring directed food sharing ^{h,j}		↑M	↓M	
Stranger-Directed Behavior					

Behavior	Details	Pro8-OT	Leu8-OT	AVP	OTA
Anxiety & Stress	Stranger-Directed prosocial food sharing task ^{d,g}	↓-	-	-	↓-
	Sexual solicitation ^b	↓	-	-	-
	Gaze ⁱ	-	-	-	-
	Social Buffering of stress response ^f	-	-	-	-
	Cortisol reactivity ^f	-	-	-	↑
Aggressive Behavior	Contact Calling ^{e,f}	-	-	-	-
	Locomotion ^{e,f}	-	-	-	-
	Scent Marking ^{c,f}	-	-	-	-
	Infant-directed aggressive vocalization ^h	-	-	↑	-

Note. ↑ indicates that behavior was increased after treatment, ↓ indicates that behavior was decreased after treatment, - indicates no effect of treatment. Results noted with ↑- or ↓- indicates at least one significant treatment effect for that behavior and one no effect. **F** indicates effect was present in females, **M** indicates effect was present in males. Parenthetical “(undisturbed)” indicates observations were conducted in home cage without any other behavioral tasks.

Superscripts indicate references: ^aSmith, et al. 2010;

^bCavanaugh, et al 2014;

^cCavanaugh, et al 2015;

^dMustoe,et al 2015;

^eTaylor & French 2015;

^fCavanaugh, et al 2016;

^gMustoe, et al. 2016;

^hTaylor, Intorre, & French 2017;

ⁱCavanaugh, et al *unpubl data*;

^jSaito & Nakamura 2011