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Opinion

Allosteric interactions via the orthosteric ligand binding sites in a constitutive G-protein-coupled receptor homodimer

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N-methyl scopolamine (PubChem CID:71183)
Nag 26 (PubChem CID:91810287)
TCS 1102 (PubChem CID:11960895)

ABSTRACT

I interpret some recent data to indicate that co-operative effects take place between the (identical) orthosteric binding sites in a G-protein-coupled receptor dimer. In the current study, the reasonability of this concept was tested by creating a mathematical model. The model is composed of a symmetrical constitutive receptor dimer in which the protomers are able to affect each other allosterically, and it includes binding, receptor activation and signal amplification steps. The model was utilized for analyses of previous data as well as simulations of predicted behaviour. The model demonstrates the behaviour stated in the hypotheses, i.e. even an apparently neutral receptor ligand can allosterically affect agonist binding or receptor activation by binding to the normal orthosteric ligand binding site. Therewith the speculated allosteric action originating from the orthosteric binding site of the dimeric receptor is a realistic possibility. The results of the simulations and curve fitting constitute a reasonable starting point for further studies, and the model can be utilized to design meaningful experiments to investigate these questions.

1. Introduction

G-protein-coupled receptor (GPCR) di- or oligomerization, especially heterodimerization, has raised much interest as a potential regulator of receptor trafficking, ligand selectivity and receptor signalling. While the current consensus is that most (all?) GPCRs, of at least families A and C, make homo- or heteromeric complexes, the significance of these complexes is yet for most of the receptors unknown [1–4]. Structural modelling has suggested that there is space for only a single G-protein heterotrimer per receptor dimer, and thus each dimer may constitute a single signal transducer (see, e.g., [5–7]). However, many questions remain open. Radioligand binding or competitive displacement sometimes shows bell-shaped or steeper than normal concentration–binding relationship or kinetic abnormalities indicative of positive co-operativity of the receptor protomers within a presumably homomeric complex [8–13]. It has indeed been possible to reproduce such behaviour with simple mathematical models involving receptor dimers (or bivalent receptors) [9,14–16] or to affect the behaviour by adjusting receptor expression level [13]. In contrast, it is not known, how di- or oligomerization affects receptor signalling, e.g. how the signalling of partially agonist-occupied (one or several but not all sites) or fully agonist-occupied (two or all sites) receptor may differ. There are many studies aiming to investigate the signalling of heteromeric receptor complexes but due to technical limitations, it is often not easy to distinguish between molecular and functional interactions in the signalling.

In this paper, I present a hypothesis concerning allosteric interactions between the protomers in a constitutive receptor dimer. If occupation of a single ligand binding site by an agonist is enough to activate the receptor, then the ligand binding site may be utilized to allosterically modulate agonist binding to or activation of the receptor by other ligands. Thus no additional binding sites for allosterically acting ligands are required but the entire action may be obtained via the orthosteric binding sites. In the mathematical model constructed in the current study, the receptor dimer can thus be activated by binding of a single agonist molecule to one protomer, while the other protomer's orthosteric binding site may act as an allosteric site, which can be utilized for homotropic positive or negative interactions or for similar

Abbreviations: AF-DX 116, *N*-2-{2-[dipropylaminomethyl]-1-piperidinyl}ethyl)-6-oxo-5*H*-pyrido2,3-b(1,4)benzodiazepine-11-carboxamide (a muscarinic receptor antagonist); CHO-K1, Chinese hamster ovary K1 (a cell line); GPCR, G-protein-coupled receptor; Nag 26, 4'-methoxy-*N*,*N*-dimethyl-3'-[*N*-3-{[2-3-methylbenzamidoethyl]amino}phenyl)sulfamoyl]-(1,1'-biphenyl)-3-carboxamide (an orexin receptor agonist); NAM, negative allosteric modulator; PAM, positive allosteric modulator; TCS 1102, *N*-biphenyl-2-yl-1-{[(1-methyl-1*H*-benzimidazol-2-ylsulfanyl]acetyl}-L-prolinamide (an orexin receptor agonist).

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heterotropic interactions, in which case the other ligand may be a positive or negative modulator or neutral.

2. Material and methods

2.1. Mathematical models

Derivation of all the equations is shown in detail in *Supplementary Material 1*. The receptor models were built according to the ternary complex model for an obligate receptor dimer (Figs. 1C and 2). The basic demands were to allow modelling of the co-operativity both at the level of the binding and the receptor activation. The equations were derived utilizing the techniques described in [17] and checked explicitly for the different steps (*Supplementary Material 1*) and by numerical iteration.

Simpler variants in Fig. 1A and B illustrate the generation of the model. The actual model used in the studies is shown in Fig. 1C. This is presented in a more comprehensible way in Fig. 1D.

For two different ligands A and B, the equations become

$$[\mathrm{RR}] = \frac{[\mathrm{RR_t}]}{\left(1 + 2\frac{[\mathrm{A}]}{K_\mathrm{A}} + 2\frac{[\mathrm{B}]}{K_\mathrm{B}} + \frac{[\mathrm{A}][\mathrm{A}]}{K_\mathrm{A}K_\mathrm{AA}} + 2\frac{[\mathrm{A}][\mathrm{B}]}{\gamma K_\mathrm{A}K_\mathrm{B}} + \frac{[\mathrm{B}][\mathrm{B}]}{K_\mathrm{B}K_\mathrm{BB}}\right) + \frac{1}{K^*}\left(1 + 2\frac{[\mathrm{A}]}{\alpha K_\mathrm{A}} + 2\frac{[\mathrm{B}]}{\beta K_\mathrm{B}} + \frac{[\mathrm{A}][\mathrm{A}]}{\alpha\varepsilon K_\mathrm{A}K_\mathrm{AA}} + 2\frac{[\mathrm{A}][\mathrm{B}]}{\alpha\beta\gamma\delta K_\mathrm{A}K_\mathrm{B}} + \frac{[\mathrm{B}][\mathrm{B}]}{\beta\phi K_\mathrm{B}K_\mathrm{BB}}\right)} \quad (1a)$$

