A Comparison of the Ability of Leu\textsuperscript{8} and Pro\textsuperscript{8}-Oxytocin to Regulate Intracellular Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-Activated K\textsuperscript{+} Channels at Human and Marmoset Oxytocin Receptors

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ABSTRACT

The neurohypophysial hormone oxytocin (OT) regulates biologic functions in both peripheral tissues and the central nervous system. In the central nervous system, OT influences social processes, including peer relationships, maternal-infant bonding, and affiliative social relationships. In mammals, the nonapeptide OT structure is highly conserved with leucine in the eighth position (Leu\textsuperscript{8}-OT). In marmosets, proline (Pro\textsuperscript{8}-OT) was more efficacious than Leu\textsuperscript{8}-OT in measures of \(G_\text{q}\) activation, with both peptides displaying subnanomolar potencies. At the hOTR, neither the potency nor efficacy of Pro\textsuperscript{8}-OT and Leu\textsuperscript{8}-OT differed with respect to \(G_\text{q}\) signaling. In both mOTR- and hOTR-expressing cells, Leu\textsuperscript{8}-OT was more potent and modestly more efficacious than Pro\textsuperscript{8}-OT in inducing hyperpolarization. In mOTR cells, Leu\textsuperscript{8}-OT-induced hyperpolarization was modestly inhibited by pretreatment with pertussis toxin (PTX), consistent with a minor role for \(G_\text{i/o}\) activation; however, the Pro\textsuperscript{8}-OT response in mOTR and hOTR cells was PTX insensitive. These findings are consistent with membrane hyperpolarization being largely mediated by \(G_\text{q}\) signaling mechanism leading to Ca\textsuperscript{2+}-dependent activation of K\textsuperscript{+} channels. Evaluation of the influence of apamin, charybdotoxin, pawilline, and TRAM-34 demonstrated involvement of both intermediate and large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels.

Introduction

Oxytocin (OT) is a nonapeptide that regulates a host of physiologic functions both peripherally (e.g., uterine contraction, lactation) and centrally (e.g., social behavior). OT is synthesized in the magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus, and OT neurons primarily project to the posterior pituitary where OT is released into the bloodstream (Ludwig and Leng, 2006). OT neurons also project to multiple regions within the “social brain” (Stoop, 2014). These latter OT projections are thought to be responsible for the modulation of many behaviors, including social recognition and memory, sexual behavior, parental care, pair-bond formation and maintenance, and cooperation and aggression (Insel et al., 2010; Johnson and Young, 2015). Dysfunction in OT signaling has also been widely reported in mental health outcomes in which social deficits are commonly observed, such as schizophrenia and depression/anxiety. Consequently, OT has received considerable interest as a therapeutic for these disorders but studies have shown mixed results (Young and Barrett, 2015; Guastella and Hickie, 2016; Parker et al., 2017).

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OT-like nonapeptides are highly conserved signaling molecules that activate G protein–coupled receptors (GPCRs). OT binds primarily to the oxytocin receptor (OTR) and, to a lesser extent, the related nonapeptide vasopressin receptors (Gimpl and Fahrenholz, 2001; Manning et al., 2008). The OTR promiscuously couples to and activates multiple G proteins producing diverse effects on cellular function, including inhibition of adenyl cyclase (Gi0), stimulation of potassium channel currents (Gk), and activation of phospholipase C (Gq) (Reversi et al., 2005). OTR activation also leads to a variety of signaling responses, which suggests that OT activation may preferentially bias specific G-protein pathways that vary across cell types both within the brain and in the periphery. For example, Gq activation mediates activation of neural OTRs that generate pulsatile OT secretion (Wang and Hatton, 2007), whereas both Gi0 and Gq activation mediate Ca2+ mobilization and GTP hydrolysis in myometrial cells (Phaneuf et al., 1993).

Despite the high degree of conservation of the OT ligand among most mammals, many New World monkeys (NWMs) possess OT sequence modifications that have evolved from the ancestral mammalian OT sequence (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly; Leu8-OT). Thus far, five additional OT-like variants have been identified with variability in amino acids mainly at position 8, but also at positions 2 and 3 (Lee et al., 2011; Wallis, 2012; Ren et al., 2015; Vargas-Pinilla et al., 2015). The most common OT variant is a Leu-to-Pro substitution at the eighth amino acid position (Pro8-OT). This substitution significantly alters the linear portion of the ligand’s three-dimensional architecture, whereby formation of the Pro-Pro polyproline helix in the linear portion of the OT ligand could potentially lead to changes in OT interaction with the OTR with attendant alteration in potency and/or efficacy (Zingg and Laporte, 2003; Geisler and Chmielewski, 2009).

