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REVIEW ARTICLE

# 100 years of modelling ligand–receptor binding and response: A focus on GPCRs

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Experimental pharmacologists rely on the application of models to describe biological observations in order to learn about a drug's effective concentration, the strength with which it binds its target and drives a response (at either molecular or system level), and the nature of more complex drug actions (allosterism/functional selectivity). Models in current use build upon decades of basic principles, going back to the beginning of the last century. Yet often, researchers are only partially familiar with these underlying principles, creating the potential for confusion due to failure to recognise the underpinning assumptions of the models that are used. Here, we describe the history of receptor theory as it underpins receptor stimulus–response models in use today, emphasising particularly attributes and models relevant to GPCRs—and point to some current aims of model development.

## 1 | INTRODUCTION

By its simplest definition, a *receptor* is the target of a substance which is responsible for initiating a biological response. Of all possible drug targets, the taxon of receptors includes ligand-gated ion channels, enzymes and enzyme-linked receptors (such as receptor tyrosine kinases), intracellular receptors (steroid hormone receptors) and GPCRs. Of this array, the largest and arguably pharmacologically most important superfamily is that of the GPCRs, which are encoded by over 800 different genes in the human genome (reviewed in Hill, 2006; Lundstrom, 2009). GPCRs may be activated by stimuli as diverse as photons, organic odorants, nucleotides/nucleosides, peptides, larger proteins and a plethora of small molecules such as hormones, lipids and neurotransmitters. As such, GPCRs have been described as the largest single group of druggable targets in the human genome (Wacker, Stevens, & Roth, 2017) and are consequently of great interest to biomedical researchers—indeed, due to their physiological relevance, as many as 30% of all Food and Drug Administration-approved medications today target GPCRs (Wacker et al., 2017).

Many aspects of contemporary receptor pharmacology hinge on foundational concepts—including the critical ongoing development of mathematical models of stimulus–response systems. However, the basis in theory of these foundational concepts is often overlooked, despite underpinning ongoing model development. A likely contributor to this problem is the fact that access to primary data is challenging, because many of the early reports date back to the early 20th century (Figure 1). This review therefore sets out to summarise this history and to briefly and accessibly integrate the foundational concepts of receptor theory with introductions to the most widely employed models of receptor function (with an emphasis on models of the attributes of GPCRs) for a general pharmacology audience. It is not the intention of this review to duplicate the seminal materials authored by Kenakin, whose systematic reviews we strongly recommend for readers seeking detailed expositions on these subjects (e.g. Kenakin, 1997, 2004, 2008a, 2008b, 2016, 2017; Kenakin & Christopoulos, 2011). Rather, our main aim with this review is to take a didactic approach to matters of theory, that is, to provide a summary of receptor theory for non-modellers. This audience may not be greatly interested in mathematical details yet may find value in an integrative summary that will help them understand the limitations and assumptions inherent within the models used to describe pharmacological experiments.

**Abbreviations:** ACh, acetylcholine; GPCR, G protein-coupled receptor; Hb, haemoglobin.

## 2 | RECEPTOR THEORY

Receptor theory is the application of receptor models to explain drug behaviour. Until the end of the 19th century, drug action was

  

