Ligands raise the constraint that limits constitutive activation in G protein-coupled opioid receptors.

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Running title: Constitutive receptor activation and reversal of ligand efficacy

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Background: Native subtypes of G protein-coupled receptors (GPCR) show different levels of constitutive activation.

Results: Using a BRET assay to detect receptor-G protein complexes, we find that constitutive activation causes a uniform reduction of the apparent efficacy of all ligands.

Conclusion: An intramolecular energy barrier separates constitutive from ligand-regulated activation.

Significance: The data suggest that GPCR activation involves both cooperative and anticooperative components.

SUMMARY
Using a cell-free bioluminescence resonance energy transfer strategy we compared the levels of spontaneous and ligand-induced receptor-G protein coupling in δ (DOP) and μ (MOP) opioid receptors. In this assay GDP can suppress spontaneous coupling thus allowing its quantification. The level of constitutive activity was 4-5 times greater at DOP than at MOP receptor. A series of opioid analogs with a common peptidomimetic scaffold displayed remarkable inversions of efficacy in the two receptors. Agonists that enhanced coupling above the low intrinsic level of MOP receptor were inverse agonists in reducing the greater level of constitutive coupling of DOP receptor. Yet the intrinsic activities of such ligands are identical when scaled over the GDP baseline of both receptors. This pattern is in conflict with the predictions of the ternary complex model and the “two-state” extensions. According to this theory, the order of spontaneous and ligand-induced coupling cannot be reversed if a shift of the equilibrium between active and inactive forms raises constitutive activation in one receptor type. We propose that constitutive activation results from a lessened intrinsic barrier that restrains spontaneous coupling. Any ligand regardless of its efficacy must enhance this constraint to stabilize the ligand-bound complexed form.

Mutant and occasionally wild-type forms of G protein coupled receptors (GPCRs) can exist in a state of constitutive (ligand-independent) activation. Some ligands show “negative efficacy” in reversing this spontaneously active state and are thus named inverse agonists or negative antagonists (1-4). Unraveling the mechanisms of constitutive activity is important for the understanding of receptors functional chemistry (5) and may suggest novel therapeutic interventions for several genetic diseases associated with naturally occurring constitutively active receptor mutations (6-12).

Constitutive activation and inverse agonism are quantitatively predictable on the basis of the theoretical background that describes the cooperative effects between two ligand binding processes (i.e. the ligand and the Gα subunit) taking place on distinct sites of the same protein (i.e. the receptor) (13-18). However, little additional progress has been made in unraveling the mechanism and the structure-activity relationships that underlie the phenomenon of receptor constitutive activation. At least two factors hamper progress in the field. One is the difficulty to quantify the extent of ligand-independent activity. Constitutive activation can be assessed as difference in basal signaling between cells expressing or not the receptor. The magnitude of this “transfection-dependent” signaling is very small, often at the lowest limit of signal detection, and requires subtracting two larger numbers (i.e., the “basal” signaling recorded in two different cell populations). Thus, quantitative biochemical assessment of negative efficacy is difficult to accomplish and varies widely across different studies.

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Constitutive receptor activation and reversal of ligand efficacy

The second problem is the rare availability of ligand congeners exhibiting gradual variations from positive to negative values of efficacy. Even when several inverse agonists are known for a given GPCR subtype, they often belong to a different chemical class than the agonist or the neutral antagonist. Thus, it is hard to evaluate how discrete modifications of structure may tune the transition from the positive to the negative region of efficacy (19).

Here we used a cell-free bioluminescence resonance energy transfer (BRET) assay of receptor-G protein interaction to measure intrinsic and ligand-dependent coupling. In this system receptor binding to endogenous Gα subunits results in reduced distance between the receptor C-terminal and the N-terminal region of the Gβγ subunit. This causes enhanced RET emission between a bioluminescent (Rluc) donor and a fluorescent (RGFP) acceptor that are genetically tethered to the respective endings of the two molecules (20-22). Binding of guanine nucleotide to endogenous Gα subunits abolishes the signal, thus allowing measuring the extent of constitutive activation as the difference in basal signal between absence and presence of GDP. We evaluated the differences in spontaneous and ligand-regulated coupling between μ (MOP) and δ (DOP) opioid receptors (23,24), using 35 analogues sharing a common peptidomimetic scaffold. This is derived from the condensation of the two unnatural amino acids, 2',6'-dimethyltryptamine (Dmt) and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic). As previously shown, substitutions within the Dmt-Tic pharmacophore can generate a vast array of changes in affinity and efficacy of ligands for the two opioid receptors (25-28).

We find that more than 40% of the studied Dmt-Tic analogues display varying degrees of inverse efficacy for the DOP receptor, but act as partial or full agonists for the MOP receptor. This reversal of efficacy appears to depend on the greater constitutive activity of the DOP receptor compared to MOP. In fact, the maximal levels of absolute coupling for most ligands (i.e., the net BRET over the GDP baseline) are remarkably similar at the two receptors. The analysis of the data suggests a new model of receptor constitutive activation. According to such a view, constitutive activation of the receptor results from the intramolecular lessening of a constraint that all ligands must oppose to stabilize the ligand-bound receptor-G protein complex.

EXPERIMENTAL PROCEDURES

Reagents and Drugs – Cell culture media, reagents, and fetal calf serum were from Invitrogen; restriction enzymes from New England Biolabs; coelenterazine and bisdeoxycoelenterazine from Biotium Inc.; DADLE from Bachem; ICI 174,864 from Tocris; GDP (Tris Salt) from Sigma-Aldrich. All DMT-Tic analogues were synthesized as reported (26-28). Their structures and abbreviations are listed in Supporting information, table s1.

Cells and membranes – Preparation of retroviral vectors coding for Rluc-tagged human MOP and DOP receptors and RGFP-fused Gβγ1 or β-arrestin 2, and the transduction of SH-5YSY human neuroblastoma cells were described previously (22). Cells were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and F-12, with 10% (v/v) fetal calf serum, 100 μg/ml hygromycin B and 400 μg/ml G418 in a humidified atmosphere of 5% CO2 at 37°C. Enriched membranes from transfected cells were obtained by differential centrifugation (22) and stored in aliquots at -80 °C before use.

