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Themed Section: Opioids: New Pathways to Functional Selectivity

REVIEW

Identifying ligand-specific signalling within biased responses: focus on δ opioid receptor ligands

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Opioids activate GPCRs to produce powerful analgesic actions but at the same time induce side effects and generate tolerance, which restrict their clinical use. Reducing this undesired response profile has remained a major goal of opioid research and the notion of 'biased agonism' is raising increasing interest as a means of separating therapeutic responses from unwanted side effects. However, to fully exploit this opportunity, it is necessary to confidently identify biased signals and evaluate which type of bias may support analgesia and which may lead to undesired effects. The development of new computational tools has made it possible to quantify ligand-dependent signalling and discriminate this component from confounders that may also yield biased responses. Here, we analyse different approaches to identify and quantify ligand-dependent bias and review different types of confounders. Focus is on δ opioid receptor ligands, which are currently viewed as promising agents for chronic pain management.

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Abbreviations
DPDPE, [D-Pen(2), D-Pen(5)]-enkephalin; Emax, maximal effect; PWRS, plasmon waveguide resonance spectroscopy; SNC-80, (1)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3- methoxybenzyl]-N,N-diethylbenzamide; TICP, Tyr-Ticpsi [CH2-NH]Cha-Phe-OH; TIPP, H-Tyr-Tic-Phe-Phe-OH; UFP-512, H-Dmt-Tic-NH-CH(CH2-COOH)-Bid

Introduction

Opioids are the most effective analgesics known but their clinical use is limited by a compromise between maintaining analgesic efficacy on the one hand and controlling side effects and development of tolerance on the other (Dworkin, 2009). Not surprisingly then, improving the side effects profile and reducing the potential for analgesic tolerance have remained major goals in opioid receptor research. Within this context the notion of 'biased agonism' has raised considerable interest as a means of separating desired actions from undesired effects of opioid analgesics. This new pharmacological concept refers to the ability of certain receptor ligands to stabilize the receptor into conformations that distinctively engage specific signalling partners, thus directing pharmacological stimuli towards desired responses. As such, biased ligands may constitute a powerful means of separating analgesic actions from undesired effects of opioid analgesics. However, to fully exploit this opportunity, we must be able to confidently identify ligands of interest and evaluate which of their responses contribute to analgesic efficacy and which support undesired actions. The development of new quantification tools (Rajagopal *et al.*, 2011; Kenakin *et al.*, 2012; Rajagopal, 2013) has not only made this identification

possible, but should allow us to verify novel hypotheses with respect to the type of signals responsible for desired and undesired effects of opioids. Here, we review how to identify and quantify ligand-dependent bias and also examine the extent to which an imbalance in signalling versus internalization responses may be a desirable property as a predictor of ligand potential for generating tolerance. Focus will be on δ opioid receptor ligands (for nomenclature see Alexander *et al.*, 2013), which are currently viewed as promising agents for chronic pain management (Gaveriaux-Ruff and Kieffer, 2011a; Gaveriaux-Ruff *et al.*, 2011b)

Biased responses versus biased agonism

'Biased agonism' is a term that describes a specific type of signalling event in which different ligands acting at the same receptor may distinctively engage responses by different signalling partners (Urban *et al.*, 2007). Initial reports of this type of signalling behaviour characteristically revealed a reversal in the rank order of ligand potency and/or maximal responses across different functional assays (Meller *et al.*, 1992; Spengler *et al.*, 1993; Berg *et al.*, 1998). An example of this type of behaviour for δ opioid receptor agonists is given in Figure 1, where maximal responses by morphine and Tyr-Ticpsi [CH2-NH]Cha-Phe-OH (TICP) do not maintain the same rank order in cAMP (Figure 1A) and ERK (Figure 1B) cascades (Audet *et al.*, 2005). These observations are in sharp contrast with what is observed when comparing δ opioid receptor ligands with respect to their relative intrinsic activities for inhibiting cAMP production and inducing conformational changes that lead to Gαi1β1γ2 activation (Figure 2) (Audet *et al.*, 2008). Indeed, maximal cAMP inhibition and changes in BRET at the interface of Gαi1 and Gβ1γ2 subunits (Figure 2B) not only maintained the same rank order but were also correlated (Figure 2C).

According to classical receptor theory, ligands that produce different maximal responses impart pharmacological stimuli of different magnitude upon the receptor (Stephenson, 1956). Stimuli of different magnitude result in the accumulation of different amounts of a single active state of the receptor such that the more efficacious the ligand the

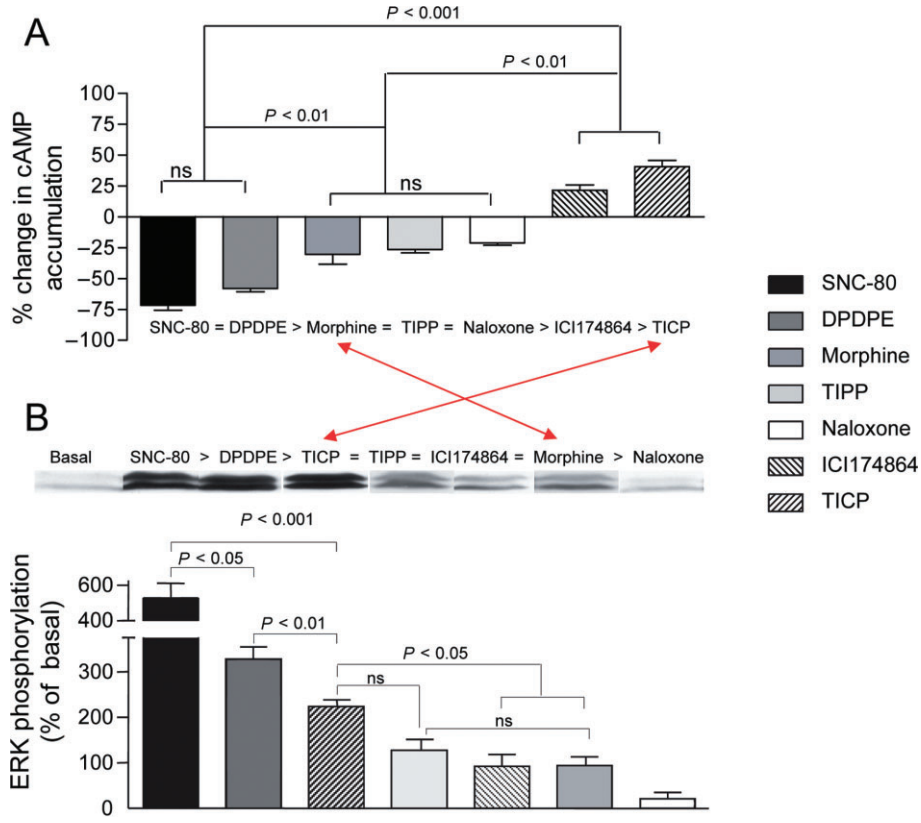


Figure 1

Maximal responses elicited by δ opioid receptor ligands do not maintain the same rank order in cyclase and ERK cascades. HEK293 cells expressing δ opioid receptor-Flag were treated with maximal effective concentrations (10 μM) of indicated ligands and (A) cAMP accumulation or (B) ERK activation were assessed. Drug effects are expressed as % change with respect to signals obtained in the absence of ligand and correspond to mean ± SEM of at least seven experiments carried out in triplicates. Rank orders are given for each graph; arrows indicate reversal in rank order in Emax values for morphine and TICP. Statistical differences that appear in the figure were established using one-way ANOVA followed by Tukey's *post hoc* test. Immunoblots above the histogram bars correspond to representative examples of results obtained for each of the indicated drugs (Modified from Audet *et al.*, 2005).