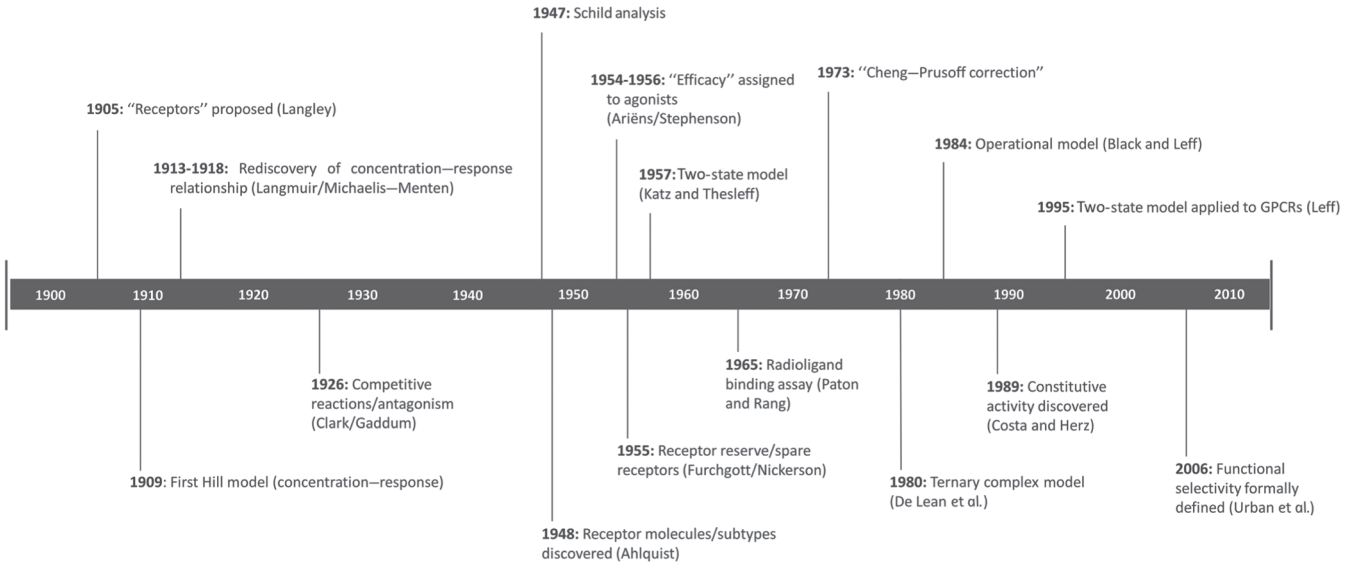


**FIGURE 1** Timeline of receptor theory, 1905–2006

described as causing tissue-level effects by acting on nerve endings per se (e.g. Langley, 1901). Langley (1905) originated the concept of receptors in a description of the mechanism by which nicotine drives contractile reactions in muscle. In his paper, Langley concluded that nicotine stimulates a "receptive substance," which is neither nerve nor muscle but which directly transfers the stimulation to, and is intimately associated with, the muscle. In a further elaboration, he proposed that heterogeneity in the opposing effects that nicotine and curare (arrowhead poison) exert on different muscles in different animals indicates species and tissue differences in these receptive substances (Langley, 1905). It is worth noting that Langley's work was clearly influenced by the *Seitenkettentheorie* (literally, *side-chain theory*, an early receptor theory for antibody production; Witebsky, 1954) proposed by his contemporary Paul Ehrlich. Langley (1905) observed:

> … it might be supposed that a receptive substance is a side chain molecule of the molecule of contractile substance ….

Ehrlich's contribution to the development of the concept of receptors is also illustrated by his maxim about the lock-and-key analogy for receptors and the molecules that bind them:

> Corpora non agunt nisi fixata [Agents will not work unless they are bound]. ( Bosch & Rosich, 2008)

AV Hill, a student of Langley, contributed the first quantitative justification (a model) for receptors. In continuing Langley's characterisation of the nature of nicotine-mediated muscle contraction, Hill discovered that the magnitude of the contractile effect could be predicted mathematically:

$$y = \frac{N}{k' + kN} - M, \quad (1)$$

where $y$ is the response magnitude, $N$ is the concentration of nicotine, $k$ and $k'$ are constants, and $M$ is the minimum value of $N$ for a response to be elicited (i.e. the minimum effective nicotine concentration; Hill, 1909). Note that assigning $k$ to $\frac{1}{E_{MAX}}$ and $k'$ to $\frac{EC_{50}}{E_{MAX}}$ yields the standard form of the $E_{MAX}$ model (Equation (9)). $y$ (1) is equal to $E$ (8); and $N$ (1) is defined as the concentration of agonist. The remaining term, $M$, exists in Equation (1) for controlling the $X$-axis starting position of the curve. This equation is the earliest expression of what has become known as the Hill–Langmuir equation for equilibrium binding (see below). Hill observed that this relationship strongly supported the idea of a combination between nicotine and "some constituent" of the muscle and that this relationship was reversible (able to be antagonised; Hill, 1909)—effectively affirming that the law of mass action applies to the relationship of nicotine with its receptor. Hill (1910) then showed the following year that this model also applies to oxygen binding to Hb.

Interestingly, this concentration–effect relationship was discovered independently no fewer than three times. A decade after Hill, Irving Langmuir (1918; who was probably unaware of Hill's earlier work) published his "adsorption isotherm" regarding the adsorption of gases onto surfaces—in fact, wide dissemination of this report resulted in Hill's contribution being overlooked for many years, with the now-named Hill–Langmuir equation referred to simply as the "Langmuir adsorption isotherm" for much of the 20th century (e.g. Paton & Rang, 1965). It is worth noting that despite the different applications of Hill's and Langmuir's original models, they share a core assumption of random interaction between molecules and their receivers (although this assumption is more explicit in Langmuir's work than in Hill's). This random movement, a function of concentration

and affinity, will drive response, and the effector will be driven by the mass (for a defined volume) of molecules. The third discovery of this relationship occurred when French–Russian physical chemist Victor Henri derived the first form of a binding model equation for enzyme kinetics (Henri, 1903), which was later generalised by Michaelis and Menten (1913) and bears their names—though perhaps this model would be better termed the Henri–Michaelis–Menten equation (e.g. Bajzer & Strehler, 2012). Considering the spontaneous appearance of the same model in three different disciplines, it is difficult to determine how the model should be attributed, though it would seem that the Hill–Henri–Menten–Michaelis–Langmuir equation may not role off the tongue so easily (note that we reverse the Menten–Michaelis name to reward Maud Menten; the student of Leonor Michaelis, for her role in its discovery).

Ultimately, credit for proving that receptors exist as specific, discrete molecular entities which mediate drug responses is attributed (Maehle, Prüll, & Halliwell, 2002) to Ahlquist (1948), in a study published some four decades after Hill's paper. In it, Ahlquist (1948) demonstrated the existence of two different types of adrenoceptor, *alpha* and *beta*, which mediated entirely different (often opposing) effects upon stimulation with adrenaline.

## 2.1 | Occupancy theory

Pre-dating Ahlquist, significant contributions developing the early ideas of Langley and Hill were reported in the mid-1920s. Clark (1926b, 1926a) published two articles on the actions of ACh and atropine on isolated frog heart, in the same year that Gaddum (1926) reported the effect of adrenaline and ergotamine on rabbit uterus. In their respective publications, Clark (1926a) and Gaddum (1926) became the first researchers to present log concentration–response plots for drug activity and both empirically demonstrated the now familiar rightward shift of agonist responses in the presence of a competitive antagonist, although neither recognised it as such and did not associate their observations with the idea that the drugs were acting on the same target. The idea of *occupancy* is first implied by Clark's (1926b) observation that the action of ACh is proportional to the degree of "combination of the drug with some element of the muscle." In his early reports, Clark (1926a, 1926b) also used the term "receptor" to describe the specific sites of drug action. At its crux, therefore, occupancy theory suggested that the magnitude of a response was directly proportional to the amount of a drug bound to its receptor and that maximum response would be the consequence of complete occupancy.