$$binding(\mathrm{A}) = \frac{[\mathrm{RR}]_\mathrm{t} \times 2\frac{[\mathrm{A}]}{K_\mathrm{A}}\left(1 + \frac{[\mathrm{B}]}{\gamma K_\mathrm{B}} + \frac{[\mathrm{A}]}{K_\mathrm{AA}} + \frac{1}{\alpha K^*} + \frac{[\mathrm{B}]}{\alpha\beta\gamma\delta K_\mathrm{B}K^*} + \frac{[\mathrm{A}]}{\alpha\varepsilon K_\mathrm{AA}K^*}\right)}{\left(1 + 2\frac{[\mathrm{A}]}{K_\mathrm{A}} + 2\frac{[\mathrm{B}]}{K_\mathrm{B}} + \frac{[\mathrm{A}][\mathrm{A}]}{K_\mathrm{A}K_\mathrm{AA}} + 2\frac{[\mathrm{A}][\mathrm{B}]}{\gamma K_\mathrm{A}K_\mathrm{B}} + \frac{[\mathrm{B}][\mathrm{B}]}{K_\mathrm{B}K_\mathrm{BB}}\right) + \frac{1}{K^*}\left(1 + 2\frac{[\mathrm{A}]}{\alpha K_\mathrm{A}} + 2\frac{[\mathrm{B}]}{\beta K_\mathrm{B}} + \frac{[\mathrm{A}][\mathrm{A}]}{\alpha\varepsilon K_\mathrm{A}K_\mathrm{AA}} + 2\frac{[\mathrm{A}][\mathrm{B}]}{\alpha\beta\gamma\delta K_\mathrm{A}K_\mathrm{B}} + \frac{[\mathrm{B}][\mathrm{B}]}{\beta\phi K_\mathrm{B}K_\mathrm{BB}}\right)} \quad (1b)$$

$$binding(\mathrm{B}) = \frac{[\mathrm{RR}]_\mathrm{t} \times 2\frac{[\mathrm{B}]}{K_\mathrm{B}}\left(1 + \frac{[\mathrm{A}]}{\gamma K_\mathrm{A}} + \frac{[\mathrm{B}]}{K_\mathrm{BB}} + \frac{1}{\beta K^*} + \frac{[\mathrm{A}]}{\alpha\beta\gamma\delta K_\mathrm{A}K^*} + \frac{[\mathrm{B}]}{\beta\phi K_\mathrm{BB}K^*}\right)}{\left(1 + 2\frac{[\mathrm{A}]}{K_\mathrm{A}} + 2\frac{[\mathrm{B}]}{K_\mathrm{B}} + \frac{[\mathrm{A}][\mathrm{A}]}{K_\mathrm{A}K_\mathrm{AA}} + 2\frac{[\mathrm{A}][\mathrm{B}]}{\gamma K_\mathrm{A}K_\mathrm{B}} + \frac{[\mathrm{B}][\mathrm{B}]}{K_\mathrm{B}K_\mathrm{BB}}\right) + \frac{1}{K^*}\left(1 + 2\frac{[\mathrm{A}]}{\alpha K_\mathrm{A}} + 2\frac{[\mathrm{B}]}{\beta K_\mathrm{B}} + \frac{[\mathrm{A}][\mathrm{A}]}{\alpha\varepsilon K_\mathrm{A}K_\mathrm{AA}} + 2\frac{[\mathrm{A}][\mathrm{B}]}{\alpha\beta\gamma\delta K_\mathrm{A}K_\mathrm{B}} + \frac{[\mathrm{B}][\mathrm{B}]}{\beta\phi K_\mathrm{B}K_\mathrm{BB}}\right)} \quad (1c)$$

α: Whether RR, ARR or RRA is more readily activated AND whether the activated receptor dimer prefers RR*, ARR* or RRA* form. $\alpha < 1$ ⇒ ARR and RRA are more readily activated AND ARR* and RRA* are preferred over RR*.

β: Whether RR, BRR or RRB is more readily activated AND whether the activated receptor dimer prefers RR*, BRR* or RRB* form. $\beta < 1$ ⇒ BRR and RRB are more readily activated AND BRR* and RRB* are preferred over RR*.

γ: Whether RR or RRB more readily binds A AND whether RR or RRA more readily binds B. $\gamma < 1$ ⇒ RRB more readily binds A AND RRA more readily binds B.

δ: Affects the equilibria of ARR* and RRA* vs. ARRB* and BRRA*; BRR* and RRB* vs. ARRB* and BRRA*; and ARRB and BRRA vs. ARRB* and BRRA*. $\delta < 1$ promotes the formation of ARRB* and BRRA*. Please observe that when there is just a single ligand (A = B) ⇒ $\delta = \alpha^{-1} = \beta^{-1}$.

ε: Whether the activated receptor dimer prefers ARR*, RRA* or ARRA* form AND whether the receptor dimer activation (*) is more efficient for ARR, RRA or ARRA. $\varepsilon < 1$ ⇒ ARRA* is preferred AND ARRA is more readily activated than ARR or RRA.

ϕ: Whether the activated receptor dimer prefers BRR*, RRB* or BRRB* form AND whether the receptor dimer activation (*) is more efficient for BRR, RRB or BRRB. $\phi < 1$ ⇒ BRRB* is preferred AND BRRB is more easily activated than BRR and RRB.

K^* sets the constitutive activity of the receptor dimer.

It should be recognized that positive *binding* co-operativity between the two binding sites is obtained by $\gamma < 1$ and the positive *activity* co-operativity by $\delta < 1$ or $d > a$; negative cooperativity is obtained for the opposite relationships.

In all calculations, the free concentrations of the ligands were assumed equal to the total concentrations.

In the presence of just a single signal transduction pathway, the receptor *activity* is calculated from

$$activity = \frac{[\mathrm{RR}]}{K^*}k_\mathrm{p}\left(1 + 2\frac{[\mathrm{A}]}{\alpha K_\mathrm{A}}a + 2\frac{[\mathrm{B}]}{\beta K_\mathrm{B}}b + \frac{[\mathrm{A}][\mathrm{A}]}{\alpha\varepsilon K_\mathrm{A}K_\mathrm{AA}}e + 2\frac{[\mathrm{A}][\mathrm{B}]}{\alpha\beta\gamma\delta K_\mathrm{A}K_\mathrm{B}}d + \frac{[\mathrm{B}][\mathrm{B}]}{\beta\phi K_\mathrm{B}K_\mathrm{BB}}f\right) \quad (2)$$

with [RR] from Eq. (1a). The term k_p comes from enzyme kinetics meaning the rate (k) of product (p) formation and the parameters a, b, d, e and f multiply k_p according to Fig. 1C. This represents the efficiency of activation of the primary signal transducers of the receptors, e.g. the heterotrimeric G-proteins.