Differences between OT and the related nonapeptide vasopressin (which differs in amino acid positions 3 and 8) show select ligand recognition with specific portions of the OTR and vasopressin receptor 1A, potentially suggesting important OTR recognition features that could change as a function of a Leu-to-Pro substitution in position 8 (Chini et al., 1995, 1996; Zingg and Laporte, 2003). OT ligand variants are also of interest because these ligands show significant coevolution with corresponding OTR sequence structures as well as a significant association with the presence of social phenotypes, including social monogamy and paternal care in primates (Ren et al., 2015; Vargas-Pinilla et al., 2015), and these social phenotypes are known to be influenced by exogenous OT (French et al., 2016). The association between OT/OTR structures with social behavior in NWMs raises the possibility that OT-related phenotypic differences might be a consequence of functional selectivity with respect to the signaling properties associated with OT analog (e.g., Pro8-OT) activation of OTRs. Currently, there is limited information regarding signaling profiles of OT analogs at human and marmoset oxytocin receptors (hOTRs and mOTRs, respectively).

To assess whether OT/OTR variability results in altered pharmacological properties of OT ligands, we stably transfected Chinese hamster ovary (CHO) cells expressing hOTRs or mOTRs and examined the resulting activation of OT-OTR signaling pathways. We evaluated whether OT ligand variation resulted in distinct activation of different G protein–mediated cell-signaling pathways (Gia and Gq) in hOTR- and mOTR-expressing cells, as assessed by elevation of intracellular Ca2+ or alteration in membrane potential.

**Materials and Methods**

**CHO Cell Cultures.** Wild-type CHO-K1 cells were purchased from American Type Culture Collection (CCL-61) and cultured in Ham’s F-12 medium (SH30026.01; Hyclone), 10% fetal bovine serum (FBS) (S11550; Atlanta Biologicals), 1.5% HEPES 1 M solution (SH30221.01; Hyclone), and 1% penicillin-streptomycin (10,000 U/ml, 15140-163; Life Technologies). hOTR-expressing CHO-K1 cell lines were purchased from Genscript (M00195). mOTR plasmid was purchased from Genscript and stably transfected into CHO-K1 cells. hOTR- and mOTR-expressing cells were cultured in Ham’s F-12 medium (SH30026.01; Hyclone), 10% FBS (S11550; Atlanta Biologicals), 1.5% HEPES 1 M solution (SH30221.01; Hyclone), 1% penicillin-streptomycin (10,000 U/ml, 15140-163; Life Technologies), and 400 mg/ml G418 (G64000-5.0; RPI Corp.). κ-Opioid receptor (κ-OR)-expressing CHO (κ-OR-CHO) cells were cultured in RPMI 1640 medium supplemented with 10% FBS (S11550; Atlanta Biologicals). Cells were cultured at 37°C in 5% CO2 and 90% humidity.

**CHO Cell Stable Transfection and Selection of Clones.** CHO-K1 cells (1 × 106) were electroporated with 1.5 μg vector encoding mOTR plasmid (Genscript). After transfection, cells were seeded on 10-cm plates and grown under antibiotic pressure with 400 mg/ml G418 (G64000-5.0; RPI Corp.) for 72 hours. The clones were picked using cloning cylinders (3166-10; Corning), and 50 mg/ml G418 (25300-054; Gibco) was added to the cells/cell colony and detached by pipetting two to three times. Dissociated cells were diluted in 100 ml media and plated in 24-well plates (~1 cell/well). The cells were allowed to grow under antibiotic pressure for 3 weeks, with media change every 72 hours. Five clones were picked for a fluorescence imaging plate reader (FLIPR) membrane potential (FMP) assay. Among all five clones, clone 2 showed maximum responses to Leu8-OT and Pro8-OT in decreasing the FMP blue fluorescence and was selected for further studies.