The fundamental idea of competitive interactions between drugs, though observed by both authors in 1926, was finally described a decade later in a very brief but consolidating non-experimental opinion piece by Gaddum (1937). In it, Gaddum (1937) affirmed Clark's (1926b) observation that pharmacological effect is proportional to the amount of drug combining with receptors and that this interaction is reversible. Noting that this means that drug–receptor interactions are governed by the law of mass action (and that this explains the sigmoid shape of log concentration–response curves), Gaddum further observed that sigmoid curves for concentration–responses of stimulating drugs (ACh and adrenaline) retained the same slope when performed in the presence of *antagonistic drugs* (atropine, Clark, 1926a, or ergotamine, Gaddum, 1926, respectively; Gaddum, 1937). This implied that the amount of stimulating drug required to produce an equivalent response was ratiometrically associated with the antagonist's concentration. Basically, in Gaddum's (1937) own words:

> … antagonistic drugs act by competing with the active drugs for the receptors and inertly blocking them up.

If Gaddum had followed this observation up with a complete mathematical proof, he would have supplanted Schild regression analysis. Schild's (1947) major contribution was thus to demonstrate that the equal effects of different drugs were antagonised equivalently by different antagonists and explained this by introducing the term "dose ratio." This is the ratio of

- the concentration of an agonist required for a half-maximal response in the *presence* of an antagonist and
- the concentration of the agonist required for half-maximal response in the *absence* of the antagonist.

Schild plots (log(dose ratio − 1) vs. log[antagonist]) were thus an early, widely transferrable method for estimating the affinities of antagonists using functional data. Given competitive binding theory (that dose ratio increases linearly as a function of antagonist concentration, consistent with Gaddum's description), the parameter $pA_2$ can be derived. This point is the abscissa transection on a Schild plot (negative log of the antagonist concentration, given by the character *p*) and is the antagonist concentration at which the agonist concentration (denoted *A*) must be increased twofold (given by the subscript "2") in order to effect an equal (submaximal) response (Schild, 1947). When Schild regression has a slope of unity (meaning that the antagonism is purely competitive), $pA_2$ is an estimate of the equilibrium dissociation constant for the antagonist (B), $pK_B$.

Schild analysis is still usually considered a valid method for the estimation of antagonist affinities, particularly in circumstances where it is not possible to estimate affinities directly by performing radioligand binding assays (as may be the case if receptor number is too low, such as in a synapse). However, several requisites exist for Schild analysis to be accurate (Colquhoun, 2007): The antagonist must be neutral; antagonist and agonist binding is mutually exclusive at any binding site; the antagonist has the same affinity for every binding site (in other words, there is only one receptor type for which the antagonist has affinity); agonist occupancy translates to equal effect irrespective of the number of sites occupied by antagonist and the measurements must be made at equilibrium (Colquhoun, 2007). In practice, these criteria are often not attainable.

Here, we must disrupt the chronological sequence. For contemporary molecular pharmacologists, due partly to the restriction of applying Schild analysis for characterisation of neutral antagonists,

direct methods for estimating ligand affinity are generally preferred. The landmark method development that ultimately enabled truly equivalent interpretations of affinity for both antagonists and agonists was the radioligand binding assay of Paton and Rang (1965). This hugely important report included the familiar derivation of the aforementioned Hill–Langmuir equation (then referred to as the Langmuir isotherm):

$$\text{Fractional occupancy} = \frac{[\text{Ligand}]}{[\text{Ligand}] + K_D}, \tag{2}$$

where $K_D$ is the equilibrium dissociation constant.

For the purposes of integrating Schild analysis and the more familiar Hill–Langmuir equation, note that despite their different derivation, the interpretation of $K_B$ (or $K_A$, in the case of an agonist, A) is the same as the equilibrium dissociation constant $K_D$. Confusingly, the reciprocal of $K_D$, the affinity constant ($\frac{k_{on}}{k_{off}} = \frac{1}{K_D}$, which could also be termed the equilibrium association constant), is also conventionally designated $K_A$.

This term can also be derived using the law of mass action, which states that the rate of a reaction (in this case, a binding interaction) will be proportional to the "mass" (concentration) of the reactants and that at equilibrium (at which the ratio of the concentrations of reactant and product is a constant), the rates of the forward and reverse reactions are equal. For governing the interaction of ligand molecules with receptors, several additional assumptions are typically associated with the law of mass action. These are best stated by Kenakin (2016):

1. "All receptors are equally accessible to ligands—violation of this assumption leads to incomplete assessment of ligand binding."
2. "The binding is reversible—violation of this assumption precludes calculation of valid $K_A$ values."
3. "Receptors are either free or bound to ligand, and there is no more than one affinity state, or states of partial binding (the ligand and receptor must exist in only two states, bound or unbound)—violation of this assumption leads to ambiguity in the assignment of potency values."
4. "Binding does not alter the ligand or receptor—violation of this assumption also leads to ambiguity in the assignment of potency values (system-dependent potency). When a ligand binds to a receptor and changes its conformation, this is an expression of pharmacological efficacy" (Kenakin, 2016).

Using the law of mass action, $K_D$ may therefore be derived:

$$[\text{Ligand}] \cdot [\text{Receptor}] \cdot k_{on} = [\text{Ligand} : \text{Receptor}] \cdot k_{off}. \tag{3}$$

Meaning that at equilibrium, ligand binding (association) is the product of the ligand and (free) receptor abundances and a constant $k_{on}$ and that this is equal to ligand dissociation, which is the product of the amount of ligand/receptor complexes and a constant $k_{off}$. Changing the subject of this expression for the ratio of $\frac{k_{off}}{k_{on}}$ (which is $K_D$, the equilibrium dissociation constant) gives:

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[\text{Ligand}] \cdot [\text{Receptor}]}{[\text{Ligand} : \text{Receptor}]}. \tag{4}$$

And when [Ligand] = $K_D$, this term cancels out, giving

$$1 = \frac{[\text{Receptor}]}{[\text{Ligand} : \text{Receptor}]}. \tag{5}$$

And therefore,

$$[\text{Receptor}] = [\text{Ligand} : \text{Receptor}]. \tag{6}$$

This means that half the receptors are free and half are bound to ligand. In other words, at equilibrium, when the concentration of ligand is equal to $K_D$, 50% of the receptors will be occupied. The same answer can be obtained by solving the Hill–Langmuir equation for [Ligand] = $K_D$. Therefore, for a purely competitive antagonist, the concentration of antagonist that causes a twofold concentration shift in agonist response = $pA_2$ = $pK_B$ = $pK_D$ = 50% receptor occupancy at equilibrium.