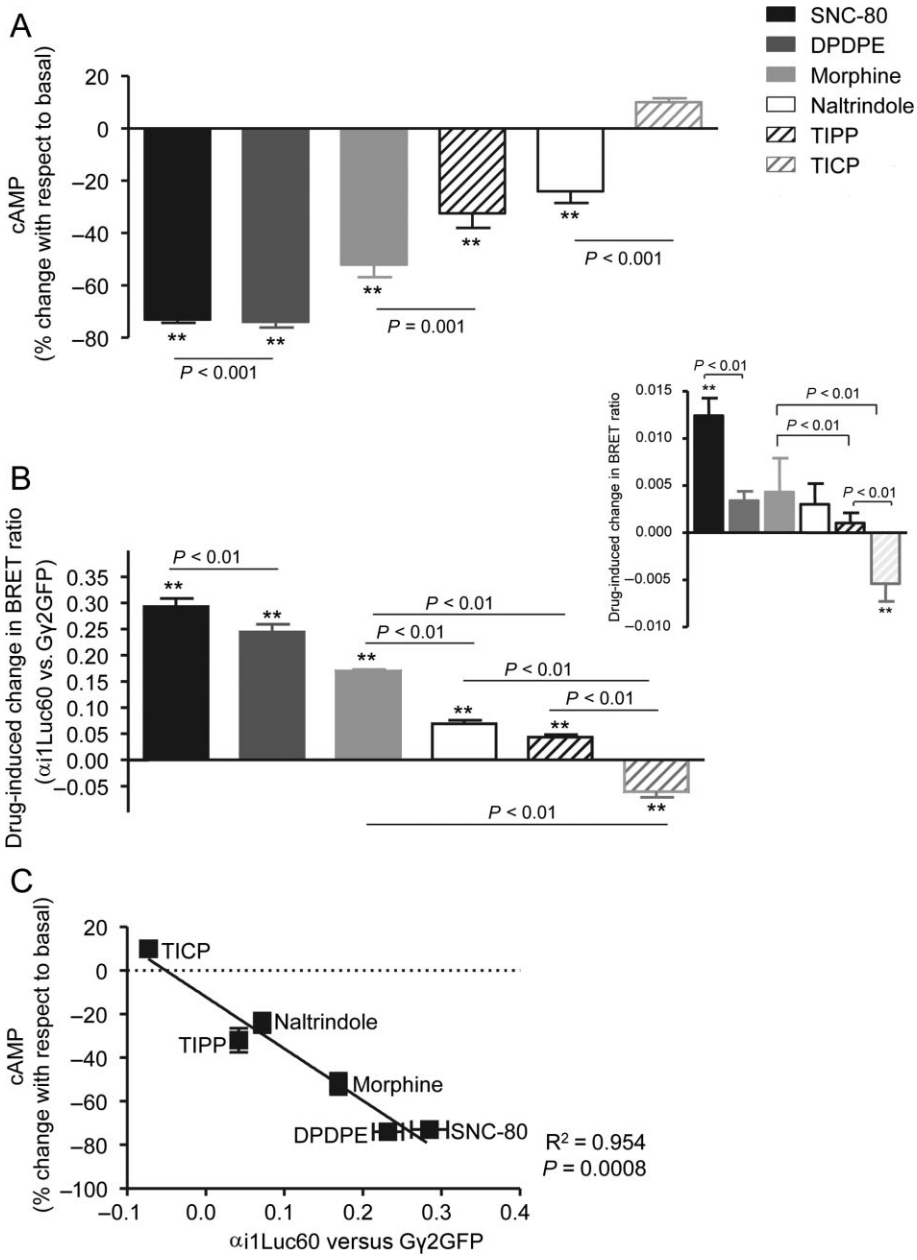


Figure 2

Ligand-induced changes in cAMP production and conformational rearrangements undergone at the Gαi1/Gβ1γ2 interface are proportional. (A) HEK293 cells expressing δ opioid receptor-Flag were treated with maximal effective concentrations (10 μM) of indicated ligands and cAMP accumulation assessed in radiometric assays. Drug effects are expressed as % change with respect to signals obtained in the absence of ligand and correspond to mean ± SEM of seven independent experiments. (B) HEK 293 cells were transfected with recombinant plasmids for δ opioid receptors, a BRET pair constituted by the Gαi1 subunit tagged with the donor at position 60 (Gαi1-Luc60), the Gγ2 subunit tagged with the acceptor at the N-terminus (GFP-Gγ2) together with untagged complementary Gβ1 subunits. Results are expressed as the difference between measures obtained in presence and absence of ligand and correspond to mean ± SEM of six experiments carried out in duplicates. Statistical comparisons were done by one-way ANOVA using Dunnett's correction to compare drug effects with basal conditions. Fisher's 'least significance difference' adjustment was used in order to assess differences among drugs. Inset: shows conformational changes induced by different ligands at the interface between δ opioid receptor-GFP and Gαi1-Luc60. (C) Correlational analysis of responses shown in (A) and (B). (Modified from Audet *et al.*, 2008).

greater the accumulation of this single active conformation, which is considered responsible for all responses controlled by the receptor. An essential prediction of this model is that ligands of different efficacy should conserve their rank order of maximal effect (Emax; and potency) at all responses assessed (Kenakin, 2002a). Hence, the direct correlation between the amplitude of conformational rearrangements elicited by Gαi1β1γ2 activation and the magnitude of cAMP

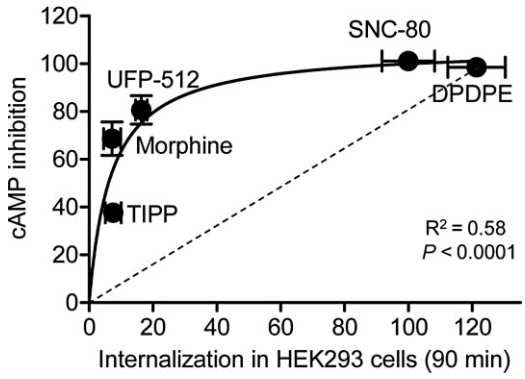


Figure 3

The relationship between maximal cAMP inhibition and internalization elicited by δ opioid receptor ligands is not proportional. HEK293 cells expressing δ opioid receptor-Flag were treated with maximal effective concentrations (10 μM) of the indicated ligands, and cAMP inhibition (n = 6–9) or receptor internalization (n = 9–12) were assessed using a radiometric or an ELISA-based assay. Data were fit to a single site hyperbola and goodness of fit is indicated in the figure. (Modified with permission from Charfi *et al.*, 2013).

inhibition is consistent with this notion, and if we were to evaluate bias from these observations, we would conclude that there is none for the responses in question. By contrast, different amounts of a single active state cannot account for one drug being more efficacious than the other in one type of response but less efficacious in another readout. Consequently, the idea that GPCRs could adopt multiple, agonist-specific receptor conformations was introduced in order to explain changes in rank order of Emax values across different responses (Kenakin, 1994; Leff, 1996; Urban *et al.*, 2007; Onaran and Costa, 2012). In the case of δ opioid receptors, the existence of agonist-specific receptor states has been proposed from functional assays and corroborated by different biophysical methods such as BRET (Audet *et al.*, 2008; 2012) or plasmon wave resonance (Alves *et al.*, 2003; 2004).

