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5-HT_{2C} constitutive receptor activity: effector pathway dependence

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Abstract

Recent evidence suggests that receptors may exist in an "inactive" conformation (designated R) in equilibrium with one or more so-called "active" conformations (R*, R**, R***, etc.) that have the ability to activate cellular effector mechanisms in the absence of an agonist (constitutive activity). In addition, many current models of receptor function suggest that different active conformations may differentially interact (qualitatively and/or quantitatively) with cellular effector mechanisms. One of the predictions stemming from these models is that constitutive receptor activity should be effector pathway dependent. We demonstrate that inverse agonism at the serotonin$_{2C}$ (5-HT_{2C}) receptor is effector pathway dependent and that the experimental observations can be described by a three-state model of receptor function. Further, we provide evidence for protean ligand activity of SB 242084. The results emphasize the importance of associating the pharmacological properties of ligands with the experimental conditions and responses measured.

Keywords: Inverse agonism; Constitutive receptor activity; Efficacy; Receptor conformations; Signal transduction

Serotonin$_{2C}$ (5-HT_{2C}) receptors are members of the seven-transmembrane spanning (7-TMS or heptahelical) receptor superfamily, frequently referred to as G protein-coupled receptors (GPCRs). Like many, if not all, 7-TMS receptors, 5-HT_{2C} receptors couple to

Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; 7-TMS, seven-transmembrane spanning; AA, arachidonic acid; CHO, Chinese Hamster Ovary; DCI, dichloroisoprenaline; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; GPCR, G protein-coupled receptor; GRK, G protein receptor kinase; IP, inositol phosphate; PDZ, PSD-95/Discs-large/ZO-1 homology; PLA_2, phospholipase A_2; PLC, phospholipase C.

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multiple cellular effector systems. Perhaps the best studied effector system is the phospholipase C (PLC) pathway. Stimulation of PLC by 5-HT_{2C} receptors is mediated by activation of $G_{\alpha q/11}$ and leads to the production of inositol phosphates (IPs) and diacylglycerol. Another effector pathway that couples to 5-HT_{2C} receptors, but is somewhat less well studied, is the phospholipase A_2 (PLA_2) signalling cascade, which leads to the liberation of arachidonic acid (AA). Receptor-mediated activation of both of these effector pathways is insensitive to pertussis toxin. Although the signalling for PLC activation is likely via $G_{\alpha q/11}$, the signalling mediator for PLA_2 activation is not known.

In addition to the more classical signalling mechanisms described above, 5-HT_{2C} receptors couple to a variety of other signalling systems. Although not generally viewed as classical effectors, desensitization mechanisms, such as G protein-coupled receptor kinase (GRK), are activated in response to receptor activation, and as such may be considered as effectors. Moreover, recent reports suggest that arrestin, which binds to receptors phosphorylated by GRK, may serve a cellular signalling role in addition to promoting uncoupling of the receptor from more traditional effectors [1]. Additionally, it has been demonstrated that 5-HT_{2C} receptors can couple to effector systems such as to pertussis toxin-sensitive G proteins (e.g., $G\alpha_{i/o}$) and to PSD-95/Discs-large/ZO-1 homology (PDZ) domain containing proteins that may have possible effector activity. Consequently, the net effect of activation of 5-HT_{2C} receptors is a coalescence brought about by the concurrent activation of several effector pathways within cells.