Significantly, the concentration of ligand in all these equations (given by [Ligand]) is more accurately described as the unbound ("free") ligand concentration. In a system of high volume and few specific binding sites, the concentration of free ligand will be equal to the concentration of bound ligand. In contrast, in conditions of low volumes and a large number of binding sites, this may cease to be true and result in ligand depletion (conventionally defined as the situation when >10% of total ligand in bound; e.g. Motulsky & Christopoulos, 2003). This limitation is particularly notable considering current trends towards assay miniaturisation—ligand depletion will occur more readily in these circumstances.

A final noteworthy development in the Hill–Langmuir equation, as applied by Paton and Rang (1965), allowed the estimation of equilibrium dissociation constants for non-radiolabelled ligands. The so-called Cheng–Prusoff correction (Cheng & Prusoff, 1973) can be employed to binding data at equilibrium, where a concentration series of an unlabelled ligand is used to displace the binding of a radioligand with known affinity ($K_D$, used at a sub-saturating concentration). $IC_{50}$ (the concentration of the unlabelled ligand that is empirically determined to displace 50% of the radioligand) can be easily obtained, and then $K_i$ (the unlabelled ligand's equilibrium dissociation constant) is the result of a calculated correction for the affinity ($K_D$) of the radioligand, using the formula (Cheng & Prusoff, 1973; Lazareno & Birdsall, 1993):

$$K_i(\text{Unlabelled compound}) = \frac{IC_{50}}{1 + \frac{[\text{Radioligand}]}{K_D\,(\text{Radioligand})}}. \tag{7}$$

This useful theory was initially met with some scepticism and the fear that the Cheng–Prusoff correction would lead to misinterpretation and misestimation of antagonist affinity (Leff & Dougall, 1993). At least for ligand affinities estimated from competitive radioligand

binding assays (as opposed to functional assays), however, these concerns are not often acknowledged—the Cheng–Prusoff correction is the prevailing approach for determining affinity for unlabelled compounds and is promoted through contemporary textbooks and applied pharmacology resources (such as GraphPad Prism) alike (e.g. Motulsky & Christopoulos, 2003).

## 2.2 | Efficacy theory: The first ideas

Competition between agonists and antagonists was an accepted concept in receptor theory by the middle of the 20th century. However, Clark's idea that effect is directly proportional to occupancy is clearly incongruent with the reality that, while they both bind receptors, agonists but not antagonists are able to elicit responses. Ariëns (1954), quickly followed by Stephenson (1956), was the first to account for this observation in ascribing the attribute of *efficacy* (Stephenson's term, as distinct from *affinity*) to agonists. The apparent different intensities of response elicited by different agonists were also a pivotal finding of both studies and originated the idea that agonists can have different extents of *intrinsic activity* (Ariëns' term for efficacy, neither of which should be confused with *intrinsic efficacy*, see below) to describe agonists that do not reach the same maximum effect as other drugs. Ariëns (1954) called these agonists "dualists" to reflect the fact that such drugs could act both as agonists and as antagonists of drugs which had greater intrinsic activity. Stephenson (1956) categorised the drugs using the now-customary name of "partial agonists." Importantly, he also went further than Ariëns in removing Clark's assumption of linear proportionality between effect and occupancy (that maximum efficacy occurs at 100% occupancy) by adding a *stimulus* function (a parameter that modifies the outcome efficacy) to indicate the amount of receptor activation by an agonist. This separation means that efficacy (in an intact system) is an agglomeration of agonist and system properties. More precisely, Stephenson (1956) issued three hypotheses that

1. "A maximum effect can be produced by an agonist when occupying only a small proportion of the receptors."
2. "The response is not linearly proportional to the number of receptors occupied."
3. "Different drugs may have varying capacities to initiate a response and consequently occupy different proportions of the receptors when producing equal responses. This property will be referred to as the efficacy of the drug."

Implicit in these hypotheses is the concept of a receptor reserve, or what Stephenson (1956) called "spare receptors," though this phenomenon was fully explained in contributions by others. First, Furchgott (1955) observed that Clark's assumption of a linear relationship between occupancy and response did not seem to hold for histamine, as response curves were left shifted relative to (and therefore not fully satisfactorily explained by) mass action-based occupancy. Furchgott (1955) opined:

> … it seems **impossible** that response should be proportional to the fraction of the total receptors combined with the drug.

Mark Nickerson (1956) also arrived at this notion in his study on β-haloalkylamine pharmacology. β-haloalkylamines were shown to act as specific irreversible antagonists of histamine-mediated ileum contractility, but Nickerson (1956) noted that while the maximum contractility induced by histamine was reduced by high concentrations of antagonist, at lower concentrations, histamine contractility was *not reduced* although a parallel right shift in the histamine response (as would be expected for competitive inhibition) was observed. To Nickerson, these apparently incompatible observations could only be explained by maximum histamine response requiring occupancy of a mere 1% of total receptors and receptor occupancy therefore could not be the limiting factor in determining magnitude of a tissue response. This theory fully explains the disconnect between affinity and potency for many different GPCRs and is important in vivo (e.g. cannabinoid receptor reserve, Gifford et al., 1999).