**Drugs.** Leu8-OT(66-0-52; American Peptide Company) and Pro8-OT (58863; Anapec) were reconstituted in dimethylsulfoxide (DMSO) (D4540; Sigma-Aldrich). Charybdotoxins (C7802; Sigma-Aldrich) were reconstituted in ultrapure water with 0.2% DMSO. Pertussis toxin (PTX) (10,000 U/ml, 15140-163; Life Technologies), and 400 mg/ml G418 (G64000-5.0; RPI Corp.) were reconstituted in ultrapure water with 5 mg/ml bovine serum albumin (BP1600-100; Fisher Scientific). Dynorphin A (1-13) amide (26-4-51A; American Peptide Company) was dissolved in 25 mM Tris at pH 7.4. Apamin (A1289; Sigma-Aldrich) was reconstituted in 0.05 M acetic acid.

**Intracellular Calcium Mobilization Assay.** The effect of OT addition on intracellular calcium mobilization was examined using Fluor3-AM fluorescence (F1241; Molecular Probes) monitored with a FLIPR2 plate reader (Molecular Devices). FLIPR operates by illuminating the bottom of a 96-well microplate with an air-cooled laser and measuring the fluorescence emissions from cell-permeant dyes in all 96 wells simultaneously using a cooled charge-coupled device camera. This instrument is equipped with an automated 96-well pipettor, which can be programmed to deliver precise quantities of solutions simultaneously to all 96 culture wells from two separate 96-well source plates.

Cells were plated at 0.3 million cells/ml in 96-well plates (P9803; MidSci) and cultured overnight in culture media at 37°C in 5% CO2 and 95% humidity. On the day of assay, growth medium was aspirated and replaced with 100 μl dye-loading medium per well containing...
4 μM Fluo-3 AM and 0.04% pluronic acid (P3000MP; Molecular Probes) in Locke’s buffer (8.6 mM HEPES, 5.6 mM KCl, 154 mM NaCl, 5.6 mM glucose, 1.0 mM MgCl2, and 2.3 mM CaCl2, pH 7.4). Cells were incubated for 1 hour at 37°C in 5% CO2 and 95% humidity and then washed four times in 180 μl fresh Locke’s buffer using an automated microplate washer (Bio-Tek Instruments Inc.). Baseline fluorescence was recorded for 60 seconds, prior to a 20 μl addition of various concentrations of Leu6-OT and Pro3-OT. Cells were excited at 488 nm and Ca2+-bound Fluo-3 emission was recorded at 538 nm at 2-second intervals for an additional 200 seconds.

To assess the role of intracellular calcium in the OT mobilization of calcium, the sarcoendoplasmic reticulum Ca2+-ATPase (SERCA) inhibitor thapsigargin was used to rapidly deplete intracellular calcium stores. Thapsigargin inhibition of calcium mobilization in prostate cancer cells is complete in less than 5 minutes (Sehgal et al., 2017). Cells were incubated in 100 μl dye-loading medium per well containing 4 μM Fluo-3 AM and 0.04% pluronic acid in Locke’s buffer (8.6 mM HEPES, 5.6 mM KCl, 154 mM NaCl, 5.6 mM glucose, 1.0 mM MgCl2, 2.3 mM CaCl2, and 0.5 mM probenecid, pH 7.4), at 37°C in 5% CO2 and 95% humidity for 60 minutes prior to washing four times in 180 μl Locke’s buffer and 10 μl addition of thapsigargin (1 μM final concentration) and incubated for an additional 5 minutes. Intracellular calcium mobilization assays were performed as described above.

Membrane Potential Assay. The FLIPR Membrane Potential Assay (FMP blue, F1241; Molecular Probes) was used to assess changes in membrane potential. Confluent cells were plated at 0.3 million cells/ml in 96-well plates (P8903; MidSci) and cultured overnight in culture media at 37°C in 5% CO2 and 95% humidity. The growth medium was removed and replaced with 190 μl final Locke’s buffer using an automated microplate washer (Bio-Tek Instruments Inc.). Baseline fluorescence was recorded for 60 seconds, prior to a 20 μl addition of log concentrations of Leu6-OT and Pro3-OT. Cells were excited at 530 nm and emission was recorded at 565 nm at 2-second intervals for an additional 200 seconds.

To ensure the veracity of comparisons of EC50 and maximum response achievable (Emax) values of OT variants, all compounds were evaluated in parallel on the same 96-well plate, with the same split of cells and with identical reagent solutions. This experimental design was used for all OT peptide comparisons throughout this study, using both hOTR- and mOTR-expressing cells. Inasmuch as all assays were performed in the same CHO cell line, we can exclude differences in cellular context as a source of observed differences in peptide potency or efficacy.