Combination of Eqs. (1a) and (2) gives

$$activity = \frac{[\mathrm{RR_t}]\,k_\mathrm{p}\left(1 + 2\frac{[\mathrm{A}]}{\alpha K_\mathrm{A}}a + 2\frac{[\mathrm{B}]}{\beta K_\mathrm{B}}b + \frac{[\mathrm{A}][\mathrm{A}]}{\alpha\varepsilon K_\mathrm{A}K_\mathrm{AA}}e + 2\frac{[\mathrm{A}][\mathrm{B}]}{\alpha\beta\gamma\delta K_\mathrm{A}K_\mathrm{B}}d + \frac{[\mathrm{B}][\mathrm{B}]}{\beta\phi K_\mathrm{B}K_\mathrm{BB}}f\right)}{K^*\left(1 + 2\frac{[\mathrm{A}]}{K_\mathrm{A}} + 2\frac{[\mathrm{B}]}{K_\mathrm{B}} + \frac{[\mathrm{A}][\mathrm{A}]}{K_\mathrm{A}K_\mathrm{AA}} + 2\frac{[\mathrm{A}][\mathrm{B}]}{\gamma K_\mathrm{A}K_\mathrm{B}} + \frac{[\mathrm{B}][\mathrm{B}]}{K_\mathrm{B}K_\mathrm{BB}}\right) + \left(1 + 2\frac{[\mathrm{A}]}{\alpha K_\mathrm{A}} + 2\frac{[\mathrm{B}]}{\beta K_\mathrm{B}} + \frac{[\mathrm{A}][\mathrm{A}]}{\alpha\varepsilon K_\mathrm{A}K_\mathrm{AA}} + 2\frac{[\mathrm{A}][\mathrm{B}]}{\alpha\beta\gamma\delta K_\mathrm{A}K_\mathrm{B}} + \frac{[\mathrm{B}][\mathrm{B}]}{\beta\phi K_\mathrm{B}K_\mathrm{BB}}\right)} \quad (3)$$

For GPCRs, any biologically relevant signal is measured after at least one step from the activated receptor; the shortest pathway may be the direct regulation of ion channels by the G-protein subunits. The steps can be thought to be composed of linear or hyperbolic equations and