In the mid-1960s, these facets of the theory of efficacy were ultimately distilled into *intrinsic efficacy*. This development by Furchgott (1966) entailed the separation of the ligand and system components of a response, yielding a characteristic pertaining purely to ligands, which has been described as representing the magnitude of "stimulus *per* receptor" (Urban et al., 2006) or equivalent (Kenakin, 2009). The term *intrinsic efficacy* should not to be confused with *intrinsic activity* (which refers to $E_{MAX}$, the maximum response an agonist is capable of eliciting). The intrinsic efficacy parameter therefore acts as a scalar for translating receptor density into effect; Furchgott (1966) originally defined it as the quotient of efficacy (*intrinsic activity*, $E_{MAX}$) and receptor density, or

$$\epsilon = \frac{E}{R_{tot}}. \tag{8}$$

In addition to the consequences for receptor activity that are mediated by the presence of ligands, constitutive activity is now also a consideration in the understanding of efficacy. This phenomenon was first reported for δ-opioid receptors (Costa & Herz, 1989), when researchers showed that the peptide antagonist ICI174864 inhibited basal receptor activity. This effect was described as "negative intrinsic activity" (Costa & Herz, 1989) to counterpoint with the "positive" activity of agonists. This description was intended to indicate that ICI174864 inhibited the receptor's activity in the absence of an agonist and was distinguished from the effects of a neutral antagonist, MR2266. Lefkowitz also contributed to the theory of constitutive activity, with the discovery that substitution of four residues in the $\alpha_{1B}$-adrenoceptor sequence resulted in receptor-mediated inositol phosphate accumulation in the absence of agonist (Samama, Cotecchia, Costa, & Lefkowitz, 1993). Similar effects were seen for cAMP accumulation caused by the β-adrenoceptor (Lefkowitz, Cotecchia, Samama, & Costa, 1993). Constitutive activity is now a

well-founded premise in molecular pharmacology and is a feature of the function of many GPCRs.

## 3 | MATHEMATICAL MODELS OF RECEPTOR FUNCTION

Mathematical models were developed to explain the translation of the components of receptor activity into effects and entail linking together the two fundamental phenomena of receptor occupancy (the binding of ligands) and efficacy (the transmission of receptor stimulation into an effect, composed of a combination of ligand and system factors). There does not yet exist a single unifying model that is capable of describing all drugs effects with an experimental system (particularly as new receptor and ligand properties are discovered). In the present day, this has led to an assortment of models, each of which is then employed preferentially for understanding specific functional properties or phenomena. These models are therefore not mutually exclusive and a number of current receptor theory models are described briefly below.

### 3.1 | $E_{MAX}$ model

The $E_{MAX}$ model is undoubtedly the simplest of the models employed to interpret receptor signalling data and is based on the Hill–Langmuir equation, given as (re-parameterised from Equation (1)):

$$E = \frac{E_{MAX}}{1 + \left(\frac{EC_{50}}{[agonist]}\right)}. \quad (9)$$

Indeed, the $E_{MAX}$ model is sometimes called the Hill model (Di Veroli et al., 2015; Gesztelyi et al., 2012), although this term is usually used to describe the model when a sigmoidicity parameter has been added (as per Hill, 1910, equation (10)). It is also the most commonly used model and is used every day by molecular pharmacologists for the interpretation of concentration–response data (and by systems pharmacologists for dose–response data). The $E_{MAX}$ model is an *empirical* model. It has high utility precisely because it does not set out to provide mechanistic insight (Gesztelyi et al., 2012) but simply describes the non-linear relationship of log[agonist] versus response using four parameters. Each of these parameters has an empirically unambiguous interpretation in relation to its definition in the (extended) $E_{MAX}$ model equation (we use the term "extended" to signal that the usual $E_{MAX}$ model now incorporates a term to account for a non-zero basal response), which is usually given as

$$E = \frac{basal + (E_{MAX} - basal)}{1 + \left(\frac{EC_{50}}{[agonist]}\right)^{Hill\ slope}}, \quad (10)$$

where $E$ is the response magnitude ("effect") and the four parameters are basal, $E_{MAX}$, $EC_{50}$ and Hill slope. In an idealised system, basal signal would be equal to the receptor's constitutive activity (*Y*-magnitude/response in the absence of agonist), though in reality only a portion of the basal parameter is related to the receptor in question. $E_{MAX}$ is then the total *Y*-axis magnitude of the drug response (which, in an ideal system, would be "agonist effect above basal"). $EC_{50}$ is the 50% maximal agonist concentration (*X*-midpoint between basal and $E_{MAX}$) and the Hill slope is a factor characterising the curve slope at its midpoint (Motulsky & Christopoulos, 2003).

The $E_{MAX}$ model with a basal component is often simplified to three parameters by constraining the Hill slope to 1 and this is typically preferred for the analysis of GPCR signalling data because, like the basal parameter, the Hill slope is a system parameter (as opposed to being a ligand-dependent factor)—it is (or should be) constant for all agonists being compared. As GPCRs generally have just one orthosteric binding site, a Hill slope >1 could imply that the agonist-induced signalling of one receptor propagates the probability that another receptor would also start signalling (a type of co-operativity). In this example, a Hill slope of 1 would mean that both agonist binding and signalling occur in accordance with the law of mass action (consistent with a 1:1 ratio of ligand:receptor binding)—a null hypothesis against which it is difficult to argue. An alternative interpretation of Hill slope might be "activation threshold" (which is relevant in contexts such as voltage-evoked action potentials in electrophysiology). Software packages, such as GraphPad Prism, tend to be vague in their recommendations about constraining the Hill slope but do emphasise that many data points ("more than a dozen") are required to characterise a non-linear regression with non-unity Hill slope (Motulsky, n.d.; Motulsky & Christopoulos, 2003). It is worth noting that in the original report where AV Hill derived Hill slope—his paper describing oxygen binding to Hb (Hill, 1910)—he specifically notes that this parameter (here Hill terms his parameter *n*) is empirical:

