Identification of dimeric and oligomeric complexes of the human oxytocin receptor by co-immunoprecipitation and bioluminescence resonance energy transfer

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Abstract

The nonapeptide hormone oxytocin exerts many important biological functions, including uterine contractions during parturition and milk ejection during lactation. The manifold effects of oxytocin are mediated by a single oxytocin receptor (OTR) type, a member of the super-family of G-protein-coupled receptors. There is accumulating recent evidence that certain G-protein-coupled receptors exist in the form of oligomeric complexes. Here we demonstrate, using two different co-immunoprecipitation strategies as well as bioluminescence resonance energy transfer techniques, that the OTR is capable of forming oligomeric complexes *in vivo* and that these complexes exist at the cell surface membrane. The human OTR was N-terminally tagged with either a Myc or Flag epitope and transiently expressed in COS-7 cells. Cell lysates were immunoprecipitated using an anti-Flag antibody and analyzed by SDS-PAGE and Western blotting using an anti-Myc antibody, or vice versa. Either strategy provided evidence for the co-precipitation of Myc- or Flag-tagged OTR respectively.

Biochemical characterization of OTR dimers showed that homodimer formation is not dependent on the establishment of disulfide bonds. The existence of OTR dimers and oligomers at the level of the cell surface was demonstrated by exposing intact living cells to an anti-Flag antibody and analyzing the immunoprecipitate by Western blotting with an anti-Myc antibody. This approach demonstrated furthermore that the presence of receptor oligomers at the cell surface is modulated by ligand in a time-dependent fashion. Finally, we obtained evidence that the OTR is forming oligomeric structures in intact living cells by observing the occurrence of bioluminescence resonance energy transfer in cells co-transfected with OTR constructs bearing at their C-terminus either a *Renilla* luciferase or the yellow fluorescent protein. Taken together, these data show that the OTR can form homodimers and oligomers in the cell model used and that these oligomers are present at the cell surface.

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Introduction

The receptor for the multifunctional cyclic nonapeptide oxytocin (OT) is a G-protein-coupled receptor (GPCR) that forms part of the OT/vasopressin receptor family. The OT receptor (OTR) is able to couple functionally to both Gq/11 and Gi proteins (Ku *et al.* 1995, Strakova & Soloff 1997). OTR activation triggers multiple molecular events, including an increase in intracellular free

calcium concentration, activation of mitogen-activated protein kinase, protein kinase-C and myosin light-chain kinase and synthesis of prostaglandin E2 (Zingg & Laporte 2003). The mechanisms by which the multiple OTR signaling cascades are specifically activated and modulated are still incompletely understood. Several studies have assessed the ligand/receptor interactions between the OTR and OT by photo-affinity labeling (Breton *et al.* 2001), site-directed

mutagenesis (Fanelli *et al.* 1999, Hawtin *et al.* 2001) or the construction of receptor chimeras (Postina *et al.* 1996).

All these studies refer to the OTR in its monomeric form. However, a growing body of evidence now indicates that certain GPCRs function as oligomeric complexes (Angers *et al.* 2002, Eidne 2002). Complexes can form between the same or different classes of receptors and the oligomerization process can be either dependent on (Patel *et al.* 2002) or independent of ligand binding (Issafras *et al.* 2002). Furthermore, many reports show the importance of GPCR oligomerization for multiple aspects of receptor biology such as intracellular signaling, trafficking, pharmacology and sequestration (Salahpour *et al.* 2000, Angers *et al.* 2002).

A complete understanding of OTR structure/function relationships requires a definition of the oligomeric state of the OTR *in vivo*. As a first step towards resolving this problem, we have applied different co-immunoprecipitation strategies as well as an *in vivo* approach. We now report that the human OTR can form dimers and oligomers as demonstrated *in vitro* by co-immunoprecipitation and *in vivo* by bioluminescence resonance energy transfer (BRET) in transiently transfected COS-7 cells. The characterization of such complexes will be a prerequisite for a full understanding of the mechanism of OTR action.

While the present paper was under review, the formation of OTR dimers during biosynthesis was described by S. Terrillon *et al.* (2003).