BRET measurement and data analysis – BRET signals were measured and analyzed as previously described (20-22). Receptor/Gβγ1 interactions were measured in 96-well white plastic plates (Packard Opti-plate) using membrane preparations (3μg of proteins) in a total volume of 100 μl PBS; receptor/β-arrestin 2 interactions were measured in intact cell monolayers. All ligands were tested using 8 log-spaced concentrations and in each assay microplate concentration-response curves for the nucleotide GDP and for the full agonist [D-Ala2, D-Leu5]enkephalin (DADLE) were included, to assess respectively the level of zero and maximal receptor activation. At concentrations ≥ 100 μM several Dmt-Tic analogues produced detectable inhibition of Rluc activity (measured as in (22)). Therefore, concentrations greater than 10 μM were avoided. Curves representing the change of BRET ratio as a function of ligand concentration were first analyzed by nonlinear curve fitting with the general logistic function (29):

$$\text{BRET}_{\text{ratio}} = d + (a - d)/\left(1 + \left(x/c\right)^{b}\right)$$

where x is ligand concentration; a and d, the curve asymptotes; c is the ligand concentration yielding half-maximal BRET change; and b is the slope factor at c (with positive or negative sign, for agonists or inverse agonists and GDP). Next, all data points were converted to fractional Receptor-G protein coupling (FRC) by subtracting the maximal inhibition of BRET produced by GDP and dividing by the maximal stimulation induced by DADLE: $\text{FRC} = (\text{BRET}_{\text{ratio}} - d_{\text{GDP}})/(a_{\text{DADLE}} - d_{\text{GDP}})$, where $d_{\text{GDP}}$ and $a_{\text{DADLE}}$ are the best fitting parameters shared across the set of fitted curves and mark the 0 and 1 levels of coupling. Transformed data were re-fitted with the same equation, to compute ligands Intrinsic Activity (IA, i.e., $E_{\text{max}}$ in FRC units) and Potency (EC50, given as negative log value, pEC50). Both parameters contain information about ligand efficacy (i.e. the intrinsic ability of each individual ligand to couple the system), but neither provides a simple proportional measure of it, since both are nonlinearly affected by the difference in binding affinity between receptor and G protein. However, while

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potency also depends on the receptor binding affinity of the ligand, IA does not. The FRC in the absence of ligand is the level of spontaneous coupling of each empty receptor. Concentration curves are shown as representative experiments (except where otherwise stated), while the corresponding parameter values averaged from at least three independent experiments performed on membranes obtained from different batches of cells are shown in the tables.

RESULTS

Apparent reversal of ligand efficacy in DOP and MOP receptors. — Ligand-mediated coupling between opioid receptors and Gβ1 via endogenous Gα subunits was described previously (22). In this study we co-expressed each luminescent receptor with the fluorescent Gβ1 subunit in the human neuroblastoma cells SH-SYSY. The relative abundance of Gαo makes these cells a better model of the neuronal environment. Two lines expressing similar levels of receptors and Gβ1 with virtually identical donor/acceptor ratios were selected for this study.

Using membrane preparations, we generated curves describing the change in fractional receptor coupling (FRC, see Exp. Procedures) as a function of ligand concentration. Computed potencies (pEC50) and intrinsic activities (IA) of the 35 Dmt-Tic analogues are summarized in Table 1. An example of such concentration-response curves is shown in fig. 1ab. Note the difference in spontaneous coupling between DOP and MOP receptors (shaded areas). ICI-174,864 (prototypic inverse agonist) inhibited DOP receptor coupling to a level very close to the GDP baseline, but enhanced FRC in the MOP receptor, although at greater concentrations (10 μM). These peptides (all tested at 10 μM) show IA values not statistically different in the two receptors; but even when significant, the differences are relatively small. The global data correlation in fig.1d is highly significant (0.88, P < 0.001), and suggests a general trend: every ligand seems to approach the same level of receptor-G protein coupling on binding MOP or DOP, despite the large difference the receptors show in the empty state.

This phenomenon is not the result of a particular level of receptor expression or G protein composition determined by the engineering of neuroblastoma cells. We used membranes from HEK-293 cell lines (22) where DOP and MOP receptors are present at ~2.5 greater level of constitutive coupling in both DOP and MOP, which is consistent with the enhanced expression, this characteristic pattern consisting of equal intrinsic activity with unequal constitutive activity was very similar in the two cell lines (data not shown).

Thus, the G protein-coupling responses of empty and ligand-bound opioid receptors deliver conflicting information about ligand efficacy. If considered as relative change from the receptors baselines, most ligands undergo reversal of efficacy on passing from one receptor to the other. But if considered as absolute changes, most ligands maintain equal efficacy in the two receptors.

Effects of ligands on receptor-arrestin coupling — We wondered whether Dmt-Tic ligands could show inverse agonism and/or biased agonism at receptor-arrestin interaction. Using a previously described BRET assay in intact cells (22), we compared the β-arrestin 2 coupling activities of Dmt-Tic analogues in both receptors. All ligands were first screened (data not shown) at saturating concentrations (10 μM). Those producing a detectable change of BRET ratio were further analyzed using concentration-response relationships. One third of the Dmt-Tic analogues had measurable effects on arrestin coupling in both receptors, but in MOP only 3 were potent enough to allow concentration response analysis. Emax and pEC50 values are listed in Table 2, curves are shown in fig. 2ab. Although the basal BRET signal was slightly greater in DOP than in MOP expressing cells, no
ligand-mediated inhibition was detected, suggesting that such a difference cannot be attributed to divergences of constitutive arrestin coupling. Thus, neither DOP nor MOP receptors display measurable levels of constitutive activation in recruiting arrestin.

The comparison of arrestin and G protein IA in the DOP receptor shows that agonists display consistently lower efficacy at the former interaction. The extent of such a discrepancy is inversely related to the level of IA (Fig. 2c). We reported the same trend before using a wider range of different opioid ligand structures (22); it suggests that the strength of receptor interaction is far lower for arrestin than for G proteins. None of the inverse agonists for G protein exhibited detectable agonism for arrestin.

**Ligand structural changes associated with modifications of efficacy** — Structure-activity analysis of the data in table 1 uncovers two modifications of the Dmt-Tic scaffold that are related to loss of efficacy and inverse agonism for DOP receptors.

One is the position of the carboxylic group in the “tripptide” series of Tic-Dmt analogues. Common motif in this class of ligands is the presence of different amino acids extending the C-terminal of the Tic residue (Table 1 and Fig. 3a). The replacement of Glu-NH2 with Gln-COOH (i.e., the transfer of the carboxylate anion from the side chain to the peptide backbone), results in a dramatic reduction of IA, which converts a strong partial agonist into an inverse agonist. A similar reduction is observed in the pair of peptides that are extended with the corresponding D-amino acids. Likewise, a corresponding pattern (neutral antagonist becoming inverse agonist) occurs on replacing Asp-NH2 with Asn-OH (Fig. 3a).