Current models of receptor activation are based on a multi-state model in which receptors are proposed to exist in equilibrium between inactive (R) and one or more active (R*, R**, R***) conformations. Active receptor conformations are said to be capable of activating cellular effector mechanisms in the absence of an activating ligand (agonist), and this activity is referred to as constitutive receptor activity or ligand-independent receptor activity. Constitutive receptor activity contributes to basal response levels in a cell. The simplest multi-state model is a two state model (Fig. 1) in which one active

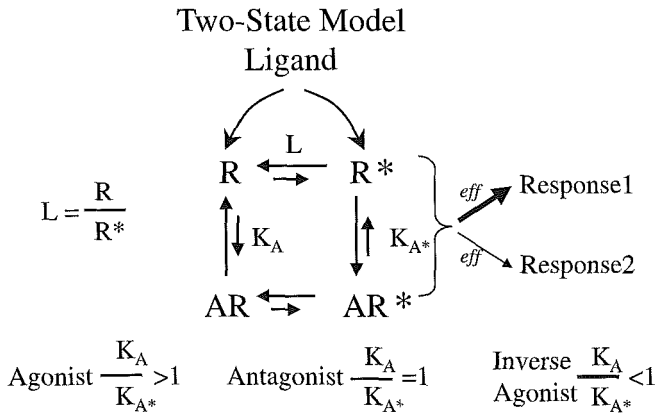

Fig. 1. Two-state model for receptor function. The model, derived from the work of Leff [2] is shown with the active form of the receptor (R*) coupling to two responses with different efficiencies (eff) that are ligand independent.

receptor conformation (R*) is in equilibrium with one inactive conformation (R) [2]. The proportion of the receptor population in a particular conformational state at any point in time is defined by an allosteric constant, L. As L decreases, the level of basal response contributed by constitutive receptor activity increases. The three-state model (Fig. 2), as described by Leff et al. [3], exhibits the next higher level of complexity. In this model, receptors exist in equilibrium between an inactive (R) and two active conformations (R* and R**). In addition to L, a second allosteric constant (M) describes the relative likelihood of finding the receptor in the inactive conformation (R), or one of the active states (R* or R**). This model was developed to accommodate the capacity of agonists to selectively activate a subset of the multiple signal transduction pathways that may be coupled to a single receptor subtype (see Refs. [4,5]), which was not permissible with the two-state model.

The multi-state models support three classes of ligand activity, which are defined on the basis of relative affinity constants (K_A, K_{A*}, K_{A**}) of the ligand for each of the various receptor species. Agonists have greater relative affinity for active conformations and thereby stabilise that population leading to increased response. Antagonists have equal affinity for all receptor conformations and therefore do not alter the equilibrium between the conformations and thus do not alter the basal response. Inverse agonists are ligands that preferentially bind to, and stabilise, the inactive conformation(s) and thus reduce the proportion of the receptor population in the active state(s), leading to reduced basal responses.

Both the two-state and the three-state models permit the maximum effect of inverse agonists to differ, depending upon the response measured. In both models, the value of the allosteric constant(s) sets the base level of constitutive receptor activity and thus the system maximum for the effect of inverse agonists. The two-state model demands that the relative efficacy of inverse agonists be independent of the response measured (i.e., the same for all responses). However, with the three-state model, inverse agonists may have different ratios of affinity constants K_A/K_{A*} versus K_A/K_{A**} and thus may display different

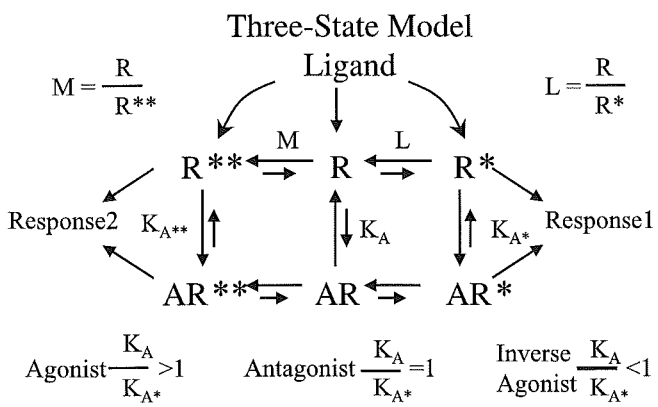

Fig. 2. Three-state model of receptor function. In this model, developed by Leff et al. [3] the receptor can adopt three conformations, two of which are active (R* and R**) and which couple to different responses.

magnitudes of inverse agonist activity toward different responses. Consequently, inverse agonist relative efficacy could be different for different responses.