A common observation attributed to the existence of ligand-specific conformations is the non-linear relationship observed between relative Emax values of opioid receptor ligands in signalling responses and in responses related to receptor regulation (Alvarez *et al.*, 2002; Pradhan *et al.*, 2010; Raehal *et al.*, 2011; Charfi *et al.*, 2013). For example, disproportion between Emax values for maximal G-protein activation and maximal β-arrestin recruitment by δ and μ opioid receptor ligands was shown to conform to a hyperbolic function (Molinari *et al.*, 2010). A similar type of graph describes the relationship between maximal cAMP inhibition and internalization by δ opioid receptor ligands (Figure 3) indicating a clear imbalance between partial agonist ability to induce maximal cAMP inhibition and δ opioid receptor sequestration (Charfi *et al.*, 2013). The question is whether such a deflection from a proportional correlation corresponds to ligand bias. As we saw from previous examples, a change in rank order of Emax values constitutes a fair indication of ligand-specific signalling. However, if we look at maximal effects in Figure 3, there is no significant change in the rank order of maximal cAMP and internalization responses for any of the ligands studied. Instead, we observe a uniform mitigation of internalization as compared with cyclase inhibition by partial agonists. Bias that similarly affects a specific response by different ligands has been called *system bias*, and should be distinguished from ligand-specific signals (Kenakin *et al.*, 2012; Onaran and Costa, 2012; Kenakin and Christopoulos, 2013b). For example, Molinari *et al.* (2010) propose that the need for β-arrestin to diffuse from the cytosol to the membrane could be a *system factor* that reduces coupling efficiency between β-arrestin recruitment and receptor activation. The situation is quite different for G-protein activation where coexistence of heterotrimeric subunits and receptors at the membrane makes them immediately available to translate receptor occupancy into response (Gales *et al.*, 2005; Rebois *et al.*, 2006; Molinari *et al.*, 2010; Onaran and Costa, 2012). Similar considerations apply to *observational bias*, which refers to how different assay conditions may influence the way ligand actions are perceived. For example, *observational bias* may explain why in some assays maximal responses by full and partial agonists are similar to one another while they are well discriminated in others. Figure 4 shows an example of this phenomenon when cAMP inhibition by the full agonist [D-Pen(2), D-Pen(5)]-enkephalin (DPDPE) and the partial agonist morphine were monitored in a radiometric (Figure 4A) and a BRET-based assay (Figure 4B). It is clear from the figure that in the radiometric assay DPDPE does not produce a measurable change in cAMP levels until the drug reaches a concentration of 10 nM while cAMP inhibition is already evident at 0.1 nM in the BRET-based assay. Consistent with these observations, responses observed at the 10 μM concentration were similar for both drugs in BRET assays but morphine's response was smaller than that of DPDPE in the radiometric one. In the latter assay, cAMP levels were measured 20 min after agonist exposure (Charfi *et al.*, 2013) while BRET readings were completed only 6 min after drugs were introduced into the medium (Tudashki *et al.*, 2014). This implies that the process of desensitization has more weight in radiometric than BRET assays. Thus, in the more desensitized system, where receptor coupling to downstream effectors has been reduced the most, maximal tested concentrations produced a smaller effect for morphine than for DPDPE. In the BRET assay, where desensitization has less time to develop, the 10 μM response is similar for both drugs. Assay conditions also influence the relative potency of both drugs. In the BRET assay, EC_{50} for DPDPE (–9.8 ± 0.2 M) was 3.6 orders of magnitude smaller than that of morphine (–6.2 ± 0.2 M), but in the radiometric assay, EC_{50} values for DPDPE were right-shifted (–8.22 ± 0.11 M) and the difference with morphine (–6.04 ± 0.14 M) was of 2.2 orders of magnitude. These differences may be interpreted as the result of two factors: (i) desensitization reduced DPDPE's potency without modification of its maximal response in the radiometric assay and (ii) even if in the BRET assay DPDPE and morphine produce similar maximal responses, DPDPE's efficacy translated into a much more potent response.

Taken together, the examples analysed in this section indicate that: (i) response bias and ligand bias are not the same, (ii) although ligand-specific signalling may favour a specific signalling pathway, system and observational bias may also contribute to the overall imbalance in maximal

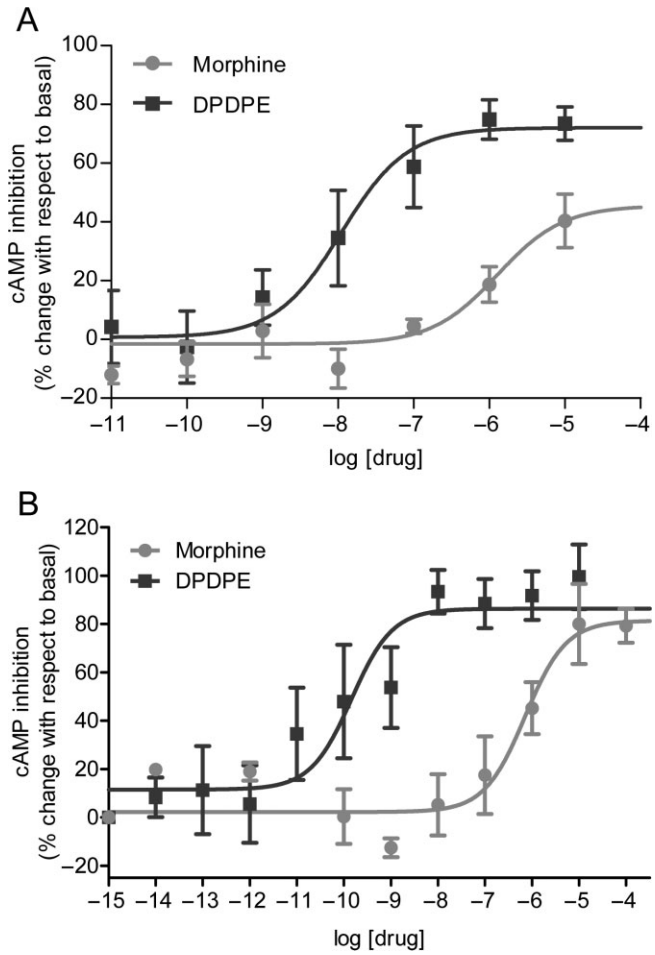


Figure 4

Concentration-response curves showing how observational bias may influence relative Emax for cAMP inhibition by δ opioid receptor agonists. (A) cAMP levels were monitored in a radiometric assays as in the previous figure and inhibition of cAMP production was expressed as % change with respect to cells that were not exposed to ligand. Values correspond to mean ± SEM of six to nine independent experiments carried out in triplicate. Statistical analyses using two-way ANOVA showed an effect of drug ($P < 0.0001$), and effect of concentration ($P < 0.0001$) and an interaction ($P < 0.0001$) (Modified with permission from Charfi *et al.*, 2013). (B) HEK293 cells were transfected with δ opioid receptors and a BRET-based cAMP biosensor that undergoes conformational changes upon cAMP binding. Results are expressed as % of maximal BRET change produced by DPDPE and correspond to mean ± SEM of four to eight independent experiments. (Modified with permission from Bagheri Tudashki *et al.*, 2013). Statistical analyses using two-way ANOVA showed an effect of drug ($P < 0.0001$), an effect of concentration ($P < 0.0001$) and an interaction ($P < 0.0001$).

responses, (iii) because of these influences comparison of maximal effects does not allow us to identify ligand bias unless there is a reversal in the rank order of Emax values in the pathways of interest and (iv) isolating the ligand-determined component within biased responses is essential if this pharmacological property is to be exploited in rational design of therapeutic agents (Kenakin, 2007; Pineyro and Archer-Lahlou, 2007; Kenakin and Miller, 2010). As discussed in the following section, shortcomings associated with maximal responses may be controlled by using a whole range of concentrations to assess bias.