Materials and methods

DNA plasmids

Plasmids encoding the wild-type human OTR or recombinant OTRs tagged at the N-terminus with either a Myc or a Flag epitope were a generous gift of Dr Bice Chini, Milan, Italy (Flag-OTR and Myc-OTR respectively). The plasmid B2R-YFP, encoding the human bradykinin B2 receptor with the coding region for the yellow fluorescent protein (YFP) added in frame at its C-terminus, was a gift from Dr S A Laporte, Montreal, Canada. The following additional constructs were derived: 3 × Flag-OTR, an OTR construct with a triple Flag epitope added to the N-terminus by inserting the OTR coding sequence in frame into the

expression vector p3 × Flag-CMV-7·1 (Sigma); OTR-luc, an OTR construct with the coding sequence for *Renilla* luciferase added in frame to the OTR C-terminus by inserting the OTR coding region into the vector pRL-CMV (Promega); OTR-YFP, an OTR construct with the coding sequence for YFP added in frame to the OTR C-terminus, obtained by inserting the OTR coding region in frame into the vector pEYFP (Clontech, Palo Alto, CA, USA). The sequence integrity of all constructs was verified by dideoxy sequencing.

Tissue culture, transfections and co-immunoprecipitations

COS-7 cells were cultured in DMEM 'high glucose' media (Gibco) supplemented with 10% fetal bovine serum. The cells were cultured in the absence of antibiotics to ensure contamination-free conditions. The day before transfection, COS-7 cells were plated at a density of 2×10^6 cells per 10 cm dish. Cells were transiently transfected with a total of 5 µg DNA using Effectene (Qiagen) according to the manufacturer's recommendations. Cell medium was changed the following day. Forty-eight hours post-transfection, cells were lysed at room temperature for 30 min in 1 ml lysate buffer (25 mM Hepes (pH 7·4), 5 mM EDTA, 50 mM NaCl, 10% glycerol, 1% Triton X-100, 50 mM NaF, 30 mM sodium pyrophosphate and protease inhibitors (1 µg/ml each of aprotinin, leupeptin and pepstatin A, and 1 mM phenylmethylsulfonyl fluoride)). To avoid any non-specific OTR aggregation caused by disulfide bond formation, the sulfhydryl alkylating reagent iodoacetamide (IOA) was present at a concentration of 10 mM during the solubilization and immunoprecipitation steps. The following steps were done at 4 °C, except where stated otherwise. The lysates were cleared by centrifugation at 13 400 *g* for 10 min and protein content of the supernatant was evaluated using a BCA Protein Assay Reagent Kit (Pierce, Brockville, Ontario, Canada). For co-immunoprecipitation, 500–1000 µg protein/ml were mixed with either 10 µg anti-Myc antibody (clone 9E10; Upstate Biotechnology, Lake Placid, NY, USA) or 4 µg anti-Flag antibody (Sigma) for 3 h. The antibody–antigen complexes were precipitated by protein G-linked Sepharose (Pharmacia) for 30 min and the beads were washed twice with lysate buffer (without IOA). The beads were

resuspended for 15 min at room temperature in 20 μ l urea buffer (9.6 M urea, 4% SDS, 250 mM Tris (pH 6.8), 10% glycerol, 130 mM dithiothreitol (DTT) and bromophenol blue). In some experiments, to avoid non-reduced immunoglobulins masking high molecular complexes, the co-immunoprecipitation was performed using the anti-Flag antibody covalently linked to protein G Sepharose according to the protocol of Schneider *et al.* (1982). The denatured co-immunoprecipitation products were subjected to SDS-PAGE analysis and then electro-blotted onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA, USA) overnight at 45 V in 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid buffer (pH 11.0) containing 10% methanol. The tagged receptors were immunodetected using the same antibodies used previously for co-immunoprecipitation in conjunction with a secondary anti-mouse antibody conjugated to horseradish peroxidase C. Bands were visualized using a chemiluminescent detection system (SuperSignal; Pierce).

Cell surface co-immunoprecipitation

Forty-eight hours post-transfection, COS-7 cells were stimulated or not by 100 nM OT for different times at 37 °C. At the end of the incubation period, the cells were washed twice with ice-cold PBS, followed by one wash in Krebs/Hepes buffer (146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl₂, 10 mM Hepes (pH 7.4) and 5.9 mM glucose). Five milliliters of Krebs/Hepes containing 0.5% BSA and 10 μ g/ml anti-Flag antibody were added to each dish and left overnight at 4 °C. The plates were extensively washed with PBS and cells were lysed as before except that no IOA was added to the lysate buffer. Whole-cell lysates were then incubated with the protein G Sepharose and co-immunoprecipitation complexes were treated and analyzed as mentioned earlier.