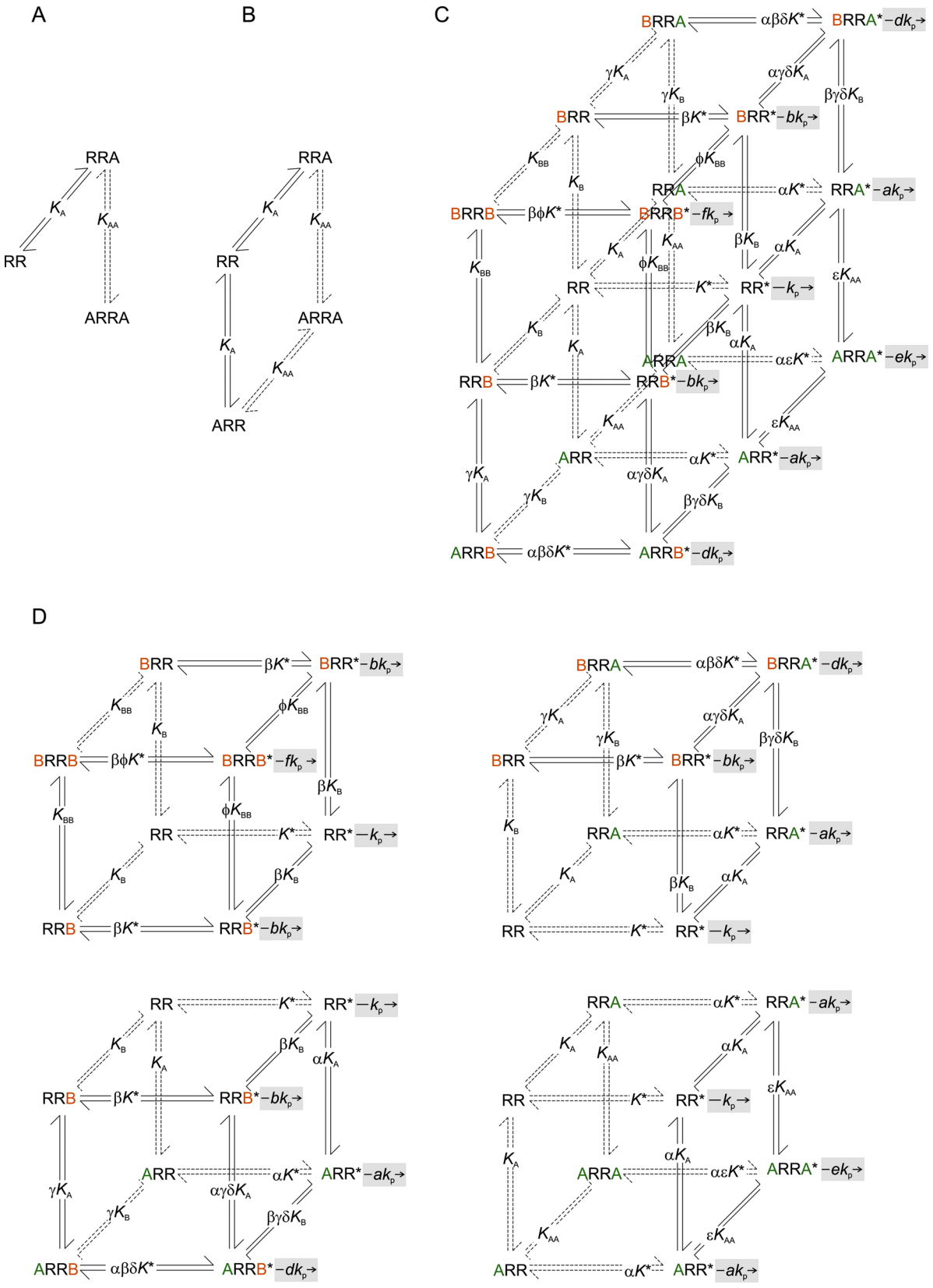

Fig. 1. The receptor model. **A**–**B**) Emergence of the dimeric receptor model. **C**) Shows the actual model used for simulations. Please also see *Supplementary Material 2* for a 3D version of the model. **D**) Presents an exploded view of **C** for clarity. Please observe that there is overlap between the cubes to show how they fit together. All the complexes within the "cubes" represent *binding* (Eqs. (1a)–(1c)) The arrows pointing out of the cubes, i.e. all multiplicatives of k_p represent *activity* (grey background) (Eq. (2)). The *response* is just a function of the sum *activity* and cannot be shown here (see Eq. (4)).

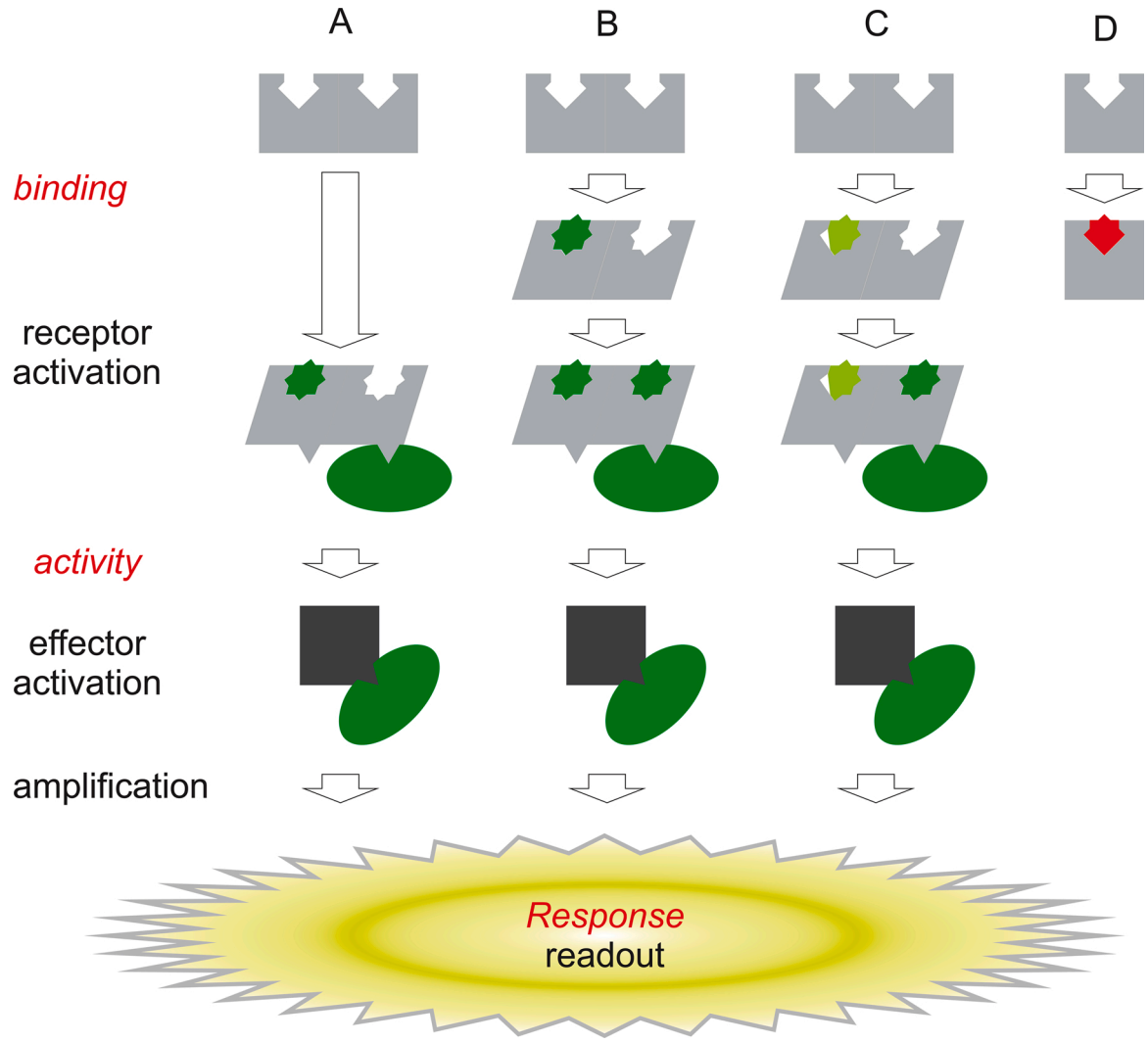

Fig. 2. The receptor dimer behaviour considered in the modelling including all three steps assessed (*binding*, *activity*, *response*; marked in red writing). All models assume that a single G-protein trimer is able to bind per receptor dimer due to steric hindrances. In each case, effects on the *binding* are rather depicted while also an effect on the receptor *activity* is possible; the equations derived can separately consider each. **A**) A single bound agonist molecule is able to activate the receptor in full. **B**) Binding of two agonist molecules is required for full activation of the receptor, but a single agonist molecule changes the receptor conformation to enhance the binding of the second agonist molecule. Something between schemes **A** and **B** could also be easily imagined (not shown). **C**) The yellow-green antagonist-PAM does not activate the receptor by itself (even when bound to both orthosteric binding sites; not shown) but changes the receptor conformation to enhance the binding of an agonist molecule. **D**) Conventional antagonist binding.

thus they – independent of their number – can be approximated by a single hyperbolic amplification step:

$$response = \frac{activity \times response_{\max}}{activity + K_R} \quad (4)$$

where $response_{\max}$ sets the maximum *response* and K_R is the *activity* producing the half-maximal *response*. This amplification was utilized in Figs. 3C and F and 4C.