Thus, the location of the C-terminal carboxylate plays a fundamental role in the efficacy of the ligands for the DOP receptor. Since the IA of such ligands could not be measured in MOP receptors, a comparative assessment of the effect of this modification cannot be made.

The second modification is the dimethylation of the Dmt amino group. Six pairs of ligands consisting of amino-free and dimethylated versions of the same molecules allowed full concentration-response curves analysis, and thus the comparison across receptors. Alkylation reduced IA in both receptors. However, given the difference in spontaneous coupling, this decrease generated inverse agonism in DOP but only reduced the extent of agonism in the MOP receptor (Table 1).

To analyze the effect, we plot DOP vs. MOP ligand IA values (fig. 3b). In this graph, the vectors tracing the distance in activity between unsubstituted and substituted analogues measure the joint variation of IA caused by ligand modification in both systems. They have different lengths, because dimethylation has a different effect on each peptide, but the slopes are similar and roughly parallel to the line of perfect correlation. This means that both direction and magnitude of the loss of IA caused by N-methylation are well conserved in DOP and MOP. Therefore, the modification produces an identical reduction of efficacy (presumably acting through an identical mechanism) in both. Yet, if we evaluate the variation with respect to the level of constitutive coupling, the same loss of efficacy converts agonists into inverse agonists at DOP but only reduces agonism at MOP receptor.

The effect of methylation on the pEC50 of the ligands was instead divergent in the two receptors. The alkylation increased potency in 5 out of 6 ligands at DOP receptor, but produced either reductions or enhancements of pEC50 at MOP receptors (Fig. 3c). These data are consistent with the previous findings that N-alkylation of the Dmt-Tic pharmacophore increases the binding affinity for the DOP receptor (25).

**The relationship between maximal coupling and the shift of GDP apparent affinity** — We used a more precise approach to assess ligands efficacy at MOP and DOP receptors. There is negative cooperativity between ligand and GDP in GPCR systems. Thus, efficacy can be deduced from the effect that the ligand exerts on the apparent inhibition constant (Ki) of GDP.

Fig. 4ab shows typical concentration-response curves for GDP-mediated inhibition of receptor-G protein coupling, obtained in the absence and at saturating concentrations of ligands. The Ki values of GDP become larger as ligands IA increases. Agonists producing the highest level of G protein coupling also raise the inhibition plateau of GDP, as expected for an allosteric interaction. These experiments also allow measuring the shift in GDP Ki induced by DOP and MOP receptors in the absence of ligand. Consistent with the difference in constitutive coupling between receptors, the GDP Ki was larger in DOP than in MOP (fig. 4c).

Using ligands covering the full range of efficacy, we examined the relationship between ligands IA and apparent Ki of GDP (plotted as negative log pKi) in both receptors. These curves are very similar in DOP and MOP (Fig. 4d). In the low ligands IA range, large differences in coupling correspond to minimal changes of pKi; vice versa in the high range (Fig. 4d). This curvilinear shape is due to the dual components of the free-energy change that underlies Ki. One - related to the binding interaction - is constant. The other - reflecting cooperativity - varies with ligand efficacy. Thus, at lower ligands IA (small cooperative effects) changes in Ki barely exceed the experimental error, but at greater values the differences become increasingly visible. Note that the changes in GDP pKi caused by empty receptors are well aligned with those induced by ligands, indicating that the level of coupling in the unbound receptor is predictable from those observed in ligand-bound receptors.

For a system consisting of two ligands interacting at different sites of the same protein complex, the shift in one ligand apparent affinity caused by saturating
concentrations of the second provides a direct measure of the free energy-coupling existing between the two binding processes (16). We computed such coupling constants (i.e. net differences in GDP pKi between absence and presence of ligand) for peptides with the same IA in both receptors. Plotting such values in MOP vs. DOP receptor yields a linear relationship with unitary slope and nonzero intercept (Fig. 4c). The unit slope indicates that for any given change of ligand structure there are identical changes of allosteric coupling (efficacy) in the two receptor/G protein systems. But the upward shift of the line on the y-axis indicates that all free-energy coupling values in the DOP receptor are uniformly reduced by a constant factor compared to the MOP receptor. The size of this shift (±1 unit of free-energy on the RT scale) is equivalent to the difference in GDP Ki between the two empty receptors (fig. 4c).

Model of constitutive activation — The overall pattern of constitutive activation and inverse agonism documented in this study is in contrast with the prediction of models such as the ternary complex model (TCM) (14) and/or the extended (ETC) (5,15) or cubic (CTC) (17) models. Assuming that DOP and MOP only differ in constitutive coupling and that Dmt-Tic with equal IA have identical molecular efficacies, we computed how that pattern should be predicted according to the TCM (fig. 5a and eq.1 in Appendix). This “TCM-fitting” analysis shows that a difference of constitutive activity (i.e. $M'_{DOP} > M'_{MOP}$) cannot coexist with equal levels of maximal activity, if the molecular efficacies of the ligands are identical in the two receptors (i.e. $\alpha'_{DOP} = \alpha'_{MOP}$). Moreover a change of constitutive activation cannot alter the rank of ligands intrinsic activities: e.g., inverse agonists inhibit spontaneous association in both the highly constitutively active and low constitutively active receptor, even if in the latter it might be difficult to quantify the effect. (see Appendix and fig.5a).

The same analysis indicates that to observe equal IA values the molecular efficacy of the ligands for DOP should be uniformly reduced by a quota of free-energy which is exactly equivalent to the difference in G protein binding affinity between DOP and MOP receptors (fig. 5bc and Appendix). Thus, both theoretical analysis (Fig. 5) and experimental measurement of GDP cooperativity (fig 4e) converge to an identical conclusion: the apparent efficacies of ligands in the constitutively active receptor are uniformly changed by the process of constitutive activation.

We developed a different extension of the TCM model, which is capable to explain the phenomenology reported in this study (see Appendix). In former models constitutive activity depends on the equilibrium between active and inactive forms of the receptor. This equilibrium is cooperatively linked to ligand binding in the same way as it is to the association between receptor and G protein.