We have studied the effector pathway dependence of inverse agonist effects at 5-HT_{2C} receptor systems. Fig. 3 shows agonist and inverse agonist actions of a series of ligands measuring two of the classical effector responses coupled to the 5-HT_{2C} receptor, PLC–IP accumulation and PLA_2–AA release in a cell line with high 5-HT_{2C} constitutive activity (see Ref. [6]). The figure illustrates that in general inverse agonist effects are greater for reduction of the IP accumulation response than for the AA response. Drugs such as SB 206553, clozapine and mianserin appear to be high efficacy inverse agonists for IP and AA, while the inverse agonist efficacies of ketanserin and mesulergine are much lower. In fact, for the AA response, these latter drugs appear to be simple antagonists as they do not alter the basal level of AA release. These differential, effector pathway-dependent properties of a ligand underscore the difficulty of labeling a ligand as an agonist, antagonist or inverse agonist without specifying the response studied. Further, Chidiac et al. [7] have nicely demonstrated that even the conditions under which a ligand is studied can alter its pharmacological characterization. In sf9 cells transfected with the β2-adrenoceptor, the ligand dichloroisoprenaline (DCI) could behave as a partial agonist in

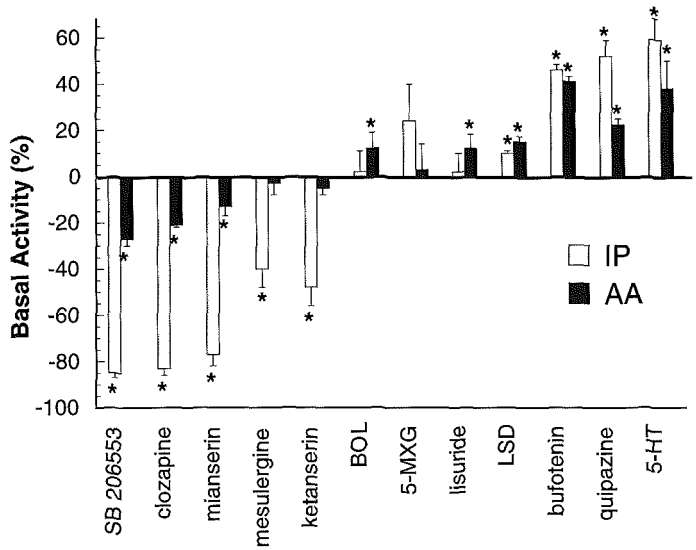

Fig. 3. Effect of 5-HT_{2C} receptor ligands on basal IP accumulation and basal AA release in high expressing Chinese Hamster Ovary (CHO)-1C7 cells. Cells, pre-labeled with [^{3}H]-AA (4 h) and [^{3}H]-*myo*-inositol (24 h), were incubated (37 °C) with the indicated drugs or vehicle (0.01% DMSO) for 25 min in the presence of 20 mM LiCl and 0.1% BSA. AA release and IP accumulation were measured simultaneously from the same multi-well. Data are expressed as the percent change in basal effector activity. Drug concentrations (100 × K_i or K_B values) were as follows: SB 206553, 300 nM; clozapine, 1 μM; mianserin, 400 nM; mesulergine, 100 nM; ketanserin, 1 μM; BOL (bromo-LSD), 700 nM; 5-MXG (5-methoxygramine), 3 μM; lisuride, 2 μM; D-LSD, 1 μM; 5-HT, 3 μM. Basal activity was 3052 ± 313 and 3835 ± 553 dpm for IP and AA, respectively. Data represent means ± S.E.M of four to eight experiments. Treatment with pertussis toxin (24 h, 50 ng/ml) did not alter basal activity or ligand-induced changes in basal activity. *$P < .05$ compared to paired vehicle. Data reproduced with permission from Ref. [6].

naive cells, whereas after exposure of cells to the full agonist isoprenaline, DCI always behaved as an inverse agonist.