2.2. Data retrieval, data analyses and simulations

Microsoft Excel (Microsoft, Redmond, CA, USA) was used for all simulation according to the equations described under 2.1. and for numerical iterations as a check for the correctness of the derived equations. Some curve fitting was performed utilizing data published by other research groups (Fig. 4D). In the lack of direct access to the original data, the average data were here retrieved from the graphs utilizing WebPlotDigitizer (https://automeris.io/WebPlotDigitizer/; [18]). The retrieved data were curve fitted in Excel as described in e.g. [19]. Please observe that the digitation is not absolutely precise due to small inaccuracies in e.g. the graphics output, and the standard deviations are difficult to estimate when different symbols and error bars overlap or the symbols totally hide the error bars. For the fitting of our own data, all data points were used whereas for the retrieved data, only the average points were used unweighted in the lack of reliable error estimates.

Some of the simulated data (Fig. 3A–C) were analyzed for cooperativity (Hill) coefficients (n) by curve fitting according to the same principal equation

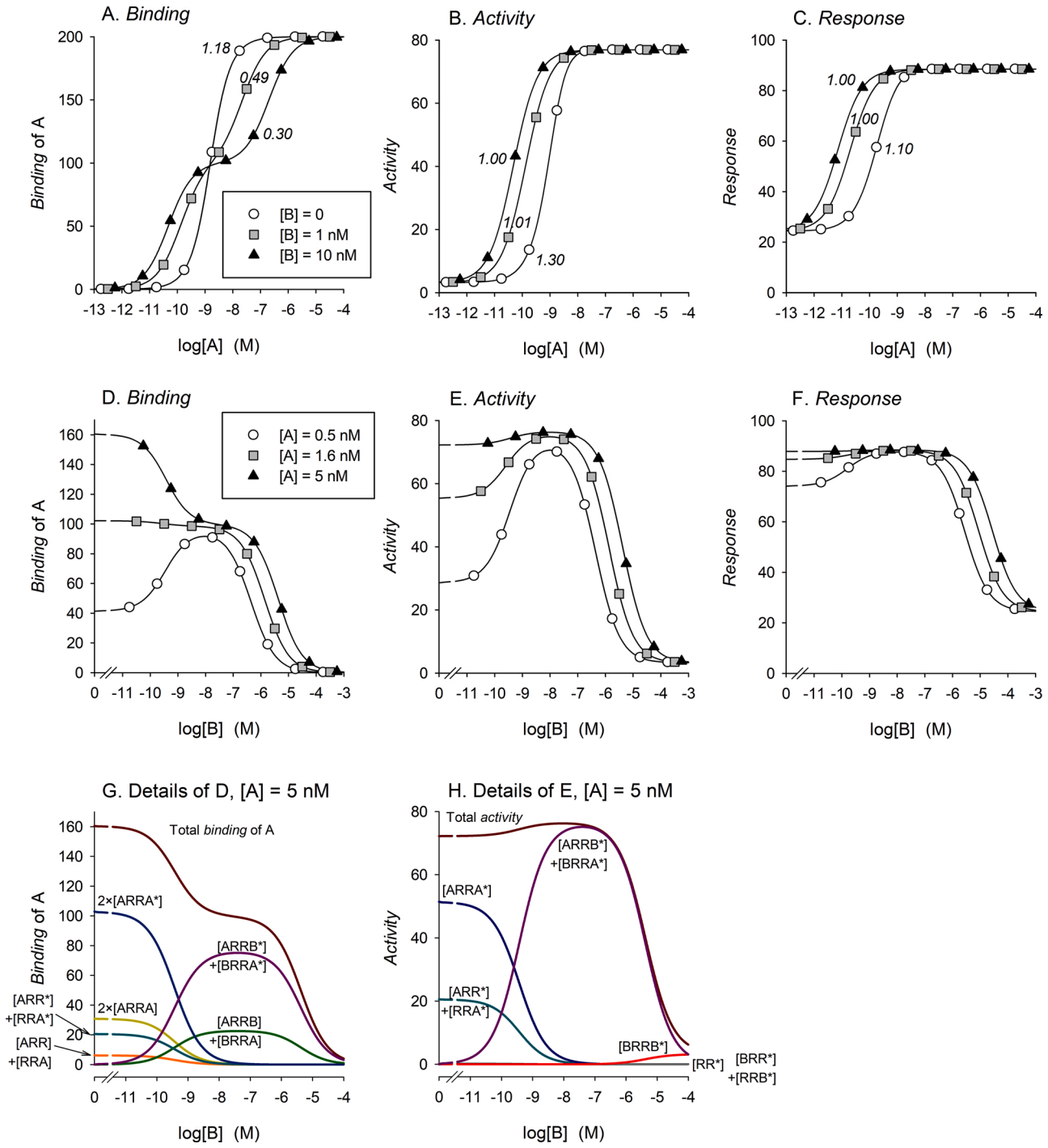

Fig. 3. Simulated behaviour of an antagonist-PAM action via the orthosteric binding site. Simulations are based on Eqs. (1b), (3) and (4). The parameters are: $[RR]_t = 100$, $K_A = K_B = K_{BB} = 1 \times 10^{-8}$ M, $K_{AA} = 1 \times 10^{-9}$ M, $K^* = 30$, $\alpha = \gamma = 0.01$, $\beta = \delta = \varepsilon = \phi = 1$, $k_p = 1$, $a = b = d = e = f = 1$, max = 100, $K_R = 10$. Thus A is the agonist and shows co-operative enhancement of its own binding (10-fold) while B is an antagonist that enhances the binding of A (100-fold) but not its own binding (and vice versa). A is able to activate the receptor whereas B is not. On the left (**A**, **D, G**) the *binding* of A, in the middle (**B**, **E**) the receptor *activity*, on the right (**C**, **F**) the amplified *response*; on the top (**A**–**C**) fixed [B], on the bottom (**D–F**) fixed [A]. The co-operativity coefficients for the curves are shown in **A**–**C**. **G**) Bound A in the different receptor–ligand complexes contributing to the total *binding* of A as in subfigure **D** for [A] = 5 nM. Please observe that [ARRA] and [ARRA*] are multiplied by two as there are two A:s bound to each. **H**) Different active receptor–ligand complexes contributing to the *activity* as in subfigure **E** for [A] = 5 nM. The curves for [RR] and [BRR*]+[RRB*] are flat and cannot be separated here.