Identifying and quantifying biased agonism from experimental data

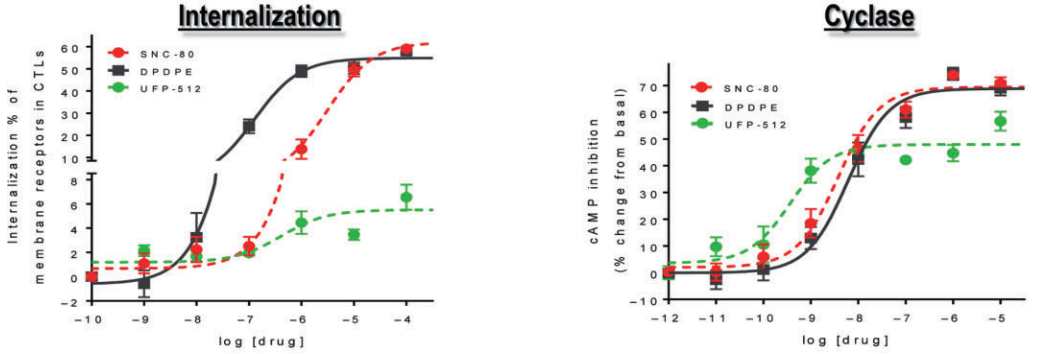
As a single active state of the receptor cannot account for a reversal in rank order of EC_{50}/Emax values for different responses, whenever this experimental observation is verified it may be considered evidence that the signals in question result from ligand-specific receptor states (Kenakin, 1995; 2002b; Onaran and Costa, 2012). However, despite being quite specific, this way of identifying biased agonists may not be sufficiently sensitive. For example, if bias is given by a reversal in rank order of potency this could be missed when only Emax changes are monitored. Ideally, a method to identify biased ligands should be one that simultaneously evaluates positional parameters and maximal responses. In addition, the method should allow meaningful quantification of ligand-dependent bias independent of system and assay confounders.

Kenakin and Christopoulos (2013a) have used the operational model by Black and Leff (1983) to develop an analytical tool that fulfils such requirements (Kenakin, 2010; Kenakin *et al.*, 2012). In the operational model, fractional response (E/Emax) at different agonist concentrations ([A]) may be calculated from the equation:

$$E/E\max = \tau^n \times [A]^n / (KA + [A])^n + \tau^n \times [A]^n$$

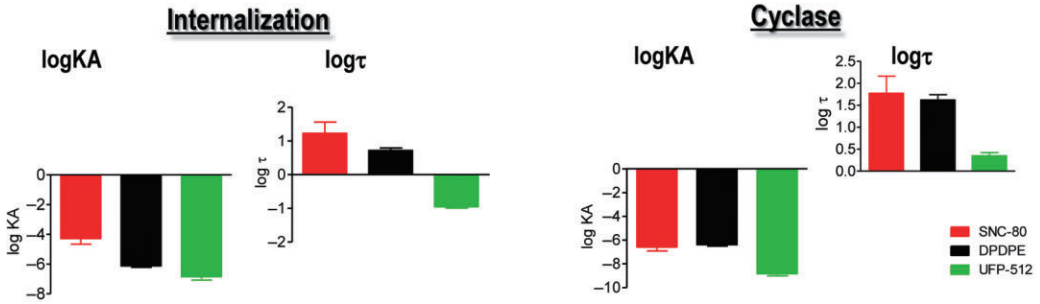
where E is drug effect, Emax is the maximal response allowed by the system and n describes the efficiency of the system to transduce receptor occupation into response. By fitting dose-response data to this equation, Kenakin and Christopoulos define two ligand-related parameters: (i) *efficacy* (τ) of the agonist to couple receptor occupancy to a specific response and (ii) '*functional affinity*' (KA) of the ligand (Kenakin *et al.*, 2012; Kenakin and Christopoulos, 2013b) (Figure 5A and B). In this model, KA is derived from functional and not binding data and it is defined as a parameter representing the tendency of the ligand to interact with the receptor state(s) mediating the response of interest (Kenakin *et al.*, 2012; Kenakin and Christopoulos, 2013b). Intuitively, its meaning may be considered in terms of the allosteric properties of GPCRs; that is if biased ligands stabilize the receptor into different conformations that preferentially interact with distinct signalling partners, allosteric properties of the receptor imply that the reciprocal is also true and therefore immediate downstream effectors condition agonist affinity for the receptor (De Lean *et al.*, 1980; Lee *et al.*, 1986; Costa and Herz, 1989; Kenakin and Miller, 2010; Kenakin, 2012). In other words, the affinity for efficacious ligands is determined at least in part by the signalling partners with which the receptor associates. Thus, taking both parameters as indicators of drug response, bias may result from ligands displaying distinct *relative efficacies* (τ) at different pathways, as well as from their differential affinity for the receptor state(s) mediating the response of interest (KA). Whatever the combination, Kenakin and Christopoulos (2013b) propose that all

A Obtain concentration-response curves



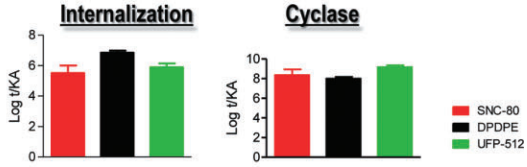
B Estimate operational parameters

	Internalization		cAMP inhibition	
	log KA ± SEM (M)	log τ ± SEM	log KA ± SEM (M)	log τ ± SEM
SNC-80 (*n*=12)	–4.28 ± 0.38	1.23 ± 0.33	–6.58 ± 0.43	1.77 ± 0.39
DPDPE (*n*=11)	–6.12 ± 0.10	0.72 ± 0.07	–6.38 ± 0.13	1.62 ± 0.12
UFP-512 (*n*=10)	–6.85 ± 0.23	–0.95 ± 0.04	–8.82 ± 0.18	0.35 ± 0.07



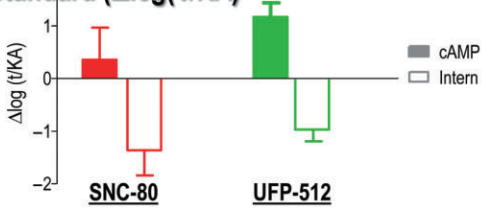
C Calculate transduction coefficients log(τ/KA)

	Internalization	cAMP inhibition
	log (τ/KA) ± SEM	log (τ/KA) ± SEM
SNC-80 (*n*=12)	5.51 ± 0.50	8.35 ± 0.58
DPDPE (*n*=11)	6.85 ± 0.13	8.00 ± 0.18
UFP-512 (*n*=10)	5.90 ± 0.24	9.16 ± 0.20



D Normalize to a common standard (Δlog(τ/KA)

	Internalization	cAMP inhibition
	Normalized coefficients Δ log (t/KA) ± SEM	Normalized coefficients Δ log (t/KA) ± SEM
SNC-80	–1.34 ± 0.50	0.36 ± 0.61
DPDPE	0.00 ± 0.14	0.00 ± 0.25
UFP-512	–0.95 ± 0.24	1.17 ± 0.27