As mentioned earlier, desensitization mechanisms can also be considered as effector pathways because they are activated in response to receptor activation. Just as activation of receptors with agonist can lead to desensitization (homologous and heterologous), constitutive receptor activity can also desensitise receptor systems. As a consequence of reducing "constitutive desensitization," 5-HT_{2C} inverse agonists can enhance both basal and agonist-stimulated responses (homologous sensitization) and also elicit heterologous sensitization (see Ref. [6]). The magnitude of the sensitization effect is dependent upon the basal degree of desensitization of the response. As shown in Fig. 4, prolonged (24 h) treatment with inverse agonists in a cell line expressing low levels of the 5-HT_{2C} receptor results in homologous sensitization that is response dependent, occurring only for the IP response and not for AA release. In these cells, there is no reduction of basal IP or AA release in response to acute exposure to inverse agonists (low constitutive receptor activity toward PLC and PLA_2), suggesting that the reduction of desensitization is quite sensitive to inverse agonist action. This is also suggested by the sensitization response to 5-MXG, which is inactive in reducing basal response in the high expressing cells (see Fig. 3). Similar results have been obtained for 5-HT_{2C} inverse agonist effects on heterologous sensitization of the IP, but not AA, response to P_{2Y} receptor activation [6].

The data presented thus far indicate that 5-HT_{2C} inverse agonists have actions that are response dependent and that the effect is greater toward the desensitization response and

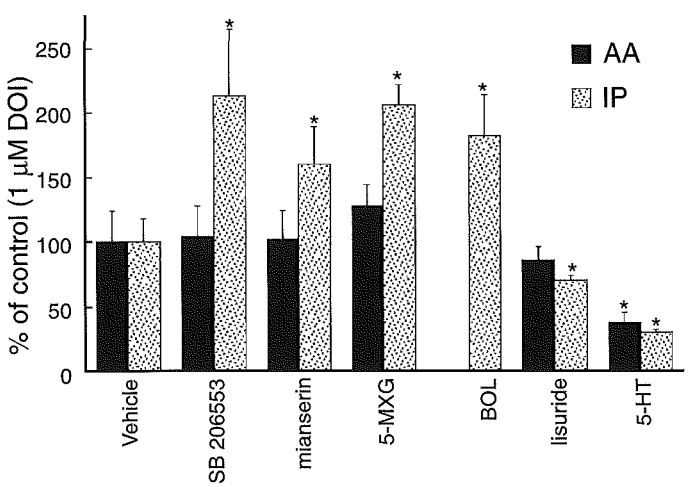

Fig. 4. Effect of 24-h treatment with SB 206553 on 5-HT_{2C} receptor-mediated AA release and IP accumulation in the low-expressing 5-HT_{2C} receptor cell line (CHO-1C19). CHO-1C19 cells were treated for 24 h with various 5-HT_{2C} ligands at concentrations (100 × K_i or K_B values) as indicated in the legend of Fig. 3. Cells were washed thoroughly and (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; 1 μM)-stimulated AA release and IP accumulation were measured. Each bar represents the mean ± S.E.M. of three to six experiments. None of the drugs tested altered basal AA or basal IP accumulation. DOI-mediated AA release was 198% above basal ± 47% and DOI-mediated IP accumulation was 352% above basal ± 63% (mean ± S.E.M, $n=6$). *$P<.05$ as compared to paired vehicle control. Data reproduced with permission from Ref. [6].