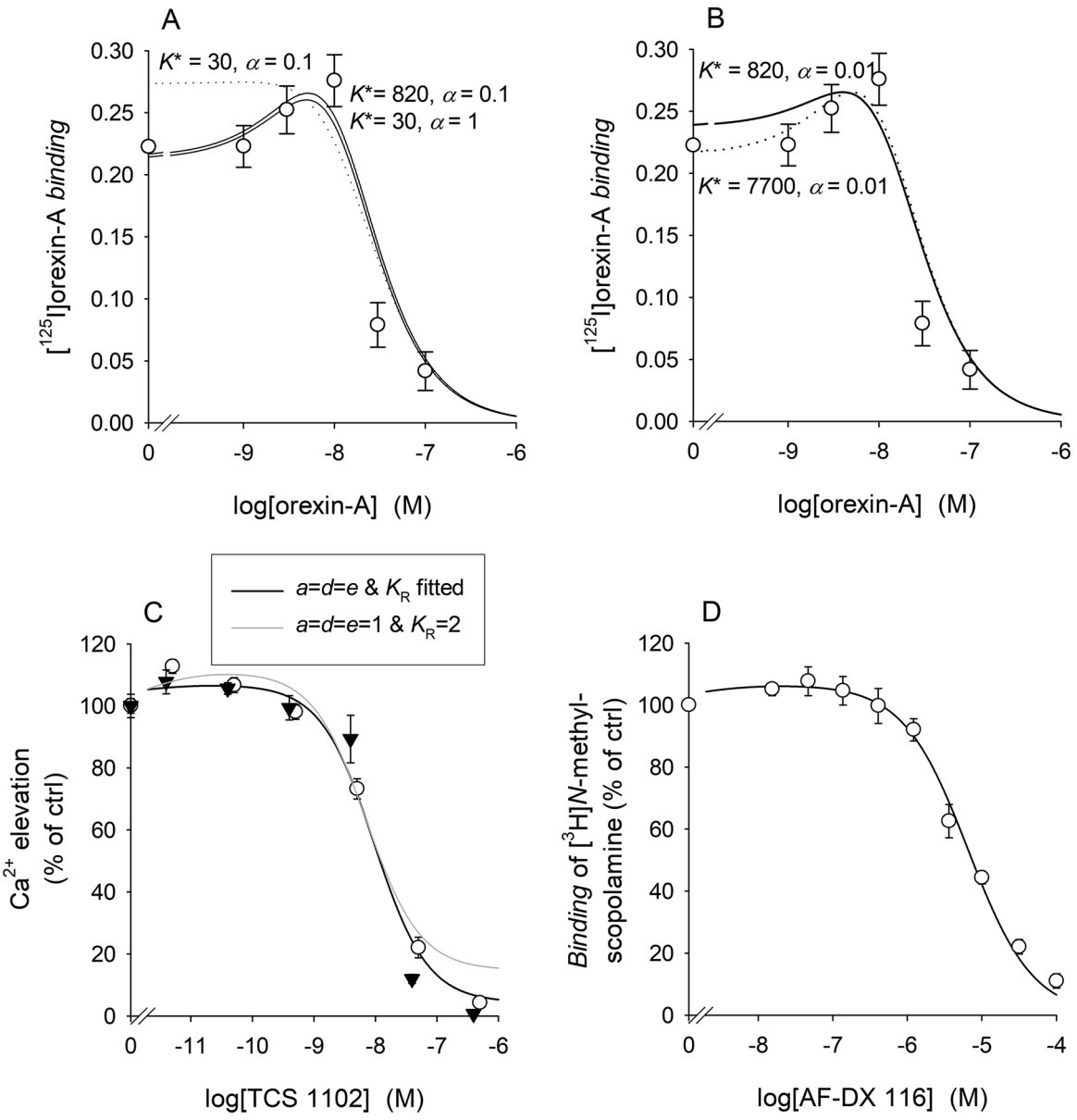

Fig. 4. Analyses of previously published data assuming allosteric action via the orthosteric binding site. **A**–**B**) *Binding* data from [45]. 0.01 nM [^{125}I]orexin-A (human) was displaced with "cold" orexin-A (human) from human OX_1 orexin receptors expressed in CHO-K1 cells. The data represent values from three independent experiments; the averaged values from each experiment were individually normalized to the binding at 0 orexin-A before averaging for the final graph. Curve fitting was performed utilizing Eq. (1b); the values from each of the independent experiments (and not their average as presented in the graph) were utilized for the fitting. The fixed parameters are: $[RR]_t = 277.83$ (based on an assumption from the experiments; please also see the text), $\varepsilon = \phi = 1$, and the binding constants for orexin-A and [^{125}I]orexin-A were kept equal ($K_A = K_B$, $K_{AA} = K_{BB}$, $\delta = \alpha^{-1}$) although the compounds are not identical. K_A and γ were fitted (values not given) and also K^* in some cases (the curves representing $K^* = 820$ or 7700). Please see text for further explanations. As the data are only for *binding*, the *activity* and *response* parameters do not contribute to the curve. **C**) The impact of the orexin receptor antagonist TCS 1102 on the Ca^{2+} response produced by the orexin receptor agonist Nag 26 on human OX_1 receptors expressed in CHO-K1 cells as two independent experiments. The data were originally published in [22]. The curve fitting (solid line) represents the average of the separate fits to each dataset utilizing Eq. (3) amplified via Eq. (4). Please observe that the curve fitting cannot be performed in a mechanistically correct way as the affinity and possible co-operativity of Nag 26 in binding to the receptors or in responding are not known; therefore, the absolute values contain no information and are not given. For the fit, TCS 1102 was assumed to be neutral with respect to agonism ($b = f = 1$). Equal fit can be obtained i) by giving Nag 26 and TCS 1102 mutual positive *binding* co-operativity ($\gamma < 1$) or ii) by giving Nag 26 and TCS 1102 mutual positive *activity* co-operativity ($\delta < 1$ or $d > a$). It should also be noted that equally good fit was obtained with high ($K^* = 30$) or low constitutive activity ($K^* = 1000$) as long as $\alpha = 0.01$ (see also **A** and **B**). **D**) Displacement of [^{3}H]*N*-methyl scopolamine with AF-DX 116 (*N*-{2-[2-({dipropylamino}methyl)-1-piperidinyl]ethyl}-6-oxo-5*H*-pyrido{2,3-b}{1,4}benzodiazepine-11-carboxamide) from human M_1 muscarinic receptors expressed in CHO-K1 cells [23]. The data were read from Fig. 5C of [23] utilizing WebPlotDigitizer. The K_D of [^{3}H]*N*-methyl scopolamine is not given in the original study so the values of the curve fitting according to Eq. (1b) are meaningless.