BRET

The oligomerization of OTR *in vivo* was assessed by BRET in transiently transfected COS-7 cells. Twenty-four hours after transfection, cells were replated onto a 96-well plate at 10⁵ cells/well. The following day, medium was replaced with 100 μ l Krebs/Hepes buffer following three washes with

the same buffer. Coelenterazine h (Molecular Probes, Eugene, OR, USA) was added at a final concentration of 10 μ M, and luminescence emission was measured immediately thereafter at both 460 nm and 530 nm using an FL 600 spectrofluorometer (Bio-Tek Instruments Inc., Winooski, VT, USA). The BRET ratio was calculated using the formula: $(\text{luminescence}_{530 \text{ nm}} - \text{Bg}_{530 \text{ nm}}) / (\text{luminescence}_{460 \text{ nm}} - \text{Bg}_{460 \text{ nm}})$ where Bg_x is the background at wavelength x nm, obtained by taking a luminescence measurement in the absence of substrate.

Results

Evidence for OTR oligomerization by co-immunoprecipitation

To investigate whether OTR can form dimers and oligomers, we first developed a co-immunoprecipitation strategy using two different OTR constructs that carried at their N-terminus either the Flag (Flag-OTR) or the Myc epitope (Myc-OTR). Co-immunoprecipitation of Flag immunoreactivity with the anti-Myc antibody or co-immunoprecipitation of Myc immunoreactivity with the anti-Flag antibody was taken as evidence for the existence of a physical association between Flag-OTR and Myc-OTR. The results of co-immunoprecipitation experiments using either of these approaches are shown in Fig. 1. Myc-immunoreactive bands were detected by Western blotting of Flag immunoprecipitates from cells transfected with both Flag-OTR and Myc-OTR plasmids (Fig. 1A, lane 1), but not from cells transfected with Myc-OTR alone (lane 2). Conversely, Flag-immunoreactive bands were detected by Western blotting of Myc immunoprecipitates from cells transfected with both Flag-OTR and Myc-OTR plasmids (Fig. 1B, lane 4), but not from cells transfected with Flag-OTR alone (lane 5). The bands detected corresponded to the molecular mass of the glycosylated form of the OTR at 70–80 kDa (Breton *et al.* 2001), the non-glycosylated form of the OTR at 38 kDa (Breton *et al.* 2001) and higher molecular mass complexes that correspond most likely to SDS-resistant dimers and oligomers.

Receptor density in this *in vitro* system was 635 $\pm$ 47 fmol/mg protein (*n* = 3), as determined by Scatchard analysis. Because, *in vivo*, OTR densities

Figure 1 Demonstration of human OTR oligomerization by co-immunoprecipitation. (A) Western blot analysis of immunoprecipitates of total lysates of COS-7 cells transiently transfected with expression vectors encoding N-terminally tagged OTR. Cells were transfected as follows: lanes 1 and 4: Myc-OTR and Flag-OTR; lane 2: Myc-OTR alone; lane 3: mixture of two cell populations transfected separately with Myc-OTR or Flag-OTR; lane 5: Flag-OTR alone. Immunoprecipitation was performed using either an anti-Flag antibody (lanes 1–3) or an anti-Myc antibody (lanes 4 and 5) and immunodetection occurred with an anti-Myc antibody. (B) Same blot as in (A) following stripping and immunoblotting with an anti-Flag antibody. Arrows indicate bands corresponding in molecular mass to the glycosylated form of the OTR. Molecular mass markers (on the left) are in kDa. The results presented are representative of two independent series of experiments. IB, immunoblot; IP, immunoprecipitate.

Figure 2 Absence of co-immunoprecipitation of abundant markers for the plasma membrane (Na/K-ATPase) or for the endoplasmic reticulum (BiP/GRP74). COS-7 cells were transfected with expression vectors for Flag-OTR and Myc-OTR (lanes 1 and 3) or Myc-OTR alone (lane 2). Total lysates (lane 1) or Flag immunoprecipitates (lanes 2 and 3) were analyzed by immunoblotting. The same membrane was immunoblotted sequentially with an anti-Na/K-ATPase antibody (Upstate Biotechnology) (A), an anti-BiP antibody (BD Biosciences, Oakville, Ontario, Canada) (B) or, as a positive control, with an anti-Myc antibody (C). Specific bands are indicated by arrows. Molecular mass markers (on the left) are in kDa. The results presented are representative of two independent series of experiments. IB, immunoblot; IP, immunoprecipitate; TL, total lysate.

can reach up to 1850 and 7300 fmol/mg protein in myometrium and endometrium respectively (Fuchs *et al.* 1992), these levels were well within the physiological range. In addition, when OTR expression levels were lowered by lowering the amount of expression plasmid used for transfection, OTR oligomers remained detectable by the co-immunoprecipitation approach at receptor densities as low as 50 fmol/mg protein.