E Calculate bias factor (ΔΔlog(τ/KA)

	Bias factor		
	ΔΔlog(τ/KA)	$10^{\Delta\Delta\log(\tau/KA)}$	*P* value
SNC-80	1.7	50	0.04
DPDPE			
UFP-512	2.12	132	<0.0001

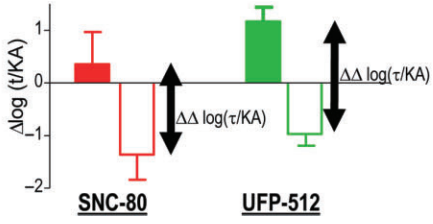
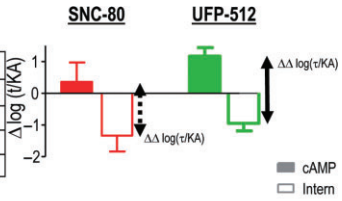


Figure 5

Quantification of signalling versus internalization bias for δ opioid receptor agonists. Concentration-response curves describing modulation of cAMP production or δ opioid receptor internalization by the indicated ligands were obtained in HEK293 cells (A). Data were analysed with the operational model to obtain efficacy (τ) and conditional affinity (KA) values (B) that were then used in the calculation of transduction coefficients log(τ/KA) (C). Note that τ values for SNC-80 are higher than those for those than UFP-512 both in cyclase and internalization responses. However, because UFP-512's KA values are smaller than those obtained for SNC-80, log(τ/KA) ratios of both ligands are not different in either response. Internalization and cyclase transduction coefficients for all ligands were then normalized to DPDPE both in cyclase and internalization readouts to yield corresponding $\Delta\log(\tau/KA)_{CYCLASE}$ and $\Delta\log(\tau/KA)_{INTERN}$ (D). The difference between these coefficients $\Delta\Delta\log(\tau/KA)$ corresponds to the bias factor. In this case, these factors indicate that compared with DPDPE, SNC-80 is 80-fold and UFP-512 132-fold more efficient in engaging cyclase inhibition over internalization (E). Statistical significance of these differences was established using two-tailed Student's *t*-test to compare normalized transduction coefficients (Δlog(τ/KA)) obtained in the two assays (Modified with permission from Charfi *et al.*, 2013).

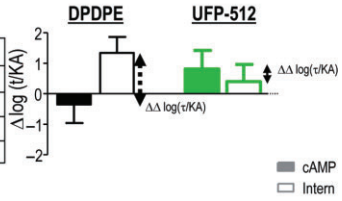
DPDPE STANDARD

		Bias factor		
	Δlog(τ/KA) ± SEM	ΔΔlog(τ/KA)	$10^{\Delta\Delta\log(\tau/KA)}$	*P* value
SNC-80	0.36 ± 0.61	1.7	50	0.04
DPDPE	0.01 ± 0.25			
UFP-512	1.17 ± 0.27	2.12	132	<0.0001



SNC-80 STANDARD

		Bias factor		
	Δlog(τ/KA) ± SEM	ΔΔlog(τ/KA)	$10^{\Delta\Delta\log(\tau/KA)}$	*P* value
SNC-80	0.00 ± 0.82			
DPDPE	−0.35 ± 0.61	−1.7	50	0.04
UFP-512	0.81 ± 0.61	0.42	2.63	0.63



UFP-512 STANDARD

		Bias factor		
	Δlog(τ/KA) ± SEM	ΔΔlog(τ/KA)	$10^{\Delta\Delta\log(\tau/KA)}$	*P* value
SNC-80	0.00 ± 0.28	−0.42	2.63	0.63
DPDPE	−1.17 ± 0.26	−2.12	132	<0.0001
UFP-512	−0.81 ± 0.61			

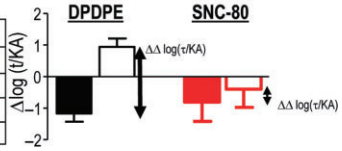


Figure 6

Bias factors heavily depend on the standard chosen for normalization. Transduction coefficients generated in Figure 5C were normalized to DPDPE (as before), SNC-80 or UFP-512 as indicated. Note the difference in the bias profile of SNC-80 when either DPDPE or UFP-512 was used as the standard. Also note that internalization versus cyclase bias displayed by SNC-80 when DPDPE was the standard is of same magnitude but opposite as the bias displayed by DPDPE when SNC-80 was the standard.

possibilities are contemplated by calculating a consolidated *transduction coefficient* (log τ/KA), which describes the *efficiency* with which a drug evokes a particular effect. This single numerical value can be used to compare ligands across different readouts, to determine whether they display bias and to quantify its magnitude (Kenakin *et al.*, 2012; Kenakin and Christopoulos, 2013b) (Figure 5C). However, it is important to note that together with an estimation of ligand efficacy, τ values also incorporate system-dependent variables (Black and Leff, 1983). As the latter may vary across different responses, these variations need to be taken into consideration when estimating the magnitude of bias using transduction coefficients. To do so, log(τ/KA) ratios are normalized to a common standard. This can be done by expressing test drug efficiency ratios in relation to the efficiency of the chosen standard, yielding normalized transduction coefficients or Δlog(τ/KA) values (Figure 5D). Δlog(τ/KA) is calculated by subtracting the transduction coefficient of the standard ($\log(\tau/KA)_{STAN}$) from that of the test drug ($\log(\tau/KA)_{TEST}$). Normalized transduction coefficients can be used to statistically compare ligand signalling efficiencies in different pathways, and to determine how much more (or less) efficient any ligand may be at inducing response in one readout with respect to another. This comparison is established by subtracting normalized transduction coefficients for each pathway (e.g. $\Delta\log(\tau/KA)_{INTERN}-\Delta\log(\tau/KA)_{CYCLASE}$) to establish a bias factor ($\Delta\Delta\log(\tau/KA)$) (Figure 5E). (Gregory *et al.*, 2012; Charfi *et al.*, 2013; Deng *et al.*, 2013). Because bias factors result from the comparison of transduction coefficients that were normalized to a common standard, bias is always expressed with respect to this standard, and varies accordingly. This difference is evident in Figure 6 where internalization versus cyclase signalling bias of δ opioid receptor agonists was obtained using different standards. This difference is evident in Figure 6 where internalization versus cyclase signalling bias of δ opioid receptor agonists was obtained using different

standards. Given that opioid analgesics compete with endogenous opioids to produce their effects, the use of enkephalins as standards would allow us to directly compare drugs of interest to the neuromodulator(s) they replace.

The operational model has also been used with alternative assumptions to quantify bias (Rajagopal *et al.*, 2011; Rivero *et al.*, 2012; Rajagopal, 2013). This alternative approach admits that ligand-specific conformations distinctively engage different effectors but assumes independence between ligand affinity for the receptor and its association with the signalling partners that support different responses of interest (Rajagopal *et al.*, 2011). Thus, the main difference between the two quantification approaches is that one of the methods does not admit allosteric interactions between ligand-occupied receptors and immediate signalling partners. In keeping with this assumption, the method proposed by Rajagopal *et al.* (2011) provides the operational model with a fixed affinity value obtained in binding assays and which is assumed to be the same for all responses. As a consequence, the method solely relies on operational efficacies (τ) to measure whether a ligand displays bias in producing two or more responses. This approach has been applied to μ opioid receptor agonists, revealing a significant bias for endomorphin 2 in G-protein activation versus β-arrestin 2 recruitment (Rivero *et al.*, 2012; Kelly, 2013). In contrast, a single affinity state did not adequately represent δ opioid receptor ligand responses in cyclase and internalization assays (Charfi *et al.*, 2013). This is illustrated in Figure 7, where cyclase and internalization data for DPDPE, SNC-80 and UFP-512 were fitted to the operational model, but this time fixing KA in both responses to a single affinity value (corresponding to binding affinity). In this case, only cyclase data points could be reasonably fit to the model, confirming that a single affinity state could not account for functional responses in both readouts. Based on these observations, it seems that at least some aspects of ligand diversity are better represented by allowing the receptor to adopt distinct functional affinities for different responses (Kenakin and Christopoulos, 2013a).