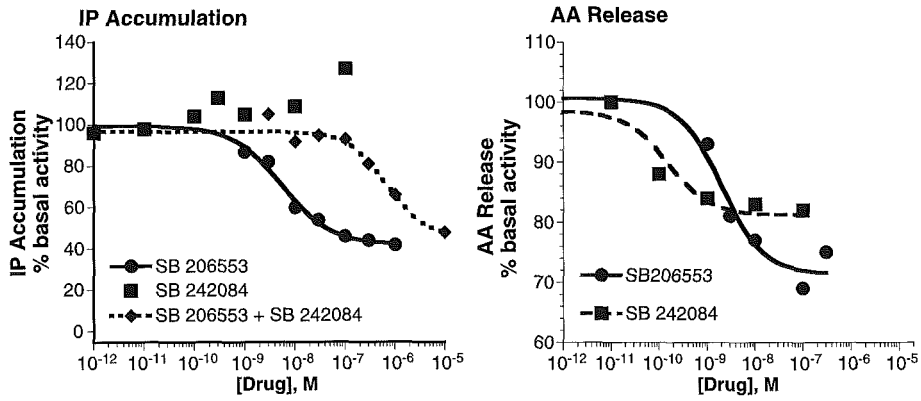

Fig. 5. Effect of SB 206553 and SB 242084 on basal IP accumulation and AA release in CHO-1C7 cells expressing high levels of the 5-HT_{2C} receptor. Left: SB 206553 reduced basal IP accumulation while SB 242084 displays weak agonist activity. The concentration–response curve to SB 206553 is shifted to the right in the presence of SB 242084. Right: Both SB 206553 and SB 242084 reduce basal AA release. Data represent the mean of triplicate determinations in a representative experiment. These results suggest that SB 242084 behaves as a protein ligand–agonist action on the PLC response and inverse agonist action on the PLA_2 response.

the PLC–IP pathway than the PLA_2–AA pathway. Such results can be explained with the two-state model by postulating that the 5-HT_{2C} receptor couples with higher efficiency to the PLC effector such that constitutive receptor activity, and desensitization, is greater for the IP response. However, results shown in Fig. 5 cannot be explained simply by greater constitutive receptor activity of the 5-HT_{2C} receptor toward PLC or by the two-state model. SB 242084 displays greater inverse agonist activity toward the 5-HT_{2C} receptor—PLA_2–AA pathway, than for the PLC–IP response. In fact, the relative efficacy of SB

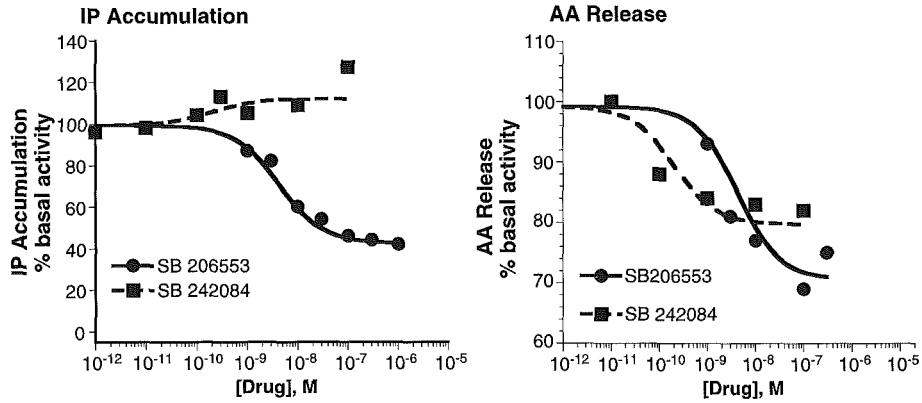

Fig. 6. Predictions of the three-state model for 5-HT_{2C} ligands. Data points shown are from Fig. 5. SB 206553 (solid line) and SB 242084 (dotted line) were calculated by substituting parameters shown in Table 1 into equations for fR* and fR** as described in the text, with fR* representing IP accumulation and fR** representing AA release. The assumption was made that fractional responses were proportional to fractional occupancy.