To assess the role Gq in OT ligand-induced membrane hyperpolarization, cells were incubated overnight with PTX to inactivate Gq (Zhou et al., 2007). Cells were plated at 125,000 cells/ml in 96-well plates. PTX (150 ng/ml) was added 24 hours after plating and incubated for an additional 24 hours. The membrane potential assay was performed as described above. To confirm the influence of PTX on a known Gq-mediated response, the effect of PTX on αOR-mediated hyperpolarization was used as a positive control (Murthy and Makhlof, 1996). αOR-CHO cells were used for these experiments. The PTX assays were performed as described above for mOTR- and hOTR-expressing CHO cells, except for stimulation with dynorphin addition of challenge with NS-1619 rather than OT analogs.

SKA-31 is an activator of IKCa channel KCa3.1 (Sankaranarayanan et al., 2009; Christophersen and Wulff, 2015). If changes in intracellular calcium are responsible for the activation of KCa3.1, the response should be SKA-31 sensitive. Cells were incubated at 37°C in 5% CO2 and 95% humidity for 35 minutes prior to a 10 μl addition of SKA-31. Cells were incubated for 5 minutes after the addition of paxilline. Membrane potential assays were performed as described above, with the exception of challenge with NS-1619 rather than OT analogs.

BAFTA-AM is an intracellular calcium chelator (Strayer et al., 1999). If changes in intracellular calcium are responsible for activation of the Ca2+-activated potassium channels, the response should be BAFTA-AM sensitive. Cells were incubated at 37°C in 5% CO2 and 95% humidity for 35 minutes prior to a 10 μl addition of BAFTA-AM. Cells were incubated for an additional 10 minutes after drug addition.

Statistical Analysis. All concentration-response data were analyzed and graphs were generated using GraphPad Prism software. EC50 and Emax values for OT peptide-stimulated increases in Fluo-3 fluorescence or decreases in FMP blue fluorescence were determined by nonlinear regression least-squares fitting of a logistic equation to the peptide concentration versus fluorescence area under the curve data. The 95% confidence intervals (CIs) for all EC50/IC50 and Emax values were used to assess differences in potency and efficacy. R2 was used to assess goodness of fit. A one-way ANOVA was performed with Sidak multiple comparisons to determine statistical significance and the adjusted P values are reported.

Results

OT Analogs Induce Gq-Mediated Intracellular Calcium Mobilization. Gq mediates intracellular calcium mobilization by activation of phospholipase C (PLC) β with
attendant inositol phosphate and diacylglycerol production (Ritter and Hall, 2009). To assess OTR activation of \( G_q \), functional assays were performed using Fluo-3 AM as a calcium indicator dye. We asked whether Leu8-OT, found in most mammals, and Pro8-OT, found in many NWMs, show differential mobilization of intracellular \( \text{Ca}^{2+} \) upon activation of mOTRs. In mOTR CHO cells, we found that the two OT ligands produced a concentration-dependent elevation of intracellular calcium with similar potencies (EC\(_{50}\)), but the cognate ligand Pro8-OT was more efficacious (Emax) than Leu8-OT (Fig. 1, A–C; Table 1). In contrast, we found that the two OT ligands showed similar potencies and efficacies in increasing intracellular calcium concentration in hOTR CHO cells (Fig. 1, D–F; Table 1). The absence of a Leu8-OT effect on calcium concentration in nontransfected CHO-K1 cells demonstrated that the OT peptide effects observed in transfected cell lines required mOTR and hOTR expression (Supplemental Fig. 1).

Thapsigargin is a potent inhibitor of SERCA, which is responsible for maintaining the gradient between the low calcium cytosol and the sarco/endoplasmic reticulum high calcium storage. Inhibition of the SERCA pump results in a depletion of intracellular calcium stores (Dravid and Murray, 2004; Quynh Doan and Christensen, 2015). To confirm the role of intracellular calcium stores in OT-mediated calcium influx, cells were pretreated with thapsigargin. In control mOTR and hOTR CHO cells, Leu8-OT and Pro8-OT again produced concentration-dependent increases in intracellular calcium; however, pretreatment with thapsigargin abrogated this response in CHO cells expressing both mOTR and hOTR for both OT analogs (Supplemental Fig. 2). Together these data demonstrated that intracellular calcium stores represent the source of OT-mediated elevation of cytosolic calcium levels.