> My object was … [not] to base any direct physical meaning on *n* …. ( Hill, 1910)

In other words, the Hill slope was initially developed in order to improve the fit of the curve Hill was modelling and was originally solved for particular values (under different assay conditions) ranging from 1 to 3.19 (Hill, 1910). It therefore seems that there has been a tendency for users of the $E_{MAX}$ model to over-interpret the Hill slope parameter—Hill may have considered that *n* could represent co-operativity in binding or effect (certainly, co-operative binding is relevant in his particular application, Bellelli, 2010), but he treated it as a mathematical constant that was necessary to improve curve fitting and was dependent on experimental conditions. Hill later noted that the equation for oxygen–Hb binding is actually an average of several similar quantities where the individual *n* values equal a range of integers—meaning that the parameter would not yield an integer overall (Hill, 1913).

The particular benefits of the $E_{MAX}$ model arise in comparison to the weaknesses of *mechanistic* models (including the three models discussed briefly below). Mechanistic models contain physicochemical constraints that allow meaningful extrapolation from empirical data to

complex biological systems (Danhof et al., 2007; e.g. disentangling the affinity and efficacy components that together result in the empirical outcome of potency), but at least two substantial problems arise because of this. First, mechanistic models require *a priori* knowledge of the complete mechanism by which an effect is elicited (and modified) and are consequently almost invariably incomplete. Second (and as a consequence of the first), mechanistic models may be poorly transferrable between biological systems and result in poorly estimated parameters (Kenakin, 2006; Motulsky & Christopoulos, 2003), especially if few (or no) parameters can be constrained. Empirical models, like the $E_{MAX}$ model, are not subject to such limitations, because they merely "geometrically" describe agonist effects (Gesztelyi et al., 2012).

It is worth noting that the next step in non-linear regression, so-called global fitting, removes some of the limitations of traditional analyses. This approach entails using automated regression software to simultaneously describe a *family* of curves, which allows sharing common parameters and therefore reduces the reliance of analysis on a single control condition or curve (Hall & Langmead, 2010). Examples of the application of global fitting might include Schild analysis (where the parameter of interest, $pA_2$, is the result of shifts in a family of concentration–response curves); and second, in global fitting of saturation binding data (in which, at each radioligand concentration, the whole non-specific binding *curve*, as opposed to individual concentration points, is subtracted from total binding for the interpolation of binding parameters).

## 3.2 | Two-state model

The first mechanistic model of receptor function was developed by Del Castillo and Katz (1957) and Katz and Thesleff (1957) working on ion channels. Both studies related to the action of ACh inducing depolarisation by acting on receptors situated in the motor endplate. The formation of an "intermediate receptor compound" (occupied receptor) was proposed, which was then arbitrarily suggested to toggle between states that either did or did not initiate depolarisation (Del Castillo & Katz, 1957). Some commentators have described this initial equilibrium as the "simple two-state model" (Rang, 2006). In the following paper, several alternative hypotheses were proposed. It was determined that the arbitrary constraint that only occupied receptors can switch into an active state was inaccurate; rather, a drug should be able to interact effectively with either active or inactive receptors and indeed would have preferential affinity for one state over another (agonists for $R^*$, inverse agonists for $R$; Katz & Thesleff, 1957), culminating in the reversible two-state model (Rang, 2006):

$$\begin{array}{ccc} R & \leftrightarrow & LR \\ \updownarrow & & \updownarrow \\ R^* & \leftrightarrow & LR^*. \end{array}$$

The two-state model thus posits that receptors exist in an equilibrium and "switch" between a resting state ($R$), in which they are uncoupled from G protein effectors, and an active state ($R^*$), where receptors gain affinity for G proteins and thereby initiate response cascades. Ligand ($L$) binding then shifts this equilibrium either towards the $R^*$ state (agonists), towards the $R$ state (inverse agonists), or are neutral in that they prefer neither state (antagonists; Bridges & Lindsley, 2008; Leff, 1995; Rang, 2006). For the first time in receptor theory, the phenomena of ligand binding and response were explicitly separated. Equilibrium constants would then govern the distribution of $R$ and $R^*$ receptor populations and the agonist's activity would be determined by equilibrium constants that were specific for the state of $R$ (Katz & Thesleff, 1957; reviewed in Leff, 1995; Kenakin, 2009). A related two-state model was applied to Hb a few years later (Monod, Wyman, & Changeux, 1965)—itself following soon after the first model of allosterism in enzyme systems (Gerhart & Pardee, 1962).

A number of the features of GPCR function are neatly explained by the two-state model. The conformational $R$–$R^*$ equilibrium (in the absence of agonist) explains constitutive activity (the part of the basal signal which can be inhibited by inverse agonism), while agonists' differing abilities to shift this equilibrium provide a basis for different agonist efficacies. Competitive, neutral antagonism was nicely explained as a ligand that does not alter the $R$–$R^*$ ratio by interacting with the receptor. The model therefore remains useful to aid in understanding the nature of simple agonism and antagonism.

Some observations, however, could not be explained satisfactorily by the two-state model. For example, in the two-state model a given agonist would be expected to drive the same $R$–$R^*$ ratio irrespective of the endpoint being measured for receptor activation. However, ultimately, research showed that agonists' rank order potencies and efficacies changed for the same receptor acting with different G proteins (e.g., Berg et al., 1998). This phenomenon has been explained by the extension of the two-state model to a so-called three-state model (Berg et al., 1998; Leff, Scaramellini, Law, & McKechnie, 1997), in which receptors exist in states of equilibrium between $R$, $R^*$ or a second active state, $R^{**}$. The finite number of receptors necessitates that if a ligand accumulates receptors into one of these states, the other states (and their specific pathway efficacies) are necessarily depleted (Leff et al., 1997). The three-state model can be naturally extended to multistate settings with ever-increasing complexity.