$$binding = \frac{[\text{agonist}]^n \times \max}{[\text{agonist}]^n + K^n} \quad (5a)$$

$$activity = \frac{[\text{agonist}]^n \times \max}{[\text{agonist}]^n + K^n} \quad (5b)$$

$$response = \frac{[\text{agonist}]^n \times \max}{[\text{agonist}]^n + K^n} \quad (5c)$$

In these, max sets the maximum *binding*, *activity* or *response* and *K* is the agonist concentration producing the half-maximal *binding*, *activity* or *response*.

3. Results

The current study was stipulated by the idea that, in a receptor dimer, a ligand might act as both an antagonist and an apparent allosteric modulator solely via the orthosteric sites. Such a ligand, when binding to the orthosteric binding site, competes with the agonist. An antagonist, *per definition*, does not activate the receptor or affect the signalling, but it may co-operatively decrease or increase the agonist's binding affinity for the other site or the agonist's ability to activate the receptor. Thus this type of a ligand could be labelled as an antagonist-PAM or -NAM (PAM or NAM = positive or negative allosteric modulator), though it exerts its effect solely via the orthosteric site. This type of behaviour is visualized in Fig. 3 (Eqs. (1b), (3) and (4)); A is the agonist and B is an antagonist-PAM. At low concentrations of A, the *binding* of A – and thus the receptor *activity* and the *response* too – is enhanced by B (Fig. 3A–C). At higher concentrations, A starts to compete with B for the binding to the other protomer. This may give shallow or even clear two-site *binding* curves for A (Fig. 3A), but this is not translated to *activity* or *response*, when $e = a$ (Fig. 3B and C). However, if $e > a$, the biphasic response is also seen in the *activity* and, at low amplification, in *response* (not shown). When the concentration of B is the variable instead of A, we may see different types of biphasic *binding* and *activity* curves (Fig. 3D, E and G). If the output is subject to high level of amplification ($K_R = 10$), most of the extreme characteristics of the curves are lost in *response* (Fig. 3C and F) while at a lower level of amplification (higher K_R) the *response* curves would look more like the *activity* curves (not shown). Please observe that here the constitutive activity is quite high ($K^* = 30$; see also the high basal *response* in Fig. 3D and F) but lower constitutive activity (i.e. higher K^*) would not change the behaviour, but only decrease the constitutive signal for *activity* and *response* (not shown).

We have previously performed binding experiments on human orexin receptors utilizing [^{125}I]orexin-A as the radioligand. One of the features revealed has been the enhancement of [^{125}I]orexin-A binding when exposed to low concentrations of a competing ligand, non-labelled (cold) orexin-A [20,21](Kukkonen et al., unpublished). (Fig. 4A and B). This suggests co-operative binding of the ligands, and thus also likely of [^{125}I]orexin-A or cold orexin-A alone. Modelling using Eq. (1b) can reproduce the result (the solid lines in Fig. 4A and B) within the limits of the experimental variation. Please observe that it is not possible to determine the total number of the receptors utilizing this radioligand, and therefore the actual curve fitting values have no significance. At high constitutive activity (low K^*) it is not possible to obtain a good fit at high α (dotted lines in Fig. 4A and B). As α clearly below 1 is likely (agonists should promote receptor activation), only lower constitutive activity (higher K^*) produces the bell-shaped curve (solid lines in Fig. 4A and B). This is in agreement with the fact that we have not found any constitutive activity of orexin receptors in any of our studies even when they are expressed at high levels.

We have also observed that some orexin receptor antagonists display similar bell-shaped behaviour when used to inhibit human OX_1 orexin receptor-mediated Ca^{2+} responses, i.e. low concentrations of the antagonist apparently potentiate the response to a fixed agonist concentration, while higher concentrations show the expected inhibition [22]. Two representative experiments with CHO-K1 (Chinese hamster ovary K1) cells expressing human OX_1 receptors are shown in Fig. 4C. TCS 1102 (*N*-biphenyl-2-yl-1-{[(1-methyl-1*H*-benzimidazol-2-yl)sulfanyl]acetyl}-L-prolinamide) is the antagonist and Nag 26 (4'-methoxy-*N*,*N*-dimethyl-3'-{*N*-[3-({2-[3-methylbenzamido]ethyl} amino)phenyl]sulfamoyl}-{1,1'-biphenyl}-3-carboxamide) is the agonist. We have not consequently investigated this and thus a mechanistic explanation is impossible, but modelling with Eqs. (3) and (4) can well describe the results independent of whether the positive co-operativity is on the *binding* or the *activity* (Fig. 4C; see the legend for details). A reasonably good fit can be obtained even when *a*, *e*, *d* and K_R are fixed (the grey line in Fig. 4C; compare to the black line).

As inferred in the Introduction, bell-shaped binding or concentration–response-curves are not uncommon but the data are often hidden in the lack of suitable mechanistic explanations. In Fig. 4D, we present one example of such binding data with human M_1 muscarinic receptors from another group [23] and the fitting of it using Eq. (1b).