**OT Analog-Induced Changes in Membrane Potential Are Dependent on \( G_q \)-Mediated Calcium Mobilization.** OT analog activation of OTR and coupling to \( G_i \) have been shown to stimulate \( K_+ \) channel conductances with attendant cellular hyperpolarization (Phaneuf et al., 1993; Ritter and Hall, 2009; Gravati et al., 2010). To assess potential OTR activation of \( K_+ \) channel conductance, we performed functional assays using the membrane potential-sensitive dye, FMP blue. FMP blue dye is a lipophilic, anionic, bis-oxonol–based dye that distributes across the cell membrane as a function of membrane potential and displays depolarization-induced increased fluorescence emission after binding to intracellular proteins or decreased fluorescence after hyperpolarization-induced egress from cells (Whiteaker et al., 2001; Baxter et al., 2002). In mOTR CHO cells, both Leu8-OT and Pro8-OT produced concentration-dependent decreases in FMP blue fluorescence consistent with a hyperpolarization response. Leu8-OT showed substantially greater potency compared with Pro8-OT in the observed changes in membrane potential, with the two OT ligands showing comparable efficacies (Fig. 2, A–C; Table 2). A similar pattern was observed in hOTR CHO cells, with Leu8-OT displaying greater potency than Pro8-OT with regard to changes in membrane potential (Fig. 2, D–F; Table 2). The absence of Leu8-OT and Pro8-OT effects on membrane potential in nontransfected CHO-K1 cells again demonstrated the requirement for mOTR

<table>
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<tr>
<th>Parameter</th>
<th>Leu8-OT</th>
<th>Pro8-OT</th>
<th>Rank Order Potency</th>
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<tbody>
<tr>
<td>mOTR</td>
<td>EC(_{50})</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>0.3–1.0</td>
<td>0.2–0.7</td>
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<tr>
<td></td>
<td>R(^2)</td>
<td>0.90</td>
<td>0.92</td>
</tr>
<tr>
<td>hOTR</td>
<td>EC(_{50})</td>
<td>0.7</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>0.4–1.5</td>
<td>0.7–3.6</td>
</tr>
<tr>
<td></td>
<td>R(^2)</td>
<td>0.87</td>
<td>0.84</td>
</tr>
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and hOTR transfection in the observed hyperpolarization responses to OT ligands (Supplemental Fig. 3).

Several classes of G-protein α subunits, including G_{i} and G_{o}, can be mono-ADP-ribosylated by the exotoxin from the Gram-negative bacterium Bordetella pertussis. PTX catalyzes the covalent transfer of an ADP-ribose from NAD^{+} to a cysteine residue four amino acids from the carboxy termini of these α subunits (Murray and Siebenaller, 1993). This ADP-ribosylation disrupts the coupling between GPCRs and PTX-sensitive G proteins and therefore potentially interferes with responses to agonists such as OT. We tested mOTR CHO cells and observed that PTX treatment partially affected Leu8-OT–mediated hyperpolarization, with a significant 31.9% reduction in efficacy. The Leu8-OT E_{\text{max}} was 3590 (95% CI, 3088–4093) in control cells compared with 2446 in PTX-pretreated cells (95% CI, 1893–2999) (Fig. 3A; Supplemental Fig. 4, A and C). In contrast, pretreatment with PTX did not significantly inhibit Leu8-OT–mediated hyperpolarization in hOTR CHO cells (Fig. 3C; Supplemental Fig. 4, E and G; Supplemental Table 1). PTX treatment did not affect Pro8-OT–induced hyperpolarization in either mOTR-expressing (Fig. 3B; Supplemental Fig. 4, B and D) or hOTR CHO cells (Fig. 3D; Supplemental Fig. 4, F and H). These data demonstrate that in mOTR CHO cells, Leu8-OT–induced hyperpolarization is partially sensitive to PTX. The insensitivity of Pro8-OT to PTX in mOTR and hOTR CHO cells indicates a lack of involvement of G_{i}–mediated activation of GIRKs in the observed changes in membrane potential. In contrast, the partial sensitivity of Leu8-OT–induced changes in membrane potential in mOTR-expressing cells suggests that both G_{i}-mediated and PTX-insensitive pathways are involved in the hyperpolarization in response to this peptide.

We used a κOR-CHO cell line as a positive control to demonstrate the ability of PTX to disrupt G-protein coupling to a GPCR. κORs couple to the PTX substrate G_{i}, Dynorphin A 1-13-NH_{2} was used as the κOR agonist for these experiments.