## 3.3 | Ternary complex model

The ternary complex model became a necessary extension to the two-state model, when De Lean, Stadel, and Lefkowitz (1980) discovered that the distribution of activity states (essentially, $R$–$R^*$ equilibrium) of the β-adrenoceptor could be altered, not just by the binding of agonists but also by interaction of G proteins (effectors). States of high agonist affinity states aligned with G protein coupling to the receptor and vice versa. A model was therefore required that incorporated a two-stage process of ligand binding and interaction with a G protein (Figure 2a). Notably, "active" and "inactive" receptor states were only implicitly included in the original form of the ternary

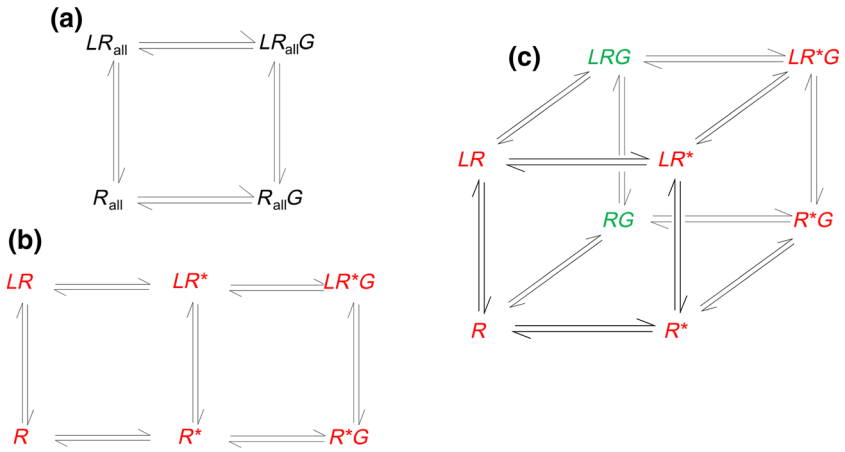


**FIGURE 2** Ternary complex model (TCM) schematics: the original TCM (a), extended TCM (b) and cubic TCM (c). The original TCM did not distinguish between two receptor activity states, but in (b) and (c), colour coding is included to highlight the progressive addition of states from the extended TCM (red) to the cubic TCM (green). Nomenclature: ligand (*L*), receptors (either "all" receptors or receptors in two states; inactive *R* and active $R^*$), and G protein (*G*). Note that *L* and *G* components are not listed when they are not "complexed" with *R*

complex model—explicit differentiation of two receptor states was not incorporated until later. This means that the original form of the ternary complex model does not directly transfer into later extensions of the model.

The failure of the original ternary complex model (Figure 2a) to account for receptors spontaneously switching between activity states (i.e. constitutive activity) necessitated model development, following on from the work of Costa and Herz (1989). This entailed a modification that apportioned to antagonists the ability to drive G protein dissociation from receptors (Wreggett & De Lean, 1984). The extended ternary complex model (Figure 2b) included an equivalent extension, incorporating spontaneous G protein activation (interaction with receptor) in the absence of agonist. This extension was the result of a study involving $\beta_2$-adrenoceptor mutations that resulted in permanent receptor activation (Samama et al., 1993). The cubic ternary complex model (Figure 2c) was constructed in order to explain the occurrence of inactive receptor interactions with G proteins. Indeed, it treats every form of the "native receptor ensemble" (which includes the G protein) as being capable of interacting with agonist (Weiss, Morgan, Lutz, & Kenakin, 1996) and it thereby becomes thermodynamically necessary for inactive receptor–G protein interactions to occur.

### 3.3.1 | Allosterism in the ternary complex model

An additional major development necessitated the consideration of allosteric behaviour—to this point, models had considered orthosteric receptor–ligand interactions only. Under the allosteric binding model, modulators are drugs which bind to sites that are topographically distinct from orthosteric binding sites and modulate, positively or negatively, the binding and/or effects of orthosteric ligands (see below, also recently reviewed in Foster & Conn, 2017). The simplest form of this model is based on the original ternary complex model and introduces separate equilibrium dissociation constants for the orthosteric and allosteric ligands, plus a co-operativity factor ($\alpha$) that represents the mutual effect of each ligand on the other's affinity for the receptor and another parameter, $\beta$, that represents the influence of the allosteric modulator on the efficacy of the orthosteric agonist (Hall, 2000, also reviewed in May, Leach, Sexton, & Christopoulos, 2007; Lindsley et al., 2016). In fact, the effects of these drugs have resulted in multiple iterations of ternary complex models, some of which are extremely elaborate—the quaternary complex model, for example, accounts for every combination of a receptor complex that includes a receptor in two states of activity, an orthosteric agonist, an allosteric modulator and a G protein (Christopoulos, Lanzafame, & Mitchelson, 1998).

Notably, however, despite the multivariate complexity of ternary complex models, all fundamentally remain two-state models, with a single $R^*$ state. Further elaboration into putative three (or more)-state models to address limitations mentioned above, that also encompass ternary complexes, would be exponentially more sophisticated (not to mention impossible to graphically represent due to a lack of dimensions).

## 3.4 | Operational model

It is worth noting that both explicit mechanistic models, with extensive parameterisation and associated challenges (see above), and more easily used/functional ("operational") semi-mechanistic alternatives each serve necessary functions. The former are, by definition, more complete but are also necessary for the development of the latter, which are needed to describe real experimental data (e.g. due to parameter identifiability constraints).