Thus, for any change in magnitude of that equilibrium, constitutive and ligand-induced complexes are invariably ordered on the coupling scale (15). In the present model, however, the allosteric transition is conceived as a change in energy state of the system, which may occur in either or both reacting proteins. This represents the energy cost of the transition from spontaneously occurring to ligand-controlled multiprotein complexes, and is linked by strong negative cooperativity to every ligand binding process. Ligands must restrain spontaneous coupling in order to drive the system to the ligand-bound associated form. Thus constitutive and ligand-driven coupling are competitive non converging paths that lead to activation of the system (see Appendix).

The simulations in figs. 6 and 7 show how the sole enhancement of the equilibrium for this intramolecular transition, without any other change in ligand-dependent parameters, can generate a pattern of constitutive activation which reproduces all the phenomenology described in this paper. Identical results with minor adjustments in parameter values can be generated using all 3 versions of the model discussed in Appendix (data not shown).

To challenge the model an experiment was first predicted “in silico”. If on adding a suitable concentration of GDP to the high constitutive active subtype we equalize the levels of spontaneous coupling in the two receptors, the correspondence of ligand intrinsic activities should be lost (Fig. 7a). We executed the same experiment in real membranes using a subset of ligands with similar intrinsic activities (Fig. 7b). Simultaneous fitting of concentration response curves obtained in parallel MOP and DOP membrane assays confirm that the ligands share indistinguishable IA values in the receptors; but this symmetry is disrupted upon addition of 200 nM GDP to DOP, which lowers its constitutive activity to a level closer to that of MOP (Fig. 7b). Consequently, the linear relationship between ligands IA in the two receptors is converted to hyperbolic (fig. 7c).

DISCUSSION

In this study we compared the activity of a congeneric series of ligands for wild-type DOP and MOP receptors using a BRET-based measurement of receptor-G protein interaction in membranes. Like GTPγS binding, this assay provides a signal that is directly related to receptor-G protein association, but brings two additional advantages: The ability to assess both receptor-G protein association and the apparent affinity of nucleotides, and the capacity to measure receptor-G protein coupling of both ligand-bound and unbound receptor. With tagged proteins expressed at similar levels, BRET allows comparing constitutive and ligand-induced activities across different receptors on the same scale.

We report several new findings on the constitutive activation and inverse agonism of the DOP receptor.
First, we find a major difference in the extent of constitutive activation between DOP and MOP receptors. Spontaneous G protein coupling is 4–5 times greater in DOP than in MOP receptor. Indeed, the wild type DOP receptor appears as a natural constitutively-active mutant of the MOP receptor.

Second, we identified 16 ligand structures that act as DOP inverse agonists and display a remarkable variation in the extent of apparent negative efficacy. This effect is mediated by occupation of the same binding site of enkephalins and other opioid ligands, as indicated by competition with a pure antagonist. Thus, inverse agonism is a frequent event in ligands based on the Dmt-Tic scaffold, making this pharmacophore the ideal structural template for investigations of negative efficacy. No ligand exhibited inverse agonism at receptor arrestin interaction, suggesting that the difference in constitutive activation between MOP and DOP is restricted to receptor-G protein interactions.

Third, we identified two main structural modifications of the Dmt-Tic scaffold that are correlated with the occurrence of inverse agonism in the ligands. These modifications engage opposite ends of the molecule and involve groups of opposite charge. The position of the anionic carboxylate at the C terminal, and the dimethylation of the N-terminal cationic amine, conferred the strongest level of inverse agonism observed for Dmt-Tic ligands in this work. This suggests that electrostatic interactions in the receptor binding pocket play a major role in determining inverse agonism. Perhaps the relatively constrained structure of the Dmt-Tic template can optimize the orientation of charged groups, which likely interact with polar residues on different domains of the TM bundle. Analysis of Dmt-Tic-bound receptor crystals should provide valuable information on the nature of such structural requirements.

However, the most important finding in this study is a surprising new feature of receptor constitutive activation. Ligands that move the level of receptor-G protein coupling into opposite directions from the ligand-free receptor baseline of DOP and MOP (thus apparently showing opposite efficacy in the two receptors) generate the same level of G protein coupling in both systems, thus exhibiting identical IA in the two receptors. Therefore, inverse agonists in the highly constitutively coupled DOP receptor appear as agonists for the low constitutively coupled MOP receptor.

One possible explanation is that the correspondence of intrinsic activity in the two receptors is fortuitous. Despite the remarkable similarity shown in atomic resolution structures (30,31), MOP and DOP receptors are different proteins. Thus, ligands can have opposite efficacy in the two receptors; yet, by chance, some might converge to similar levels of “absolute” coupling. Several indications make this explanation inconsistent with experimental evidence. One is probability. Nearly half of the ligands show statistically indistinguishable IA values at MOP and DOP, and the global correlation among all values was highly significant. Thus, the odds in favor of a random generated equivalence of intrinsic activities are extremely low. In addition, two mechanistic arguments point to the same conclusion: the identical loss of IA that N-alkylation of the ligands causes in the two receptors, and the overlapping relationships between changes of GDP affinity and ligands effects. Both support the notion that ligands efficacy and IA are strictly related. Thus, Dmt-Tic analogues have identical or very similar efficacies at DOP and MOP receptors, despite the divergent direction in which G protein association is changed from the level of spontaneous coupling in each receptor.

Our data seem to suggest that ligand-induced and constitutive receptor activation result from different mechanisms. Yet, there is quantitative agreement between the effects of empty and ligand-bound receptors on GDP affinity. One clue comes from the study of the ligand/GDP free-energy coupling values in the two receptors (fig. 4c). This analysis shows that ligands with equal IA in MOP and DOP display an equal diminution of allosteric effect in the constitutively active DOP. Measured as free energy units, this constant loss is identical to the shift of GDP affinity that the two receptors show in the unbound state. This suggests that the mechanism causing constitutive activation in the DOP receptor can also collectively reduce the allosteric effects of all ligands, regardless of their molecular efficacy. Put simply, constitutive activation can cut a common energy cost that all ligands pay when driving receptors to the G protein-associated form.

Based on the above analysis, we developed a new extension of the TCM model (see Appendix), which is capable to explain the phenomenology reported in this study. Two interesting mechanistic implications can be drawn from this modeling analysis.

First, the nature of linkage between ligand-dependent and independent activation. Unlike the concerted shift towards a common allosteric conformation of previous models, this alternative view predicts that all ligands exert negative cooperativity against the process of constitutive activation. Therefore, no ligand-bound state of the system can be energetically equivalent to the ligand-free state. This agrees with a recent single-molecule force-spectroscopy study of β2-adrenoceptors bound to ligands of differing efficacies. As shown there, no ligand-bound receptor form can exactly match the energetic, kinetic, and mechanical pattern of the empty receptor (32).