Dynorphin A 1-13-NH_{2} produced a robust hyperpolarization response in control κOR-CHO cells, and this response was abrogated in PTX-pretreated cells (Supplemental Fig. 5). These data demonstrate the effectiveness of PTX in disrupting GPCR coupling to G_{i}.

PTX disrupts GPCR interaction with sensitive G proteins, thereby interrupting downstream G_{a}- and G_{bg}-dependent signaling. To further assess the partial G_{i} mediation of Leu8-OT–induced changes in membrane potential in mOTR CHO cells, the G_{bg} inhibitor M119K was used. M119K binds to G_{bg} with high affinity, and in vitro studies demonstrate that it inhibits G_{bg} function (Bonacci et al., 2006; Kirui et al., 2010). G_{bg} subunits can directly activate GIRK channels, and reassociation with the G_{a} subunit terminates this signaling (Petit-Jacques et al., 1999; Lin and Smrcka, 2011). In mOTR and hOTR CHO cells, pretreatment with M119K did not produce a statistically significant reduction in Leu8-OT–induced membrane hyperpolarization (Supplemental Fig. 6; Supplemental Table 2). Together, these data suggest that in mOTR-expressing cells, but not hOTR CHO cells, Leu8-OT modulation of membrane

### TABLE 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ligand</th>
<th>Rank Order Potency</th>
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<tr>
<td></td>
<td>pM</td>
<td>nM</td>
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<tr>
<td>mOTR</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td>R^{2}</td>
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</tr>
<tr>
<td>hOTR</td>
<td>EC_{50}</td>
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</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>4.4–150.0</td>
</tr>
<tr>
<td></td>
<td>R^{2}</td>
<td>0.53</td>
</tr>
</tbody>
</table>

The table shows the potency of Leu8-OT and Pro8-OT at inducing membrane hyperpolarization in mOTR and hOTR CHO cells.
potential is partially mediated by GIRK channels, but a role for Gβγ-dependent signaling was not established.

Given that Pro8-OT–induced changes in membrane potential were insensitive to PTX and Leu8-OT–induced changes were only partially sensitive in mOTR-expressing cells, we next considered the possibility that OT peptide-induced hyperpolarization may involve coupling to Gq with activation of PLCβ and calcium-dependent K1 channel activation. To explore the role of Ca2+-activated K1 channels in OT analog-induced changes in membrane potential, we used a pharmacological approach with compounds that discriminate between subtypes of Ca2+-activated K1 channels. To assess the role of SKCa channels in OT-mediated membrane hyperpolarization in mOTR and hOTR cells, cells were pretreated with the SKCa-selective blocker apamin. Molecular modeling and mutational studies suggest that apamin functions to block SKCa channels through an allosteric mechanism rather than a classic pore block (Lamy et al., 2010). In mOTR and hOTR CHO cells, apamin produced very modest inhibition of Leu8-OT–induced changes (16.4% and 6.6%, respectively) (Fig. 4, A and C; Supplemental Figs. 7A and 8A, Supplementary Table 3) and did not affect Pro8-OT–induced changes in membrane potential (Fig. 4, B and D; Supplemental Figs. 7B and 8B, Supplementary Table 3). To confirm that OT-analog vehicle DMSO (0.02%) and apamin solvent acetic acid (5 mM) did not affect membrane hyperpolarization, additional controls of DMSO vehicle and acetic acid solvent were performed. Neither DMSO nor acetic acid vehicles alone affected membrane potential in mOTR- and hOTR-expressing cells (Supplemental Fig. 9). These data indicate that the acetic acid vehicle did not substantially affect membrane hyperpolarization and that SKCa channels provide minimal contribution to OT analog-induced changes in membrane potential in either mOTR- or hOTR-expressing CHO cells.