Table 1
Values for variables substituted into Eqs. (1) and (2) to simulate experimental data

Agonist	$L=M=0.1$		
	K_A	K_{A*}	K_{A**}
SB 206553	4.40e − 10	9.90e − 09	5.94e − 09
SB 242084	1.18e − 10	1.76e − 10	2.47e − 10

242084 for the PLA_2–AA response is 0.66 with respect to the inverse agonism of SB 206553 but SB 242084 appears to have weak agonist activity for the PLC response.

As shown in Fig. 6, computer simulations using the three-state model agree well with the experimental data. Equations for fR* (Eq. (1)) and fR** (Eq. (2)) were derived from the work of Leff et al. [3]. The values of L and M were set to 0.1 to provide for constitutive receptor activity. Values of K_A, K_{A*} and K_{A**} were chosen, which resulted in simulations most closely matching the experimental observations (Fig. 5; Table 1).

$$\mathrm{fR}^* = \frac{\frac{1}{L} + \left(\frac{1}{LK_{A}*}\right)[A]}{\left(1 + \frac{1}{L} + \frac{1}{M}\right) + \left(\frac{1}{K_A} + \frac{1}{LK_{A}*} + \frac{1}{MK_{A}**}\right)[A]} \quad (1)$$

$$\mathrm{fR}^{**} = \frac{\frac{1}{M} + \left(\frac{1}{MK_{A}**}\right)[A]}{\left(1 + \frac{1}{L} + \frac{1}{M}\right) + \left(\frac{1}{K_A} + \frac{1}{LK_{A}*} + \frac{1}{MK_{A}**}\right)[A]} \quad (2)$$

These results demonstrate that inverse agonist action and constitutive receptor activity is dependent upon the response measured. Furthermore, they emphasize the importance of associating ligand pharmacological characteristics with the experimental conditions and responses measured.

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Discussion 10

A. Newman-Tancredi

All these data were derived using the INI isoform?

W. Clarke

They were all done with the INI. We've looked at the VGV but not for multiple effector activation. We've started those experiments but we haven't completed them yet.

A. Newman-Tancredi

Because these other editing isoforms exhibit less constitutive activity. So you might see different patterns with these different drugs.

W. Clarke

The 5-HT_{2C} receptor is an interesting receptor in that its message undergoes a process called RNA editing, and there are different isoforms of the receptor produced as a result of RNA editing. If one studies the effect of changes in receptor density on basal inositol phosphate accumulation the edited isoforms are generally less active towards inositol phosphate than for INI, the nonedited receptor. We've reproduced that data doing the slope values, but we have not looked at other effector responses, such as PLA2, for example.

A. Newman-Tancredi

Because if we are looking at opportunities for drug development, in fact, I think it's the VSV which is most highly expressed in humans. So, in fact, all of this needs to be redone on VSV.

W. Clarke

I think one of the other things that we also miss is that those receptor isoforms are likely co-expressed in the same cells. We are going to have INI and VGV, the different edited isoforms, because the mRNA is being edited, so there's going to be unedited mRNA present along with edited mRNA; whether there are dimers forming between the molecules is an interesting question.

R. Bond

How we get a compound to be more potent as an inverse agonist than an agonist? Because that's what you showed.

W. Clarke

No actually, SB 242084 is more potent than the SB 206553 compound.

R. Bond

No, I mean the one SB compound that behaved as a partial agonist, as an inverse agonist, and it looked like the IC_{50} was 0.1 nM. And the EC_{50} was above 1 nM.

W. Clarke

I don't know, I don't think so. It was initially—in fact, I contacted you about this because I had originally thought that it might be the case.

R. Bond

I wanted to get two curves on it, one going down, another going up.

W. Clarke

I think the SB compound should be exactly the same, 1 nM. I think the scale is mislabelled. There was some hint in our original experiments, and Richard might be remembering this because I asked him about it, if he thought there could be differences in potency between inverse agonist or agonist for two different responses. I mean, for agonist one can guess that that's true based on differences in receptor reserve, but for inverse agonist it wasn't quite clear to me. And I'm sorry about that.