Additional experiments were undertaken to assess the specificity of these observations. To exclude the possibility that the observed oligomerization of OTR could be due to artifactual oligomerization occurring after solubilization, we mixed, prior to immunoprecipitation, lysates from two cell populations, each expressing one of the two tagged receptor types. No specific bands were detected in this case (Fig. 1A, lane 3). To test whether the observed co-immunoprecipitation could be due to incomplete membrane solubilization or non-specific protein aggregation, we assessed whether abundant and ubiquitous proteins were co-immunoprecipitated. To this end, we

determined whether Na/K-ATPase (a marker of the plasma membrane) or the chaperone protein BiP/GRP74 (a marker of the endoplasmic reticulum) was present in immunoprecipitates. Neither of these proteins was detected in immunoprecipitates (Fig. 2A and B), whereas OTR co-immunoprecipitation remained readily detectable (Fig. 2C).

OTR oligomers are insensitive to reducing conditions

There is no consensus in the literature whether GPCR oligomerization involves the formation of disulfide bonds. Whereas some GPCR oligomers have been reported to be sensitive to reducing conditions, others have been shown to be insensitive. To test the involvement of disulfide bonds in the formation of OTR oligomers, we compared the band pattern obtained by Western blotting of co-immunoprecipitation samples treated or not with the reducing agent DTT. In the absence of DTT treatment, a high molecular mass

Figure 3 OTR oligomers are insensitive to reducing conditions. Lysates of COS-7 cells transiently transfected with Myc-OTR and Flag-OTR were immunoprecipitated using an anti-Flag antibody covalently linked to protein G Sepharose, in either the presence or absence of the sulfhydryl alkylating agent IOA (as indicated). The immunoprecipitates were denatured in either the presence or absence of the reducing agent DTT (as indicated) and immunoblotted using an anti-Myc antibody. Molecular mass markers (on the left) are in kDa. The results presented here are representative of two independent series of experiments. IB, immunoblot; IP, immunoprecipitate.

aggregate was seen (Fig. 3, lane 3). If *de novo* formation of disulfide bonds *in vitro* during the extraction and precipitation steps was prevented by addition of IOA, only bands responding to receptor

monomers, SDS-resistant dimers and some higher-order oligomers were detected (Fig. 3, lane 2). This indicated that the high molecular mass aggregates seen in lane 3 were due to disulfide bond formation that had occurred during the solubilization and precipitation steps in the non-reducing *in vitro* environment. As shown in lane 1, addition of the reducing agent DTT after co-immunoprecipitation resulted in the same band pattern as the one observed in lane 2, indicating that receptor dimerization and oligomerization that occurs *in vivo* is not sensitive to reducing agents and thus is not dependent on the formation of disulfide bonds.

OTR oligomers are present at the cell surface and are modulated by ligand addition

The experiments described above provided evidence for the existence of OTR oligomers; however, they did not provide any information with respect to the subcellular distribution of these oligomers. To answer specifically the question whether OTR oligomers were present at the cell surface, the antibody used for co-immunoprecipitation was added directly to intact living cells, thus limiting antibody access to cell surface molecules, as described (Zeng & Wess 1999). To this end, cells expressing Myc-OTR as well as an OTR endowed with a triple N-terminal Flag tag (3 × Flag-OTR) were exposed overnight to an anti-Flag antibody and, following extensive washing, antibody-tagged complexes were immunoprecipitated using protein G Sepharose beads and analyzed by immunoblotting using an anti-Myc antibody. As shown in Fig. 4, immune complexes containing Myc immunoreactivity could be efficiently precipitated by this strategy, indicating the presence of 3 × Flag-OTR/Myc-OTR dimers or oligomers at the cell surface.

The role of receptor ligands in the formation of receptor oligomers is controversial. For some receptor systems, oligomerization is reported to be induced by agonists (Patel *et al.* 2002), suppressed by agonists (Cvejic & Devi 1997) or independent of ligand addition (Ayoub *et al.* 2002). Therefore we investigated the role of OT addition on the amount of cell surface oligomers detectable with the present approach. The fact that, as described in the previous experiments, OTR oligomers were detected independently of ligand addition indicates that OTR oligomerization can occur in the absence

of agonist. However, as shown in Fig. 4C, addition of agonist led to a rapid and significant decrease in the amount of detectable surface oligomer complexes recovered by co-immunoprecipitation.