Charybdotoxin exhibits blocking effects on both IKCa and BKCa (Anderson et al., 1988; MacKinnon and Miller, 1988; Ishii et al., 1997; Qiu et al., 2009). Charybdotoxin binds to the BKCa channel in either the open or closed conformation and dissociation from the BKCa channel is voltage dependent (MacKinnon and Miller, 1988). In mOTR CHO cells, charybdotoxin did not affect changes in membrane potential produced by either Leu8-OT (Fig. 4A; Supplemental Fig. 7C, Supplementary Table 3) or Pro8-OT (Fig. 4B; Supplemental Fig. 7D, Supplementary Table 3); however, in hOTR CHO cells, charybdotoxin modestly reduced Leu8-OT– and Pro8-OT–induced hyperpolarization by 17.0% (Fig. 4C; Supplemental Fig. 8C, Supplementary Table 3) and 24.3% (Fig. 4D; Supplemental Fig. 8D, Supplementary Table 3), respectively. These results suggested that IKCa and/or BKCa channels may partially contribute to OT-mediated changes in membrane potential. To further assess the role of BKCa channels, mOTR and hOTR cells were pretreated with the BKCa blocker paxilline. Paxilline produces inhibition by stabilizing the BKCa channels in the closed conformation (Zhou and Lingle, 2014). In mOTR CHO cells, paxilline did not affect Leu8-OT–induced changes in membrane potential (Fig. 4A; Supplemental Fig. 7C, Supplementary Table 3), whereas the Pro 8-OT response was reduced by 40.5% (Fig. 4B; Supplemental Fig. 7D, Supplementary Table 3). In hOTR CHO cells, paxilline modestly inhibited hyperpolarization by both Leu8-OT (20.6%) (Fig. 4C; Supplemental Fig. 8C, Supplementary Table 3) and Pro8-OT (26.5%) (Fig. 4D; Supplemental Fig. 8D, Supplementary Table 3), suggesting that BKCa channels do contribute to OT-mediated changes in membrane potential by hOTR. To confirm the involvement of BKCa channels in hyperpolarization of mOTR and hOTR CHO cells, we next used the BKCa activator NS-1619. In mOTR and hOTR CHO cells, paxilline inhibited NS-1619–induced membrane
inhibited the Leu8-OT response by 59.2% (Fig. 4A; Supplemental Fig. 8E, Supplementary Table 3). Similarly, in hOTR CHO cells, the combined exposure of paxilline and TRAM-34 inhibited both Leu8-OT and Pro8-OT–induced hyperpolarization by ~85% (Fig. 4; Supplemental Figs. 7, G and H and 8, G and H, Supplementary Table 3), indicating an additive effect. These data confirm that BKCa and IKCa channels are largely responsible for OT-induced changes in membrane potential.

To directly assess the role of calcium in OT-mediated membrane hyperpolarization, cells were pretreated with the intracellular calcium chelator BAPTA-AM. In both mOTR and hOTR CHO cells, BAPTA-AM exposure blocked hyperpolarization of membrane potential with either Leu8-OT or Pro8-OT (Supplemental Fig. 11, A, B, E, and F). Interestingly, in BAPTA-AM–treated hOTR CHO cells, a Leu8-OT–induced depolarization was observed (Supplemental Fig. 11E), indicating a possible dual modulation of K+ channel currents by the OTR (Gravati et al., 2010).

We next confirmed the role of intracellular calcium stores in OT-mediated changes in membrane potential by pretreating cells with thapsigargin and measuring membrane potential responses to OT analogs in mOTR and hOTR CHO cells. As expected, pretreatment with thapsigargin eliminated hyperpolarization produced by either Leu8-OT
played significant OT peptide-specific differences in potency. The membrane-hyperpolarizing responses dis- and hOTR-expressing cells in a concentration-dependent OT ligands induced membrane hyperpolarization in mOTR- and hOTR-expressing CHO cells. The observed EC50 values were consistent with results from hOTR-expressing HEK cells in comparable to results from hOTR-expressing cell lines (Busnelli et al., 2017). The results of these previous studies with hOTR were extended in this investigation by comparing mOTR and hOTR signaling responses. The partial PTX sensitivity observed at the mOTR with Leu8-OT, but not Pro8-OT, appears to represent an agonist functional selectivity where the two OT ligands activate a single receptor but produce distinct signaling outcomes (Rankovic et al., 2016). A variety of hormones and neurotransmitters acting at GPCRs are capable of producing [Ca2+]i elevation typically mediated by Ca2+-release from the endoplasmic reticulum via the Gq/phosphoinositide-PLC pathway. This Gq signaling pathway affords another potential mechanism for hyperpolarizing responses through activation of Ca2+-dependent potassium channels. A role for Ca2+-activated K+ channels in the hyperpolarization responses observed in mOTR- and hOTR-expressing CHO cells was therefore assessed using BKCa (KCa1.1), IKCa (KCa3.1), and SKCa channel blockers. Paxilline selectively blocks BKCa channels, and pretreatment with this inhibitor resulted in a significant reduction in the hyperpolarization response observed with Pro8-OT in mOTR cells and the response to both Leu8-OT and Pro8-OT in hOTR-expressing cells. Paxilline also inhibited the hyperpolarizing response to the BKCa channel opener NS-1619 in both mOTR and hOTR CHO cells, further supporting a role for a BKCa channel contribution to the observed membrane hyperpolarization. These results agree with those of an earlier report demonstrating that Leu8-OT hyperpolarized myenteric intrinsic primary afferent neurons by activating BKCa channels via the OTR-PLC-inositol trisphosphate-Ca2+ signaling pathway (Che et al., 2012).