R. Bond

It is worth noting that, and it might be related to what you said about that, if you chronically treat with inverse agonist, it's easier to reveal inverse agonist activity. I mean, it's really a nice Yin-Yang thing that continues.

W. Clarke

I think that actually there may be also multiple things going on there (with chronic treatment). One is the turning off of desensitisation mechanisms. But also probably turning off other changes, like posttranslational modifications that occur at the receptor. Maybe if the receptor is phosphorylated its ability to switch between R and R* or R**, R***, would have different propensity. Then one might see, in a desensitised state, different levels of constitutive activity that would be brought out only under desensitised conditions.

G. Milligan

I know you're measuring a lot of endpoints already, but clearly one of the trendy things to consider is the role of arrestin scaffolding in generating signals. And you've already shown some nice examples of varying efficacy for a number of G protein-coupled endpoints. Presumably an arrestin scaffolding endpoint must be G protein independent if these things are competing for each other. Have you tried to look into that area? Because I have always thought that you might see vastly and very interestingly different pharmacologies, if you truly looked at the G protein-mediated versus a 7-TM but not G protein-mediated endpoint.

W. Clarke

Absolutely, that's the message that I've been trying to get across to people studying trafficking of receptor signalling. We are more likely to see differences in receptor conformation based between two different signalling mechanisms that are not G protein mediated, necessarily. We have thought about this, and I have plans for experiments along those lines. The ability to generate concentration response curves towards that pathway, however, is somewhat difficult, and I suspect we will have to just go with maximal effects, or maximal occupancy of ligands at this stage. That will be an important aspect. Also, there certainly are 7-TM receptors that have PDZ domains that can also produce activation

of the sodium–hydrogen exchanger regulatory factor (NHERF), which would be another interesting study for response to measure.

T. Schwartz

I should like to point to the fusion proteins that we have in the NK_1 receptor. There's hardly any constitutive activity in the NK_1 receptor, whatever way you look at it. However, in relation to coupling and ligand binding, the NK_1 receptor appears to be able to go into two different types of complexes with two different G proteins, and that agonist binding to these two different complexes in the whole cell membrane, is not readily exchangeable. It looks very much like you have two separate micro-domains of different populations of receptors, depending on the G protein context they're in. So, it looks like certain receptors can really display different pharmacology at least in respect of agonism.

W. Clarke

Sure, I think that's an important issue as to whether or not micro-domains and micro-compartmentation of receptors for specific signalling molecules exist. If so, they may certainly have a strong impact on the way in which they're able to signal, and then what ligands will do. An important issue is if ligands can move those receptors away from those micro-domains.

A. Newman-Tancredi

I picked up from your work aspects of heterologous sensitisation, which was really appealing to me. Is that something that's true for the $5\text{-}HT_{2C}$ receptor only? You show that for the ATP response in the same cells, but I'd love to hear your comments now that we are 2 years further on.

W. Clarke

I didn't show it, because we haven't explored it too much further. We've been looking at the mechanisms for the heterologous desensitisation or sensitisation, and I was saying that can be for constitutive desensitisation of the $5\text{-}HT_{2C}$ receptor as a result of constitutive receptor activity. One of our early papers showed that there was constitutive desensitisation of the responsiveness of a second receptor expressed on the same cells. A heterologous desensitisation, if you will. And with prolonged treatment with inverse agonist, we're able not only to promote homologous sensitisation, but produce changes in the responsiveness of this other receptor system. Somewhat it may be analogous to what Leurs was showing before, but in the reverse direction. The interesting aspect is that in order to see homologous sensitisation we need to remove the inverse agonist. If it's still present, the agonist will be inhibited from binding, because of the presence of the inverse agonist. For the heterologous sensitisation we were able to show that prolonged treatment with clozapine, for example, or SB compound, was able to enhance purinergic receptor-mediated phospholipase C activity without changing phospholipase A activity, without having to remove the inverse agonist. And that had great implications for therapeutics, because sometimes, what inverse agonists may be doing is, not only affecting the receptor that they're targeting, but affect other receptor systems in cells. Also, we've been studying the mechanisms for the heterologous desensitisation. We had suggested that mechanism for homologous desensitisation involved changes in the level of expression of $G_{\alpha q/11}$. We actually thought that that would be the mechanism also for the changes in purinergic