Evidence for OTR oligomerization *in vivo* using BRET

The co-immunoprecipitation experiments described so far were based on the detection of receptor oligomers in cell extracts. Thus they provided strong evidence for, but no direct proof of, receptor oligomerization occurring *in vivo*. Therefore, we next investigated the existence of receptor oligomers in real time within living cells using BRET. Briefly, BRET is a technique used to detect protein/protein interaction in intact cells and is based on a non-radiative transfer of energy between the luminescent donor *Renilla* luciferase and the fluorescent acceptor YFP (Xu *et al.* 1999). Whereas light emission by luciferase is induced by substrate addition, the characteristic fluorescence emission of YFP is strictly dependent on its close proximity to luciferase upon luminescence emission. This technique has recently been used to assess oligomerization of GPCRs in intact cells (Angers *et al.* 2000, Eidne 2002). We assessed OTR oligomerization by BRET by transiently expressing in COS-7 cells two versions of the OTR carrying at their C-terminus either the *Renilla* luciferase protein (OTR-luc) or the YFP (OTR-YFP). Cells were exposed to the membrane-permeable *Renilla* luciferase substrate coelenterazine h, and bioluminescence was measured using a spectrofluorometer. As shown in Fig. 5, co-transfection of cells with a luciferase-containing OTR-luc and OTR-YFP led to a marked and reproducible increase in the BRET ratio compared with OTR-luc-only expressing cells (bar 3 vs 1). Furthermore, no signal was detected when OTR-YFP was expressed alone, excluding any luciferase-independent fluorescence emission by YFP (bar 2). Three additional control experiments further validated these observations. First, no significant increase in the BRET ratio was observed when OTR-luc was co-expressed with free YFP, not attached to the OTR (bar 4). Secondly, no change in the BRET ratio relative to control was observed when a mix of two cell populations was used, each expressing separately OTR-luc and OTR-YFP (bar 5), demonstrating that both tagged receptors have to be co-expressed

in the same cell in order to induce a change in the BRET ratio. Thirdly, no significant change in the BRET ratio was observed when the OTR-YFP plasmid was replaced by B2R-YFP, a plasmid encoding the human bradykinin B2 receptor fused to YFP (bar 6).

Finally we assessed the effect of agonist addition on the observed BRET ratio. In contrast to our findings using the co-immunoprecipitation approach, no significant change in the BRET ratio was observed upon the addition of agonist (bars 7 vs 8). However, the fact that a significant effect on the BRET ratio was observed in the absence of agonist is consistent with the previous observation that OTR oligomers were found in the absence of ligand.

Discussion

The present report provides evidence using two independent approaches that the OTR exists in dimeric and oligomeric forms at the cell surface *in vivo*. First, using a co-immunoprecipitation approach, we demonstrated the presence of SDS-sensitive and -resistant dimers and oligomers. By the use of sulfhydryl alkylating agents, we excluded the possibility that the observed co-immunoprecipitation was due to artefactual aggregation during receptor solubilization or immunoprecipitation. Moreover, OTR oligomerization does not involve the formation of disulfide bonds. Secondly, the concept of OTR oligomerization is further supported by the detection of BRET in intact living cells. Oligomerization was observed at receptor densities that were well within the physiological range.

The constitutive nature of OTR oligomers suggests that oligomerization may be involved in receptor folding and trafficking (Angers *et al.* 2002). Using a cell surface co-immunoprecipitation strategy, we determined that OTR oligomers are indeed present at the cell surface. Treatment of cells with OT led to a decrease of surface OTR recovered by co-immunoprecipitation, implying a change in receptor/receptor interactions. Because this phenomenon correlates, in time, with receptor activation, it is possible that it may be part of the receptor activation process. A transition of the receptor from an oligomeric to a monomeric state was recently proposed to be involved in the

Figure 5 OTR oligomerization in living cells detected by BRET. COS-7 cells were transfected with either OTR-luc or OTR-YFP alone (lanes 1 and 2) or with both vectors in combination (lanes 3, 7 and 8). As a control, BRET was also assessed in cells co-transfected with OTR-luc and an expression vector for the YFP protein alone (lane 4) or in a mixture of two cell populations transfected separately with either OTR-luc or OTR-YFP (lane 5) and in cells co-transfected with OTR-luc and B2R-YFP, an expression vector encoding the human bradykinin B2 receptor fused to YFP (lane 6). To test the possible influence of agonist on the BRET ratio, cells were pretreated with 100 nM OT or vehicle for 5 min at 37 °C prior to BRET analysis (lanes 7 and 8). Each bar represents the mean $\pm$ S.E.M. of BRET ratios determined in four independent experiments. Receptor densities were 425 and 310 fmol/mg protein for the OTR-YFP and B2R-YFP respectively.

activation of the thyrotropin receptor (Latif *et al.* 2002).