Second, the dual allosteric process underlying molecular efficacy. There is a ligand-specific cooperative effect that stabilizes the receptor-transducer complex (α), but also a shared anticooperative “binding effect” (β) that every ligand exerts in raising the free-energy barrier for spontaneous coupling. This adds to and may cancel the
free-energy change of the first. Therefore, a ligand with unchanged ability to stabilize the receptor-transducer complex can show agonism in a low intrinsically coupled receptor but inverse agonism when a reduction of the energy barrier generates constitutive coupling. It follows that the direction in which ligands steer basal receptor activity is not a reliable indicator of molecular efficacy. We don’t know how prevalent the mechanism of inverse agonism observed here might be among GPCRs. Of the two additional types of inverse agonists we tested (table 1), the naltrexone derivative BTNX displayed close IA values in the two receptors, with the blend of DOP inverse agonism / MOP partial agonism that is typical of Dmt-Tic peptides. But the pentapeptide ICI-174864, even if the \( E_{\text{max}} \) value at MOP was unmeasurable, clearly showed a different trend, suggesting a true reversal of molecular efficacy in the two receptors. Thus, it is possible that the phenomenon described in this paper depends on a particular way in which certain structural classes of ligands interact with the binding pocket. Obviously further studies on additional congeneric series of ligands in several GPCRs will be required. However, the anticooperativity that opposes ligand-induced to spontaneous coupling, which the behaviour of Dmt-Tic ligands unveils, is likely to depict a general feature of GPCRs, and may bring more insight in the functional chemistry of these molecules.

APPENDIX

Agonism and inverse agonism in the ternary complex model (TCM).- The TCM depicts the interactions among ligand (H), receptor (R) and G protein (G) (fig. 8) with 3 independent parameters: two affinity constants \( K' \) and \( M' \) govern the formation of HR and RG complexes in the absence of G or H, while an allosteric constant \( \alpha' \) describes the thermodynamic coupling between H and G binding to R (the prime symbol stands for effective constants as it will be explained later). For each receptor/G protein system, \( \alpha' \) encapsulates the molecular efficacy of ligands, and \( M' \) controls constitutive coupling. Let’s consider 2 receptors (R1, R2) that differ in affinity for a common G protein (i.e. \( M'^1 > M'^2 \)) and interact with a set of ligands having equal molecular efficacies in the two receptors (i.e. \( \alpha'^1 = \alpha'^2 \)). Ligand intrinsic activities (i.e. the maximal level of ligand-induced coupling \( Y_{\text{max}}^{\text{lig}} \)), is given as:

\[
y_{ij}^{\text{max}} = \lim_{[H] \to \infty} \left[ ([R,G]+[H,R,G]) \right] = \\
\frac{1}{2} \left[ \frac{R'_i + G_t + \frac{1}{\alpha'_j M'^j}}{\alpha'_j M'^j} \right]^2 - 4R'_i G_t \\
\left( \text{Where, } i \text{ and } j \text{ label different ligands and receptors, respectively; } t \text{ total reactant concentration}. \right)
\]

As shown in fig. 5a, given: \( M'^{\text{DOP}} > M'^{\text{MOP}} \), \( R_{\text{DOP}} = R_{\text{MOP}} \), \( G_t=\text{constant} \), and \( \alpha'_{\text{DOP}} = \alpha'_{\text{MOP}} \), eq. 1 predicts that ligands IA cannot be equal at the two receptors, nor can the increase in \( M' \) generate an apparent reversion from positive to inverse agonism. Thus, the TCM cannot explain the pattern of inverse agonism in Dmt-Tic ligands, unless we postulate that a peculiar change in the \( \alpha' \) value of each ligand can generate by chance a linear IA relation between the two receptors. Yet eq. 1 also defines which condition is required to observe equal IA (\( Y_{\text{DOP}}^{\text{max}} = Y_{\text{MOP}}^{\text{max}} \)) with unequal constitutive coupling (\( M'^1 \neq M'^2 \) ): i.e., \( \alpha'^1 M'^1 = \alpha'^2 M'^2 \). Written in log form this yields:

\[
\log(\alpha'^1) = \log(\alpha'^2) + \log(\frac{M'^2}{M'^1}) \quad \text{eq. 2}
\]

This means that equal maximal responses in the two receptors (\( Y_{\text{DOP}}^{\text{max}} = Y_{\text{MOP}}^{\text{max}} \)) are possible if the allosteric coupling free-energy of the ligands (i.e. \( \log(\alpha'_g) \)) in the highly constitutive active receptor is diminished by a constant amount, which is equal to the free energy difference (i.e. \( \log(M'^2/M'^1) \)) for the formation of RG complex by the two empty receptors (fig. 5bc). This analysis implies a thermodynamic linkage between RG affinity (\( M' \)) and ligand efficacy (\( \alpha' \)), so that a change in the first can uniformly change the second. Such a covariance of \( M' \) and \( \alpha' \) cannot be defined within the macroscopic framework of the TCM.

Minimal models that link apparent ligand efficacy to constitutive activity - The 3 parameters of the TCM must be considered apparent or “effective” constants. Although both \( K' \) and \( M' \) include an intra- and inter-molecular free-energy component, they are defined as pure bimolecular associations because the intra-molecular contribution is not experimentally measurable (33). Also, the ligand-induced perturbation \( \alpha' \) can only be appraised as “additive” free-energy of the bimolecular interactions. In this sense the 3 parameters are independent. If however we find covariance between \( \alpha' \) and \( M' \) (as we do here), it means that the intra-molecular perturbation underlying RG binding does not simply adds to, but interacts with ligand-induced perturbations. In previous work (15) a different covariance between \( \alpha' \) and \( K' \) was made explicit in the model assuming an allosteric switch of the receptor between functional states, since the experimental readout in those studies was the signaling activity of receptor mutants.

In this study we measure the assembly of R-GalphaY complexes in the absence or presence of ligands or nucleotide. Moreover, the linkage between \( \alpha' \) and \( M' \) shows as an equal energy cost that affects all ligand-induced perturbations regardless of their entity. Thus, to make explicit this intrinsic link we postulate an intramolecular change between two different energy states (\( S_1 \) and \( S_2 \)) controlled by a first-order constant \( J \). Since this
Constitutive receptor activation and reversal of ligand efficacy

Transition can occur with equal probability in R, G, or RG, we analyzed in parallel all 3 possible versions of the model, and named them accordingly: ACM (allosteric complex model), ARM (allosteric receptor model) and AGM (allosteric G protein model) (fig. 8).