The use of functional affinities as a means of describing ligand ability to bias pharmacological stimulus is quite recent (Kenakin and Miller, 2010; Kenakin, 2012; Kenakin and Christopoulos, 2013a,b). By contrast, the concept that affinity is conditioned by ligand efficacy and the association of the receptor to downstream signalling partners has been accepted for many years as a central notion of the ternary complex model (De Lean *et al.*, 1980; Kent *et al.*, 1980). In this model, the ligand, the receptor and a nucleotide binding protein X (today identified as the G-protein) were proposed to associate into a complex where the ligand's intrinsic activity directly determined the affinity of the agonist-receptor complex for the G-protein. Conversely, the presence of the G-protein warranted higher affinity between the receptor and agonist ligands (De Lean *et al.*, 1980; Kent *et al.*, 1980). In the case of δ opioid receptors, the use of plasmon waveguide resonance spectroscopy (PWRS) has not only validated this prediction (Alves *et al.*, 2003) but has also shown that the association with different Gα subunits distinctively modifies receptor affinity for different agonists (Alves *et al.*, 2004; Hruby *et al.*, 2010). The studies showed that δ opioid receptor affinity for DPDPE was ~10-fold higher when the receptor was associated to Gαi2 than Gαi1, but the conformation stabilized by morphine displayed 10 times higher affinity for Gαi1 than for Gαi2 (Alves *et al.*, 2004). This change in agonist affinity when the receptor is associated with different Gα subunits confirms that downstream signalling partners condition receptor affinity. Consequently, observations obtained with PWRS provide direct experimental support for the use of the operational model as proposed by Kenakin and Christopoulos (2013b), where KA values are not fixed but conditioned by receptor association with different effectors. Recent crystallization studies carried out on β_2-adrenoceptors further underscore the extent to which allosteric interactions with heterotrimeric G-proteins may influence agonist-receptor association (Nygaard *et al.*, 2013). These studies revealed that agonist

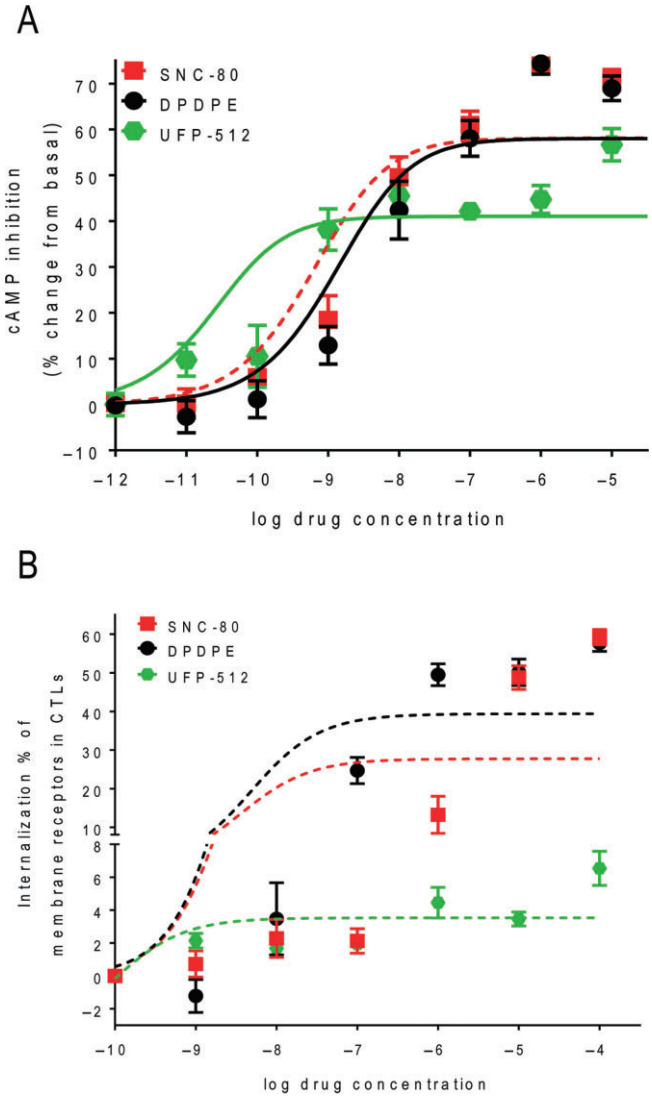


Figure 7

A single affinity state could not account for cyclase and internalization responses of δ opioid receptor agonists. (A) δ opioid receptor internalization and (B) cAMP accumulation data shown in Figure 5 were fit with the operational model, except that KA values for the agonists were fixed from published references as follows: KA_{SNC-80}: 5.75 nM; $KA_{UFP-512}$: 100 pM; and KA_{DPDPE}: 12.02 nM. (Modified with permission from Charfi *et al.*, 2013).

binding by itself was not enough to fully stabilize an active conformation of the receptor, requiring the presence of the corresponding Gαβγ heterotrimer (Chung *et al.*, 2011; Rasmussen *et al.*, 2011b) or of surrogate antibodies (Rasmussen *et al.*, 2011a) to consolidate an active state and make it amenable for crystallization.

There is now considerable evidence that in addition to G-proteins, GPCRs may signal via non-canonical pathways, which involve β-arrestin interaction with the receptor (Shenoy and Lefkowitz, 2003). As with the canonical complex, β-arrestin interaction with the receptor leads to its stabilization into conformations that display high affinity for the agonist. This has been observed in reconstitution systems where the addition of purified β-arrestin increased agonist affinity for biogenic amine receptors (Gurevich *et al.*, 1997) and in live cells, where fusion of peptide-binding receptors to β-arrestin was shown to produce a similar increase in agonist affinity (Martini *et al.*, 2002; Jorgensen *et al.*, 2005). Recent characterization of the sodium allosteric site in δ opioid receptors indicates that, at least in the case of this receptor, the switch between G-protein and β-arrestin signalling is modulated by sodium-coordinating residues in transmembrane domains II, III and VII (Fenalti *et al.*, 2014).