4. Discussion

Apparent co-operative and allosteric actions in proteins have been modelled ever since the work of Hill [24] and Changeaux, Monod and Wyman [25] on haemoglobin. Other essential proteins contributing to the knowledge have been enzymes and receptors (see e.g. [26–29]). The terms allosteric and co-operative are used in somewhat different meaning in different sources but, on a conceptual level, we may separate the allosteric or co-operative effects of substrate or other ligand binding via the active site (e.g. O_2 effect on O_2 binding to haemoglobin) from the effects obtained via sites distinct from the active sites (e.g. 2,3-bisphosphoglycerate effect on O_2 binding to haemoglobin). For receptors, the tradition names the binding site of the endogenous ligand as the orthosteric site while any other site would be an allosteric site. The situation is somewhat complicated by the fact that ligands competitive with the endogenous ligands can do this by binding to a non-orthosteric site and ligands binding to the orthosteric site may cause allosteric effects, as modelled in the current study and suggested in the studies cited here. In this respect, terms orthosteric binding, which is competitive with the endogenous ligand independent of the binding site, and allosteric binding, which is not competitive with the endogenous ligand binding, might be more unequivocal choices. The current study makes no statement as concerns the naming, but as the simulations in the current study also demonstrate, even the orthosteric and allosteric binding defined as above may become quite intertwined.

Current work arose from our recent findings when trying to develop small molecule orexin receptor agonists. These molecules were based on the idea of mimicking the peptide C-terminus, known to be most important for orexin receptor binding and activation [30–33]. In this process, we indeed managed to discover some compounds that activated orexin receptors, but these were altogether very weak as receptor activators [20,21,34]. In addition, a few compounds were able to potentiate both a) the binding of [^{125}I]orexin-A to the OX_1 receptor and b) the ability of orexin-A to activate the receptor [20,21,34]. Furthermore, the compounds c) displaced [^{125}I]orexin-A at higher concentrations and d) had no efficacy of their own [21,34]. We knew since before that orexin receptors are well able to make homomeric complexes [35,36], and we had even observed that there is apparent co-operative binding of cold orexin-A and [^{125}I]orexin-A to the orexin receptors ([20,21] Kukkonen et al., unpublished), suggesting that orexin receptors exist in complexes, in which the protomers exert co-operative interactions. There have been previous reports of molecules with simultaneous competitive

antagonistic and allosteric activities [37]. Although the binding sites for many of these molecules cannot be known, the usual conclusion from the experimental results and molecular modelling is that these molecules partially bind to the orthosteric ligand binding site but partially also to another site, i.e. a site overlapping with the orthosteric site (reviewed in [29,38]). That certainly is a reasonable model for the findings, but also a simpler and fully generalizable model for the receptors, as presented here, is equally thinkable. In this model, the ligands bind just to the orthosteric site but to "less" of it than the agonists, i.e. they make less interactions, which does not allow them to activate the receptor by themselves. Yet they still change the receptor conformation so that the activation of the receptor by the agonist is enhanced either due to enhanced agonist binding or enhanced receptor activation, making them effectively as antagonist-PAMs. For antagonist-NAMs, the agonist binding or receptor activation would be correspondingly reduced. The competitive antagonism would arise from the ability to block agonist binding when the antagonist-PAM or- NAM would occupy both (dimer) or a sufficient number of (oligomer) binding sites in the receptor complex. A PAM binding to the orthosteric site of the receptor would always work in this way, unless it showed strong negative co-operative effect on its own binding, in which case it might look like just an actual PAM. Conversely, a neutral antagonist without a PAM or NAM activity would just bind to the orthosteric binding site without inducing any conformational change or instability in the receptor. I hypothesize that antagonist-PAM/NAM-behaviour could be expected from certain ligands in any receptor di- or oligomer system.

Thus the current model was developed. I wanted to have a simple but sufficiently versatile model to keep the number of the parameters at a minimum but yet to be able to model the behaviour adequately. I thus chose to model the receptor as an obligate dimer. Several different scenarios, such as positive or negative co-operativity on the level of the binding, receptor activation or response generation (coefficients α, β, γ, δ, ε, ϕ, a, b, d, e and f), can be modelled. The ligands can be the same one (A = B) or different (A ≠ B), the latter of which allows different activity profiles for each one.

The idea of (co-operative and other types of) interactions between the protomers of di- or oligomeric GPCRs is of course obvious, and similar has been drafted or presented in several primary studies and reviews (reviewed in [29,38,39]). For most receptors and molecules, the di- or oligomerization status and the identity of the binding sites, respectively, cannot be known. The analyses become more complicated when there is a possibility of receptor heteromerization, involvement of several ligands, dynamic regulation of the di- or oligomerization and several, possibly competitive signal transduction pathways (see, e.g., [17,40]). Mathematical modelling nevertheless is able to reproduce several findings with the assumption of just a homomeric obligatory receptor dimer with two orthosteric binding sites, as previously shown for binding at some class A GPCRs [9,14–16]. In the current study, the allosteric action (e.g. antagonist-PAM or -NAM) can be reproduced – together with several other types of previously shown behaviours – with these equations, which solely include orthosteric ligand binding sites.

Naturally, it is important to recognize that what is presented here is just a hypothesis and the simulations do not offer any mechanistic proof. There are not enough of experimental binding and receptor activation data published by other groups or even for our "own" ligands [20,21,34] to allow mechanistically realistic simulations. Actually, many of the parameters needed for mechanistic analysis are not experimentally measurable even though the methods are improving (see e.g. the nanoluc-based methods for receptor binding and activation measurements and the pepducins to block receptor dimerization [41–44]). Quite possibly there may be alternative explanations to some of the data published. Nevertheless, the hypothesis is reasonable and the model follows the principle of Occam's razor by being as simple as possible. At the current state of knowledge, we may thus conclude as much as that the model follows our current knowledge of receptor and its behaviour is reasonable in the view of the previous limited experimental data. The final value of the model is determined in the experimental work based on it.

The ability to modulate the receptor activity via the orthosteric ligand binding sites would offer an accessible way for the development of new types of receptor ligands. While the orthosteric ligand binding sites of most GPCRs are not mapped at the structural level, there is quite much more information about the pharmacophore determinants, which should allow easier discovery of novel ligands with modulatory function. In addition to ligands fine-tuning receptor action, i.e. PAM and NAM molecules, we can fathom ligands that change the preferred signal pathway of the receptor and ligands with selective action on receptor heterodi- or oligomers.

Declarations of Competing Interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.105116.

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