The interaction among the two protein species (R and G), each binding a distinct ligand (peptide H and nucleotide N), leads to the formation of the coupled forms with and without ligands (i.e. the BRET-emitting species) and depends on the J-driven state transition (S1→S2) and its cooperative linkage to the binding events. Although all 3 model versions describe quite complex reaction schemes, we use two simplifications to analyze how parameter configurations predicts the BRET response. First, all parameters in the 3 versions can be reduced by exact functions to the “effective” parameters α’ and M’ of a “macroscopic” TCM equivalent scheme; thus, we can use this approach to analytically define which variations in model parameters lead to a joint variation of α’ and M’.

Second, in line with the course of the experiments presented here (conducted in the absence and presence of GDP) we analyze model behavior first in the absence, next in the presence of ligand N, which greatly simplifies the task.

Parameter space in the absence of GDP. - In all 3 model versions (fig. 8), the coupling constants β and γ indicate the cooperativity between the state transition (S1→S2) and the binding interactions H→R and R→G, respectively, while α (which gauges ligand efficacy in all versions) is the direct coupling between those binding events. In the absence of N (reaction schemes in fig. 8), the relationship between model parameters and the effective parameters of the equivalent TCM are:

\[
(ACM) \quad M' = M(1 + J) \quad \alpha' = \frac{1 + \beta J}{1 + J}
\]

\[
(ARM) \quad M' = M(1 + \gamma J) \quad \alpha' = \frac{1 + \beta J(1 + J)}{1 + \beta J(1 + \gamma J)}
\]

\[
(AGM) \quad M' = M(1 + \gamma J) \quad \alpha' = \frac{1 + \beta J}{1 + \gamma J} \quad \text{eqs. 3}
\]

As shown before in eq. 2, the condition to maintain equal ligands IA across receptors is: \(\alpha_1 M_1^2 / \alpha_2 M_2^2 = 1\). Using eqs 3, this constraint can be rewritten in terms of the parameters in the 3 model versions:

\[
(ACM) \quad \frac{\alpha_1 M_1}{\alpha_2 M_2} = \frac{1 + \beta J_1}{1 + \beta J_2} = 1
\]

\[
(ARM) \quad \frac{\alpha_1 M_1}{\alpha_2 M_2} = \frac{1 + \beta_1 J_1}{1 + \beta_2 J_2} = 1 \quad \text{eqs. 4}
\]

\[
(AGM) \quad \frac{\alpha_1 M_1}{\alpha_2 M_2} = \frac{1 + \beta_1 J_1}{1 + \beta_2 J_2} = 1
\]

This can be further simplified as follows:

(1) Since α does not contribute to M’ nor does M to α’, we can set \(\alpha_1/\alpha_2 = 1\) and \(M_1/M_2 = 1\).

(2) There is also no contribution of β to M’, thus the change of M’ should be caused by a variation in J and/or γ. To change M’ significantly either J (in ACM) or γJ (in ARM or AGM) must be >>1.

Hence, eqs. 4 reduces to:

\[
(ACM) \quad \frac{1 + \beta_1 J_1}{1 + \beta_2 J_2} = 1
\]

\[
(ARM) \quad \frac{1 + \beta_1 J_1'}{1 + \beta_2 J_2'} = 1 \quad \text{eqs. 5}
\]

\[
(AGM) \quad \frac{1 + \beta_1 J_1}{1 + \beta_2 J_2} = 1
\]

This final result underscores the symmetry and equivalence of the 3 versions both in terms of algebraic manipulation and predicted output. Also, the above rules provide guidance for a mechanistic interpretation of the parameters. It is clear from point (2) that J and/or γ look like the major free-energy constraint that limits constitutive coupling in the system. Likewise, the boundaries of β values (point 3) indicate that all receptor ligands regardless of their efficacy (α) must invariably exert a strong negative cooperative effect against the state transition driven by J.

Parameter space in the presence of GDP. - The presence of guanine nucleotide N (which binds to G with affinity L) does not change the constraints for the parameters discussed above (M, α, J, β, and γ), but introduces an additional coupling constant δ. This describes the cooperative interaction between the binding of R and N to G (fig. 8). Since we know that guanine nucleotides (both GTP and GDP) disrupt the stability of the RG complex (34), δ must lead to a reduction of the effective affinity M’ (δ <1). In the model, however, both γ and M contribute to the value of M’. Thus, to reduce M’ the binding of N could be negatively coupled either to the state transition (S1→S2) or to the intermolecular association R+G. We reasoned that the correspondence of ligands IA between receptors would be preserved in the first case but not in the second. Based on the effects of GDP shown in figure 7b, we choose the second option. This means that GDP can change via cooperativity (δ) the stability of the RG complex (just like H does via α), but cannot directly alter the state transition of the system.

Simulations of experimental data. - Simulations according to the 3 model versions were made using a previously described (35,36) numerical algorithm (a Microsoft Excel file with embedded code that allows to explore the models is freely available upon request from H.O.O.). Parameters were varied according to the rules discussed above and chosen to best fit the experimental
data. The difference in constitutive coupling between DOP and MOP was emulated by increasing \( J \) in ACM or \( \gamma \) in ARM and AGM. This is an arbitrary and inconsequent choice, since the values of \( J \) and \( \gamma \) can be reciprocally scaled as long as eqs. 5 are obeyed. To simplify, \( \beta \) was kept constant across ligands in the shown simulations, although we found that small random variations in \( \beta \) can produce similar scatter in the relation of ligands IA between receptors as we measured experimentally (fig.1d). In summary, the sole increase of \( \gamma \) or \( J \) in DOP with no change of other parameters can perfectly “fit” all the observed phenomenology: apparent reversal of ligands IA between receptors, disrupted by GDP (fig. 7) and the uniform decrease of free-energy coupling values for ligand and GDP measured in DOP receptor (fig. 6).