Biased signalling by δ opioid receptor agonists

Although spectroscopic studies provide clear evidence that association with different agonists stabilized purified δ opioid receptors into conformations that distinctively interact with $G\alpha_{i/o}$ subunits (Alves *et al.*, 2004; Hruby *et al.*, 2010), the drawback is that PWRS gives no information about the physiological consequences of these distinct interactions. Studies in live cells have provided this type of information, showing that δ opioid receptors behave as pleiotropic receptors capable of activating different Gα subunits (Allouche *et al.*, 1999; Alves *et al.*, 2004; Pineyro and Archer-Lahlou, 2007). In most cases in which ligand ability to activate different G-proteins was assessed, experiments were done at maximal effective concentration and, as we previously saw, for maximal responses to unequivocally indicate the existence of bias, there must be a clear reversal in ligand rank order to activate the different subunits (Kenakin, 2002a,b). Results fulfilling such conditions have been obtained in SK-N-BE neuroblastoma cells endogenously expressing δ opioid receptors. Herein, the rank order of maximal GTPγS binding by DPDPE was $\alpha_{i2} > \alpha_o$ and was reversed to $\alpha_o > \alpha_{i2}$ for deltorphin I (Allouche *et al.*, 1999). Etorphine, a non-selective μ/δ opioid receptor agonist, did not stimulate α_o but activated α_{i2} much more than α_{i3}. As the SK-N-BE neuroblastoma cell line used in this study only expressed δ opioid receptors (Polastron *et al.*, 1994), the specific profile of Gα activation by these ligands cannot be attributed to concomitant activation of other opioid receptor subtypes therefore indicating that ligand-specific δ opioid receptor responses may be observed at endogenous levels of expression.

The way Gα and Gβγ subunits reorganize with respect to one another upon activation of the G-protein by the receptor may also be ligand-specific. In particular, SNC-80, DPDPE and morphine reduced the distance between position 60 of the Gαi1 subunit and the N-terminus of Gγ while the same regions were drawn apart by TICP (Figure 2B) (Audet *et al.*, 2008). Moreover, ligand differences in conformational rearrangements could be similarly traced upstream in the way the receptor C-terminus interacted with Gαi (Figure 2B; inset), and downstream as functionally selective responses in cAMP and MAPK pathways (Audet *et al.*, 2008). SNC-80, DPDPE and morphine inhibited cAMP production and promoted ERK activation while TICP behaved as an 'inverse agonist' that enhanced cAMP production but also stimulated the MAPK cascade (Audet *et al.*, 2005; 2008). Thus, it is possible to conclude that the conformation stabilized by TICP was active for ERK but inactive for cyclase signalling, while the conformations stabilized by the other ligands were active in both pathways.

DPDPE and H-Tyr-Tic-Phe-Phe-OH (TIPP), a tetrapeptide related to TICP (Schiller *et al.*, 1999), were also found to engage ERK activity through different mechanisms suggesting ligand-specific modulation of the MAPK pathway by δ opioid receptor ligands. The mechanism triggered by DPDPE involved promoting a Gβγ-PLCβ3-cSrc complex that led to Raf1-mediated stimulation of the MAPK while its activation by TIPP relied upon β-arrestin 1/2 (Xu *et al.*, 2010). However, despite these striking differences, some caution is warranted before concluding that the two mechanisms were triggered by ligand-specific conformations. Indeed, together with the characterization of the two activation pathways, the authors showed that TIPP was a partial agonist that failed to induce δ opioid receptor phosphorylation at Ser^{363}. Mutating Ser for Ala at this position abolished phosphorylation by DPDPE together with the ligand's ability to engage the PLCβ3/Src/Raf1 pathway, causing it to shift to the β-arrestin 1/2 pathway (Hong *et al.*, 2009; Xu *et al.*, 2010). Thus, as the two different modalities of ERK activation were determined by differential phosphorylation of Ser^{363} and given that the partial agonist failed to induce the required phosphorylation for one of the pathways to be engaged, we cannot exclude the possibility that the differences between TIPP and DPDPE were not simply related to the full agonist producing δ opioid receptor phosphorylation while the partial agonist did not. Making this distinction is of importance to exploit the observed differences for therapeutic use.

Ligand-specific regulation of δ opioid receptors

Activation by agonists not only promotes signalling, but it also triggers homologous desensitization. The process is initiated by receptor phosphorylation and is followed by β-arrestin recruitment, receptor internalization and its ulterior sorting to degradation or reinsertion at the membrane. As for signalling, numerous reports point to the presence of biased responses along the different steps of the regulatory cascade. Some examples of this type of behaviour by δ opioid receptor agonists are considered in terms of identifying the specific contribution of ligand-specific signalling to observed response bias.

Ligand-specific patterns of δ opioid receptor phosphorylation

Phosphorylation usually involves more than one amino acid and the specific pattern of Ser/Thr residues that undergoes this type of modification has been considered a means of transferring the information codified in ligand-specific conformations to downstream regulatory proteins (Liggett, 2011; Just *et al.*, 2013). For example, μ opioid receptors have been shown to undergo hierarchical, ligand-specific phosphorylation patterns of their C-terminus and the combination of phosphorylated residues was predictive of distinct internalization profiles by different agonists (Just *et al.*, 2013). The δ opioid receptor C-tail also undergoes hierarchical phosphorylation of its Ser/Thr residues, Ser^{363} being critical for the process (Guo *et al.*, 2000; Kouhen *et al.*, 2000) and Thr^{358} contributing as an accessory (Guo *et al.*, 2000). Ser^{363} is phosphorylated by highly efficacious agonists like DPDPE (Guo *et al.*, 2000; Kouhen *et al.*, 2000), SNC-80 (Pradhan *et al.*, 2009), etorphine, deltorphin II (Marie *et al.*, 2008) and (+)BW373U86 (Bradbury *et al.*, 2009). In contrast, maximal effective concentrations of partial agonists like TIPP and morphine fail to produce any phosphorylation at this site (Bradbury *et al.*, 2009; Xu *et al.*, 2010). Given that failure to induce Ser^{363} phosphorylation corresponds with low intrinsic activity, these differences do not provide sufficient information as to whether post-translational modification at this residue is ligand-specific. Like morphine and TIPP, ARM100390 fails to produce Ser^{363} phosphorylation (Pradhan *et al.*, 2009), while the effect of TAN-67 is present but minimal (Bradbury *et al.*, 2009). What distinguishes these two ligands from morphine and TIPP is that GTPγS binding by TAN-67 and ARM100390 reached similar maximal responses as full agonists (Bradbury *et al.*, 2009; Pradhan *et al.*, 2009). Thus, the question is whether these four ligands that fail to induce Ser^{363} phosphorylation but differ in their maximal GTPγS responses display biased signalling in these two possible outcomes. One alternative is that the disproportion between GTPγS binding and phosphorylation elicited by TAN-67 and ARM100390 as compared with morphine and TIPP could be caused by factors such as the non-linear relationship between receptor occupancy and drug response (see quantification section). Using the operational model to control for this type of confounder, we found that ARM100390s transduction coefficient (log(Tau/KA)) for Gαo protein activation was ~eightfold lower than that of SNC-80 but ~1000 higher than that of TIPP (unpublished observation), indicating that both, ARM100390 and TIPP behaved as partial agonists as compared with SNC-80. By comparing G-protein activation by TAN-67 and SNC-80, Quock *et al.* (1997) arrived to a similar conclusion, namely that TAN-67 also behaved as a partial agonist and was almost 10-fold less efficacious than the standard SNC-80. In this case, the relationship between occupancy and response was taken into account by using the equation derived by Ehlert (1985), which allows us to estimate relative efficacies from empirical data such as the drug's half-maximal response ($0.5 \times Emax_{drug}/Emax_{system}$), its affinity for the receptor (Kd) and its potency (EC_{50}):

$$0.5 \times Emax_{drug}/Emax_{system} \times (1 + Kd/EC_{50}) = e_{rel}.$$

Hence, after controlling for system factors TAN-67 and ARM100390 displayed partial signalling efficacy, but it is not possible to rule out that disproportion in GTPγS versus phosphorylation responses is not simply due to more efficient stimulus-response coupling for the signalling than phosphorylation event. Quantification of bias for G-protein activation versus Ser^{363} phosphorylation should yield an unequivocal answer as to whether this residue is involved in ligand-specific regulation of δ opioid receptor signalling.