However, the possibility cannot be ruled out that agonist binding does not induce monomerization *per se* but induces a conformational change in the receptor structure that leads to a decrease in the stability of receptor oligomers and, consequently, to

receptor monomerization during the immunoprecipitation step. This hypothesis could explain why, after agonist addition, we observed an apparent decrease in OTR cell surface oligomers with the co-immunoprecipitation approach but were unable to find any significant changes in the BRET ratio.

Figure 4 OTR oligomers are present at the cell surface and modulated by agonist exposure. (A) COS-7 cells transiently transfected with Myc-OTR and 3 $\times$ Flag-OTR were treated at 37 °C with 100 nM OT for different times (as indicated) and incubated overnight at 4 °C with an anti-Flag antibody. As a control, cells transfected with Myc-OTR alone were also used (lane C). Immunocomplexes were isolated using protein G Sepharose. Co-immunoprecipitated Myc-OTR was visualized by immunoblotting with an anti-Myc antibody. Molecular mass markers (on the left) are in kDa. (B) As a control for the efficiency of the immunoprecipitation reaction, the blot shown in (A) was immunoblotted using an anti-Flag antibody. (C) Quantitative analysis of Myc-OTR recovered by co-immunoprecipitation at different times after ligand addition. Bands indicated by arrows in (A and B) were quantitated densitometrically and the ratio of Myc immunoreactivity (as shown in A) vs Flag immunoreactivity (as shown in B) was plotted. Each bar represents the mean $\pm$ S.E.M. of three independent experiments. Values are expressed as a percentage of the value at time 0. * P < 0.05 vs control as determined by ANOVA. IB, immunoblot; IP, immunoprecipitate.

Among the members of the OT/vasopressin family, only the vasopressin V2 receptor has so far been shown to form oligomers. Both wild-type and a C-terminally truncated V2 receptor mutant can form SDS-resistant dimers (Hebert *et al.* 1996) and the functionality of inactive C-terminally truncated V2 receptor mutants can be rescued by co-expression of a C-terminal receptor fragment (Schoneberg *et al.* 1996, 1997). On the other hand, certain N-terminally truncated mutants can act as dominant negatives and prevent the wild-type receptor from reaching the cell surface (Zhu & Wess 1998). To what extent V2 receptor oligomers are present at the cell surface has remained controversial, however. Sadeghi *et al.* (1998) reported that V2 oligomerization was restricted to immature receptors present in the endoplasmic reticulum and that the function of the wild-type receptor remained unaltered, when co-expressed with a mutant V2 receptor lacking G-protein-coupling activity.

In the present study, we provide evidence that the OTR, another member of the OT/vasopressin family of receptors, forms oligomers that exist at the cell surface. Based on this finding, it is tempting to speculate that the OTR may form, in addition to homodimers, also heterodimers with other members of the GPCR family, including other members of the vasopressin receptor family. It remains to be determined to what extent the formation of heterodimers between the OTR and other members of the GPCR family may account for OT binding sites with altered pharmacological characteristics, such as the ones reported in the uterus (Chen *et al.* 1994).

The question remains, however, whether OTR oligomers and possible heterodimers are functional and what role, if any, they may play in the signaling process. The present observation of ligand-induced changes in the oligomerization status may indicate that, at least at the cell surface, the oligomerization process may be dynamic and thus represent an additional level of regulation of ligand-induced signaling processes.

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NOTE ADDED IN PROOF

While the present paper was under review, the formation of OTR dimers during biosynthesis was described by S Terrillon *et al.* (Terrillon S, Durroux T, Mouillac B, Breit A, Ayoub MA, Taulan M, Jockers R, Barberis C & Bouvier M 2003 Oxytocin and vasopressin V1a and V2 receptors form constitute homo- and heterodimers during biosynthesis. *Molecular Endocrinology* **17** 677–691).

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