**Relationship with previous models.** – The model presented here is very similar or even mathematically identical (e.g. the ARM version in fig.8) to previous extensions of the TCM (15,17,37). The major difference is the intrinsic state transition, which goes in concert with ligands-induced perturbations in ETC (15) or CTC (17) but it is opposed by ligands in this model. Consequently, ETC or CTC cannot explain the \( M' \) and \( \alpha' \) covariance discussed here, nor can this model account for the \( K' \) and \( \alpha' \) covariance observed there. Rather than a contradiction, this indicates that change in function and change in energy state of the system cannot be described with the same allosteric transition. A more general theoretical framework to interpret the full repertoire of allosteric receptor behavior is needed. BRET studies on constitutively activated receptor mutants are under way in our lab, and may help us to make a step forward in that direction.
Constitutive receptor activation and reversal of ligand efficacy

References


Constitutive receptor activation and reversal of ligand efficacy


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FOOTNOTES

Abbreviations: FRC, fractional receptor-G protein coupling; IA: intrinsic activity; Dmt: 2’,6’-dimethyl-L-tyrosine; Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylate; Bid: 1H-benzimidazol-2-yl; Tib: 3-(1H-benzimidazol-2-yl)-1,2,3,4-tetrahydroisoquinolin-2-yl; dMe: dimethyl.
Legends to figures.

**Figure 1** Effect of ligands on opioid receptor-G protein coupling. **a,b)** Concentration-dependent increase of fractional receptor coupling (FRC, see Exp.Procedures) in DOP and MOP receptors by the indicated ligands. The level of constitutive coupling in the two systems is shaded. Data are representative of 3 independent experiments (means of fitted parameters in table 1). **c)** Agonist (UFP-512) and inverse agonist (Tic-Lys(Ac) curves in the absence or presence of the neutral antagonist UFP-515 (50 nM) in DOP receptor. The experiment was repeated twice with comparable results. **d)** Intrinsic activities of ligands (as FRC units) in DOP (y-axis) and MOP receptor (x-axis). Grey symbols indicate values that are not statistically different in the two receptors (P ≥ 0.05, T test). The correlation coefficient among all data is 0.88 (P < 0.001).

**Figure 2** Effect of ligands on opioid receptor-β-arrrestin 2 coupling. **a,b)** Concentration-response curves for ligand effects on BRET ratio in DOP (a) and MOP (b) receptors. The means of fitted parameters from 3 independent experiments are reported in table 2 **c)** The relative E_max values measured in G protein and β-arrrestin 2 coupling are compared.

**Figure 3** Modifications of Dmt-Tic ligands that generate inverse agonism in DOP. **a)** Concentration-response curves of ligands with C-terminal Glu/Gln or Asp/Asn residues (see caption) in DOP receptor (means of 3 experiments ± SE). The cartoon on the right illustrates the shift in position of the free carboxyl group in such ligands. **b)** Intrinsic activity of amino-free and N,N'-dimethylated peptides in MOP and DOP receptors. Data plotted as in fig.1d are means of 3 experiments; the shaded ellipsoids were drawn around the S.E in both axes. Arrows mark the loss of intrinsic activity in the two receptors. **p**EC_50 (neg. log of EC_50) values of the same ligands of b) are plotted with arrows indicating the change in potency.

**Figure 4** Effect of ligands on apparent GDP affinity. **a,b)** Concentration-response curves for GDP-mediated inhibition of DOP and MOP receptor coupling induced by the indicated ligands (10 μM). Y-axis data are the measured BRET ratios. **c)** GDP inhibition of DOP and MOP constitutive coupling. Curves were averaged (± SE) from 15 experiments, after normalizing the data as fraction of the net effect in the absence of nucleotide. The difference in Ki values is significant (ANOVA, F = 40.2, P < 1x10^-6). **d)** The negative log of Ki (pKi) in DOP and MOP computed from GDP curves (as those shown in panels a,b) are plotted as a function of the ligands intrinsic activities (table 1). Data are means (± SE) from 3 experiments. **e)** Free-energy coupling values (ΔΔG in RT units) for the negative cooperativity between ligands and GDP in DOP and MOP, calculated from the difference in the natural logarithms of GDP Ki between absence and presence of ligand. Data point are means (± SE) of 3 experiments. Linear regression (solid line) yields: slope 0.96 ± 0.11 and intercept 0.99 ± 0.22.

**Figure 5** Relationship between ligands IA in DOP and MOP: comparison of experimental observations and TCM-predictions. **a)** Simulated data represent Y_max values calculated (with the TCM parameter values indicated in the picture) using eq.1 (see Appendix), for ligands sharing equal α values (ranging from 0.01 to 1000) at two receptors (R_1, R_2) that have different M. Y_max is normalized with respect to total receptor concentration. The. Theoretical data (——) are shown with the experimental data replotted from fig. 1 (O; note: only ligands IA with non statistically different MOP and DOP values are shown); (x), projections of experimental data onto the predicted curve; (●), level of constitutive coupling (empty receptor). The shaded areas indicate the range of inverse agonism (i.e. where ligands with α’<1 are expected to inhibit spontaneous coupling). **b)** A set of logα values chosen according to eq.2 (Appendix) for the two receptors. Values of M_1 and M_2 are shown. **c)** Calculated net responses induced by the ligands that are identified with the α values shown in panel b. Net response is given as the difference between total concentrations of RG complexes (i.e. RG+HRG) calculated in the absence or presence of saturating ligand. The calculated response is normalized as in panel b. Experimental net responses (●) for the ligands are calculated from panel a.

**Figure 6** Results of simulations made according to the allosteric model illustrated in fig. 8 and Appendix. Two systems with high and low spontaneous activity (called DOP and MOP) were simulated. Fractional receptor-G protein coupling (FRC) is the sum of constitutive and ligand-bound coupled species ([RG]+[HRG]) / [R_total]). The two systems only differ in the coupling factor γ (γ = 280 in DOP and 5 in MOP). All other parameters values are equal in DOP and MOP: R-total=G-total=10^-10; M=10^2; K=10^-5; L=10^4; J=0.05; β=10^-4; δ=10^-2; ligand efficacy (α) varying from 0.1 to 14000. **a)** Simulated curves for GDP inhibition of FRC induced by the ligands in DOP and MOP. Thick lines indicate no ligand (compare with fig, 4ab). From such curves we calculated: **b)** the normalized inhibition of constitutive coupling in DOP and MOP (compare IC_{50}’s shifts with fig. 4c); **c)** the simulated relationship between GDP pKi and ligand intrinsic activities together with the ligand/GDP coupling ΔΔG’s (inset) in DOP and MOP. Solid curves are simulated data superimposed by experimental data (●) replotted from fig. 4d and 4e.
Figure 7 GDP breaks the correspondence of ligands intrinsic activity between DOP and MOP receptors. a) Results of simulations made according to the models presented in figure 8. Simulated FRC and parameter values are like in figure 6. An increase of $\gamma$ (as shown) is used to generate enhanced basal coupling in DOP (the $x$-axes of the second and third panel are ordered in the opposite directions to facilitate $E_{\text{max}}$ comparison across). The third panel shows DOP as in the first panel, but simulated in the presence of of ligand N at sufficient concentration to lower basal coupling to the same level of MOP. Note the break of $E_{\text{max}}$ symmetry. b) Curves obtained in DOP and MOP receptor membranes for the indicated ligands (without GDP) were globally fitted (first two panels). Sharing the same $E_{\text{max}}$ for each ligand in DOP and MOP did not change significantly ($P = 0.23$) the goodness of fit according to extra-sum of square principle (29) The third panel shows the same ligands assayed in DOP with 200 nM GDP (which lowers the basal activity of DOP close to that of MOP). Shaded areas show basal couplings; concentration axes are ordered as in panel a. The experiments were repeated twice with identical results. c) Observed and predicted $E_{\text{max}}$ values in DOP and MOP with or without GDP, as indicated in the picture. Predicted and observed values are taken from panel (a) and (b), respectively. Note the quantitative agreement between experimental observations and predictions.