Unlike functional approaches, mutagenesis studies have clearly established that δ opioid receptor phosphorylation can be ligand-specific. These studies show that SNC-80, but not DPDPE, phosphorylates and down-regulates a receptor truncated at Gly^{338} (Okura *et al.*, 2003), observations that have been taken as an indication that more phosphorylation sites are accessible for protein kinases in δ opioid receptors bound to SNC-80 than DPDPE (Varga *et al.*, 2004). While the idea that DPDPE and SNC-80 stabilize δ opioid receptors into different conformations has been verified (Audet *et al.*, 2012), the region and identity of the residues involved in this difference remains to be determined. The third intracellular loop seems a likely candidate as it contains numerous Ser/Thr residues which contribute to β-arrestin binding (Cen *et al.*, 2001a,b) and this response is also distinctively engaged by DPDPE and SNC-80 (Audet *et al.*, 2012).

Agonist-specific internalization of δ opioid receptors

Maximal internalization and signalling responses elicited by δ opioid receptor agonists are not proportional, an observation that has been repeatedly obtained in heterologous expression systems (Bradbury *et al.*, 2009; Charfi *et al.*, 2013), cultured neurons (Charfi *et al.*, 2013) and *in vivo* brain samples (Pradhan *et al.*, 2009). Although this type of observation may be interpreted as the result of ligand-specific responses in the absence of a clear reversal in the rank order, the imbalance in signalling versus internalization Emax values could also be due to differences in stimulus-response coupling or to system factors. Transduction coefficients, on the other hand, allow a controlled quantification of bias. Data in Figure 5 illustrate how Emax values and transduction coefficients distinctively inform us with respect to signalling versus internalization bias by different DOP agonists. In the example, in question Emax values reveal no disproportion between maximal internalization and cAMP inhibition induced by DPDPE or SNC-80, but relative cyclase inhibition by UFP-512 was much higher than its relative internalization response. From these data, no bias would be predicted for DPDPE or SNC-80, but Emax differences for UFP-512 responses would be frequently interpreted as an indication of preferential cyclase versus internalization signalling. In contrast, bias factors calculated from transduction coefficients (Figure 5E) indicate that both UFP-512 and SNC-80 are capable of preferentially engaging cAMP over internalization. Bias for UFP-512 is mainly determined by low internalization efficacy and high 'conditional affinity' for cyclase-inhibiting receptor states. Bias for SNC-80 is associated to its low 'conditional affinity' for internalizing conformations of the receptor, a property that would have been overlooked by only comparing maximal responses.

The preferential engagement of cyclase over sequestration observed for SNC-80 (Charfi *et al.*, 2013) is in contrast with internalization bias predicted from the *in vivo* actions of this ligand (Pradhan *et al.*, 2009). Different reasons could explain this divergence. Firstly, the signalling response monitored in one study was G-protein activation (Pradhan *et al.*, 2009) and cyclase modulation in the other (Charfi *et al.*, 2013). Secondly, both reports compared SNC-80 with different standards. Thirdly, quantification of internalization was different, relying on kinetic parameters in the *in vivo* study (Pradhan *et al.*, 2009) and endpoint measurements *in vitro* (Charfi *et al.*, 2013). Fourthly, one set of conclusions was based on comparison of maximal responses (Pradhan *et al.*, 2009) and the other relied on transduction coefficients (Charfi *et al.*, 2013). Fifthly, cellular backgrounds used in both studies were different, involving HEK cells in the *in vitro* study (Charfi *et al.*, 2013) and brain tissue in the other (Pradhan *et al.*, 2009). Importantly, not all regulatory proteins involved in δ opioid receptor internalization are conserved across these cellular phenotypes. For example, while β-arrestins contribute to δ opioid receptor internalization in HEK cells and neurons, PKCs and G protein–coupled receptor kinase 2 play a role in the latter but not the former (Charfi *et al.*, 2013). At the same time, the molecular basis of biased responses is given by the distinct way ligands may interact with the receptor when the latter is associated with different signalling partners (Kenakin and Miller, 2010; Kenakin *et al.*, 2012; Kenakin, 2012). Thus, the very existence of cell-specific internalization partners implies that ligand bias may not be conserved across different cell types or even in different neuronal populations mediating different effects of opioids. If this is the case, cellular background constitutes a source of variation that cannot be simply controlled by the use of the operational model, but should be taken into account if ligand bias is to be used in the rational design of novel therapeutic agents.

Distinct *in vivo* internalization profiles of δ opioid receptor agonists have been considered of predictive value with respect to ligand potential for generating tolerance (Pradhan *et al.*, 2010). However, tolerance seems to be related not only to internalization but also to the specific type of response assessed. For example, repeated administration of the non-internalizing agonist ARM100390 over a 6-day period developed tolerance for analgesic but not for locomotor or anxiolytic actions (Pradhan *et al.*, 2010). Over a similar period of time, the internalizing agonist SNC-80 produced tolerance to all of these effects (Jutkiewicz *et al.*, 2005; Pradhan *et al.*, 2010) but not to antidepressant-like effects associated with its administration (Jutkiewicz *et al.*, 2005). Numerous possibilities may explain why sequestration profiles fail to predict the occurrence of tolerance for all responses mediated by a given agonist. At the molecular level, one possible reason could be that neurons, which mediate different responses rely upon different proteins for internalization, and consequently the ability of a given ligand to engage δ opioid receptor sequestration may vary in different neuronal populations. Alternatively, internalization may not equally limit all responses mediated by the same ligand. Thus, if δ opioid receptors in different neuronal populations produce their effects through different effectors, internalization may shut down one type of signal but not the other. For instance, by separating the receptor from Ca^{2+} channels or removing both from the membrane (Altier *et al.*, 2006) internalization may limit depolarization. However, internalization may not necessarily shut down cyclase (Bagheri Tudashki *et al.*, 2013) or MAPK signals (McLennan *et al.*, 2008). Finally, the predictive value of internalization with respect to tolerance may also be influenced by the fact that δ opioid receptors internalized by different ligands do not necessarily follow the same post-endocyctic pathway (Marie *et al.*, 2003; Audet *et al.*, 2012). For example, deltorphin II and DPDPE are more efficient than SNC-80 to induce internalization (Bagheri Tudashki *et al.*, 2013; Charfi *et al.*, 2013), but unlike the latter they do not develop acute analgesic tolerance (Beaudry *et al.*, 2009; Bradbury *et al.*, 2009). A major difference between DPDPE and SNC-80 is that δ opioid receptors activated by DPDPE efficiently recycle back to the membrane, while those activated by SNC-80 are targeted for degradation (Lecoq *et al.*, 2004; Audet *et al.*, 2012). As mentioned in the preceding section, DPDPE stabilizes δ opioid receptors into a conformation that is exclusively phosphorylated at the C-terminus while the conformation stabilized by SNC-80 also induces phosphorylation outside the C-terminal domain (Okura *et al.*, 2003). While receptors activated by SNC-80 remain locked in a stable association with β-arrestin 2, those activated by DPDPE establish a transient interaction with this regulatory protein which allows them to recycle back to the membrane once the complex dissociates (Audet *et al.*, 2012).

In conclusion, here, we have revised evidence indicating that the presence of biased responses may not always correspond to ligand bias. Making the difference between both situations is essential to understand how ligand-specific responses may support desired and undesired effect of opioids in view of exploiting functional selectivity with therapeutic purposes.

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Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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