TRAM-34 inhibited between 58% and 73% of the hyperpolarizing responses to both OT ligands, suggesting that
K_{Ca}3.1 is largely responsible for membrane hyperpolarization produced by OT peptides in mOTR- and hOTR-expressing CHO cells. TRAM-34 also inhibited the hyperpolarizing response to K_{Ca}3.1 opener SKA-31, further demonstrating the involvement of K_{Ca}3.1 in observed membrane hyperpolarization. The critical role of [Ca^{2+}]_i elevation in hyperpolarization was demonstrated using BAPTA-AM to chelate intracellular Ca^{2+} (Strayer et al., 1999). Pretreatment with BAPTA-AM eliminated membrane hyperpolarization in response to both OT analogs in mOTR and hOTR cells. Similarly, passive depletion of endoplasmic reticulum Ca^{2+} stores by the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (Dravid and Murray, 2004; Quynh Doan and Christensen, 2015), also inhibited OT-induced membrane hyperpolarization produced by both OT analogs in both cell lines. These data confirm that the observed OT ligand-induced membrane hyperpolarization in mOTR- and hOTR-expressing CHO cells was primarily mediated by intracellular Ca^{2+} mobilization with subsequent activation of Ca^{2+}-dependent K^{+} channels, including K_{Ca}3.1.

OT is a fundamental mediator of sociobehavioral processes, including social cognition (Crespi, 2016), interpersonal trust (Kosfeld et al., 2005; Baumgartner et al., 2008), anxiety (Missig et al., 2010), and stress response (Light et al., 2000; Cavanaugh et al., 2016), generating interest in OT as a potential therapeutic mediator of sociobehavioral deficits in conditions such as autism spectrum disorder (Andari et al., 2010; Anagnostou et al., 2012), post-traumatic stress disorder (Frijling, 2017; Sack et al., 2017), and schizophrenia (Pedersen et al., 2011; Brambilla et al., 2016). One major challenge is connecting pharmacologic signatures to sociobehavioral processes. Identification of the mechanisms by which OT analogs affect OTR-mediated signaling is crucial to translating signaling activation at the cellular level to effects of OT ligands on social behaviors. In clinical trials for sociobehavioral deficits, intranasal OT is used because peripheral administration does not cross the blood-brain barrier (Born et al., 2002). Intranasal OT appears to be safe and well tolerated (Anagnostou et al., 2012) and imaging evidence suggests that OT induces increased activity in the “social brain” (Bethlehem et al., 2013). However, clinical trials for OT treatment of sociobehavioral deficits with various dosing schedules (single vs. multiple) and routes (intravenous vs. intranasal) have shown mixed results (Alvarenga et al., 2017), suggesting that greater understanding of OT-triggered signaling pathways downstream of the OTR could facilitate interpretation of sociobehavioral effects and lead to more refined therapeutic targeting.

These results show that Leu^8-OT and Pro^8-OT display functionally distinct responses when activating either the mOTR or hOTR. These distinct characteristics included peptide potency and efficacy as well as G-protein subtype coupling. Pro^8-OT was shown to be more efficacious than Leu^8-OT in activating the G_{q}Ca^{2+} mobilization assay in mOTR cells. Uniquely, Leu^8-OT was much more potent than Pro^8-OT in producing a hyperpolarization in both mOTR and hOTR. A final salient difference in the observed pharmacologic signatures of the two peptides was that the Pro^8-OT–induced hyperpolarization responses in both mOTR and hOTR were PTX insensitive, whereas the response to Leu^8-OT in mOTR was partially sensitive. Further functional characterization of OT analogs may therefore provide insight into the structural requirements for functionally selective or biased agonists that open new possibilities for drug discovery and the advancement of OT-mediated therapeutics.

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Authorship Contributions

Participated in research design: Pierce, Mehrotra, Murray.
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