Figure 8 The three allosteric model versions that can equivalently explain the joint variation between RG affinity and efficacy. The original TCM, which such models extend, is shown in the leftmost column. First row: Standard reaction schemes are shown by omitting ligand N and related pathways, to simplify the drawings. Second row: Schematic representation of thermodynamic couplings and allosteric equilibria (35): Receptor (R) and G protein (G) are shown as shaded boxes, binding sites as circles, binding associations as dotted lines, and thermodynamic linkages as solid lines. The allosteric transition between the two energy states $S_1$ and $S_2$ (superscripts in 1st row, and boxed-equilibria in the 2nd row) is differently located in the 3 versions: R-G interface (in ACM), R (in ARM) or G (in AGM) (Acronyms are given in the text). In all versions the allosteric equilibrium constant $J$ is defined as the ratio $[s2]/[s1]$. 
Constitutive receptor activation and reversal of ligand efficacy

Table 1. Intrinsic activity (IA) and potency (pEC<sub>50</sub>) for Dmt-Tic ligands effect on Fractional receptor-G protein coupling (FRC). Exp. E<sub>max</sub> indicates the FRC observed at 10 μM. See Supplemental data, Table S1 for ligand abbreviations and structures.

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<th>Ligand</th>
<th>DOP receptor</th>
<th>MOP receptor</th>
</tr>
</thead>
<tbody>
<tr>
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<td>IA (FRC ±S.E.)</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt; (-log±S.E.)</td>
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<tr>
<td>Empty receptor</td>
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<td>12</td>
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<tr>
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<td>ICI 174,864</td>
<td>0.07 (0.013)</td>
<td>7.20 (0.032)</td>
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<tr>
<td>Tic-Ala</td>
<td>0.34 (0.027)</td>
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<td>Tic-Asn</td>
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<td>7.92 (0.094)</td>
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Table 2. Maximal effects (E_max) and potency (pEC_{50}) of Dmt-Tic ligands for receptor-arrestin 2 coupling in intact cells. Experimental E_max is the effect measured at 10 μM of the ligand.

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<th>MOP/βARR2</th>
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<td>E_max (±S.E.)</td>
<td>pEC_{50} (±S.E.)</td>
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<td>Exp. E_max (±S.E.)</td>
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Constitutive receptor activation and reversal of ligand efficacy

Figure 1
Constitutive receptor activation and reversal of ligand efficacy

**Figure 2**

**Panel a**

<table>
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<th>Ligand</th>
<th>DOP Activity</th>
<th>β-arrestin 2 Coupling (BRET ratio)</th>
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</thead>
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<tr>
<td>DADLE</td>
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<tr>
<td>Tic-Glu</td>
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<td>UFP-512</td>
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<td>dMe-UFP-502</td>
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<td>Tic-Gly-Ph</td>
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**Panel b**

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<tr>
<th>Ligand</th>
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<td>DADLE</td>
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<td>UFP-505</td>
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<td>Bid-Propen</td>
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<td>Tic-Gly-Ph</td>
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**Panel c**

<table>
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<th>Ligand</th>
<th>Relative Emax</th>
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<td>Bid-Bzl</td>
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<td>C1-Bid</td>
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<td>Tic-Dglu</td>
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<td>Tic-Glu</td>
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<td>Tic-Gly-Ph</td>
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<td>Tic-Gly-Ph</td>
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<td>UFP-502</td>
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<tr>
<td>UFP-512</td>
<td></td>
</tr>
</tbody>
</table>

log [ligand]
Constitutive receptor activation and reversal of ligand efficacy

Figure 3
Constitutive receptor activation and reversal of ligand efficacy

Figure 4

A. BRET ratio vs. log [GDP] for DOP (a) and MOP (b).

B. Empty receptor coupling vs. log [GDP].

C. Intrinsic Activity (FRC) vs. log [GDP] for GDP.

D. pK_i of GDP vs. Intrinsic Activity (FRC).

E. GDP/ligand free energy coupling.

- MOP GDP pK_i = 7.8 (± 0.05)
- DOP GDP pK_i = 7.4 (± 0.04)
Constitutive receptor activation and reversal of ligand efficacy

Simulation Parameters

\[ M_1 = 19 \]
\[ M_2 = 1.2 \]
\[ R_1 = 0.1 \]
\[ G_1 = 0.1 \]
\[ \alpha_1 = \alpha_2 \]

\( \alpha \) Varies as shown

\[ \log(\alpha_i) \]

Figure 5
Constitutive receptor activation and reversal of ligand efficacy

Figure 6

a

DOP

FRC

log[GDP]

MOP

FRC

log[GDP]

b

Empty receptor coupling

log[GDP]

DOP or MOP

pK of GDP log

Intrinsic Activity (FRC)

0 0.2 0.4 0.6 0.8 1

DOP ∆(ln K_i)

MOP ∆(ln K_i)

0 2 4 6

0 5 5.5 6 6.5 7 7.5 8

Figure 6
Constitutive receptor activation and reversal of ligand efficacy

**Figure 7**
Constitutive receptor activation and reversal of ligand efficacy

Figure 8
Ligands raise the constraint that limits constitutive activation in G protein-coupled opioid receptors.
Vanessa Vezzi, H. Ongun Onaran, Paola Molinari, Remo Guerrini, Gianfranco Balboni, Girolamo Calo' and Tommaso Costa

J. Biol. Chem. published online July 8, 2013

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