responsiveness, but it seems that the changes in purinergic responsiveness are sensitive to tyrosine kinase inhibitors. So there's likely another pathway, going between the 5-HT_{2C} receptor and the purinergic receptor that seems to be tyrosine kinase sensitive.

M. Brann

We've done a lot of work with 5-HT_{2C} and various other 5-HT receptors, and one of the paradoxical things that we've seen there, and which is also in the published literature from others, is that in the case of the 5-HT_{2C}, you do not have an agreement between the potencies of inverse agonists in inverse agonist assays, with what you see in competitive antagonist and radioligand binding assays. Having said that, we do see perfect correlation in almost every other system we look at. And the two state model predicts that the K_i should be approximately equivalent to the EC_{50} of an inverse agonist, but for some reason, 5-HT_{2C} violates that almost totally, and the binding assays don't agree with anything. So, does anybody have any insight about it?

W. Clarke

The inverse assays are shifted way over to the right, versus what you see in competition or in saturation binding, and also the saturation binding and the competition binding don't agree very well with 5-HT_{2C}. So I was just wondering if there's any insight about what's strange about 5-HT_{2C}.

A. Newman-Tancredi

As a partial answer, we recently did a study on 5-HT_{2C} receptors expressed in CHO cells, and we looked at receptor reserve, and we found very nice correlations of potency for stimulation of G_q, and independently of stimulation of $G_{\alpha_{i3}}$ by this one receptor, once we'd knocked down the receptor expression level fairly substantially. So we did the EDDQ treatments, to take the receptor expression right down, and then we obtained very nice correlations in that expression system.

M. Brann

The issue we have for that is that the inverse experiments shouldn't be related to the reserve question, so invoking reserve doesn't really help, because of the affinity for R and R*. You should have very subtle effects of receptor reserve on inverse potency, but you have this fiftyfold shift of inverse potency, which I don't think receptor reserve works to explain. But I think there is something else.

W. Clarke

I think that's hard. Affinity values are a collection of micro-constants. They're constants that are observed affinities of a population of affinity values. And it's conceivable that some are associated with different receptor conformations that are differentially stabilised by different inverse agonists and those that are differentially stabilised by different agonists. Another important point is that the model that I presented, which is a simple model, predicts the effects of the protean ligand and was based on the idea that an active conformation for one response was inactive for another. So R** was inactive, then, for the response that was mediated by R*. So a ligand that enriched R** would do so at the expense of both of R and R*. So it's conceivable that some of this complicated pharmacology is what we've been looking at for a long time. When everything seems to have reasonable pharmacology, why do we have to invoke some of these more complex systems, when R and R* seemed to work very well? Maybe for the 5-HT_{2C} receptor, there are more complex model systems.

T. Schwartz

One of the things we assume when we're seeing all these things is that the effect and the affinity we measure occur to the same populations of receptors that are easily exchangeable with each other, and I would like to again stress the point that in many systems, we have population of receptors in a membrane that do not exchange readily with each other, and you may have a large population on one and a small on the other one, and in a radioligand binding assay, you may pick up one, so if you do an off-rate, on-way rate, it might occur in one population, and, actually, the effect in another one, depending on micro-domains and so forth, determined by scaffolding proteins and so on. So that could be very well an explanation. At least it explains a lot in the NK_1 system, where you see exactly these type of things.