Theory employed through the early part of the 20th century was adequate for the description of single agonists (including studies involving competitive antagonism), because an agonist-mediated effect was always compared with itself. This enabled characterisations of agonists and antagonists using ratios (such as in Schild analysis, where the ratio in question gives rise to $pA_2$), and "post-receptor" events that influenced a response could be assumed to be equal and therefore cancelled out. As early as 1953, the inability to compare *between* agonists (due to the inability to assume equality of post-receptor events) was recognised as a significant issue. As Gaddum (1953) observed:

> Comparative assays have no satisfactory logical basis … If other factors (species, type of effect, etc.) were varied, the results of most comparative assays could be shown to be invalid.

The ideas put forward by Stephenson (1956) and Furchgott (1966; particularly the stimulus *per* receptor/intrinsic efficacy concept) delineated the agonist-specific part of efficacy (composed of binding, i.e. affinity and stimulus, i.e. intrinsic efficacy/$\epsilon$), leaving a system-dependent part (receptor density and pathway efficiency) which was assumed to remain equal between agonists. Black and Leff (1983) note that comparative assays then depend on two assumptions: equal stimuli elicit equal effects and stimulus is the product of intrinsic efficacy and receptor density (Furchgott, 1966). However, these assumptions are unprovable because "stimulus" is an abstract concept; intrinsic efficacy does not relate to any specific measurable entity (Black & Leff, 1983), which had been suggested earlier by the finding that only relative (ratios), not absolute, values of intrinsic efficacy could be determined (Mackay, 1966).

In the context of these issues of interpretability, Black and Leff developed the operational model to create a mechanistic model that did *not* necessitate an empirical constant for intrinsic efficacy. Instead, it is included in the model implicitly, in the form of the saturable relationship between receptor stimulation and response (Black & Leff, 1983; Kenakin, 2004). In other words (Weiss et al., 1996), the operational model created a phenomenological parameter that *behaves like* efficacy.

The operational model is initially based on the observed hyperbolic relationship between receptor occupancy and response, where agonist-occupied receptor (AR) initiates a response cascade with an equilibrium dissociation constant of $K_E$ (Black & Leff, 1983; a composite parameter representing transduction efficiency). The operational model in this form therefore initially resembles the Hill–Langmuir equation:

$$\frac{E}{E_M} = \frac{[AR]}{[AR] + K_E}, \tag{11}$$

where $E_M$ is the maximum possible effect in the system (and is not equal to the agonist's empirical $E_{MAX}$). The more effective the AR complex is at driving a response, the smaller the $K_E$. Assuming the occupied receptor forms according to the law of mass action, the equation can be restated as

$$\frac{E}{E_M} = \frac{[A] \cdot [R_{\text{tot}}]}{[A] \cdot ([R_{\text{tot}}] + K_E) + (K_A \cdot K_E)}, \tag{12}$$

where $[R_{\text{tot}}]$ is the density of receptors (or total receptor number) and $K_A$ is the equilibrium dissociation constant of the agonist from AR. A "measure of the efficiency of transduction" of occupied receptors into effect, $E$ (the so-called transducer ratio, $\tau$) was then defined as the ratio of $R_{\text{tot}}$ and $K_E$ (Black & Leff, 1983), giving

$$\frac{E}{E_M} = \frac{[A] \cdot \tau}{[A] \cdot (\tau + 1) + K_A}. \tag{13}$$

Changes in the value of $\tau$ therefore reflect changes in the responsiveness of the system to agonists (both potency and maximum agonist response, efficacy), meaning that effect ($E$) is influenced by both tissue and ligand factors—indeed, $K_E$ is defined (Black & Leff, 1983) as including both the agonist's intrinsic efficacy and as a parameter to adjust this stimulus for the system's ability to transmit it.

The $\tau$ parameter is therefore an efficacy indicator (but is not equivalent to any aforementioned measure of efficacy) and is the uniquely practical ("operational") contribution of the operational model. By another definition, it is equal to the ratio of the total concentration of receptors in the system and the concentration of receptors that need to be occupied by agonist to provoke a half-maximal tissue response. Therefore, $\tau$ is also the inverse of the fraction of receptors that must be occupied to produce a half-maximal response (Motulsky & Christopoulos, 2003; so, e.g., $\tau$ = 20 indicates that $EC_{50}$ will occur at 5% occupancy).

Later developments of the operational model include a variant that could account for non-unity slope factor (i.e. variable slope concentration–responses; Black, Leff, Shankley, & Wood, 1985) and more recently still to account for non-zero basal activity (Van Der Westhuizen, Breton, Christopoulos, & Bouvier, 2014):

$$E = \frac{\text{basal} + (E_M - \text{basal})}{1 + \left( \frac{\left( \frac{[A]}{10^{\log K_A}} + 1 \right)}{\frac{10^{\log R}}{[A]}} \right)^n}, \tag{14}$$

where $n$ is a slope factor and basal represents the $Y$-magnitude of constitutive activity in the system. Note, however, that in this context, the basal parameter is itself empirical (or, at best, semi-mechanistic) in that it does not distinguish between "true" constitutive activity (i.e. receptor activity in the absence of agonist) and factors that could produce non-zero basal in the biological system (e.g. receptor-independent constitutive activity or even the presence of endogenous ligand acting at the same receptor). Mechanistic elaborations that do distinguish between these types of activity (Hall & Giraldo, 2018) tend to suffer from over-parameterisation (a state of having more parameters than can be estimated empirically), thereby losing their usefulness.

The operational model has been used recently to scaffold contemporary methods for the quantification of functional selectivity (Kenakin, Watson, Muniz-Medina, Christopoulos, & Novick, 2012; Van Der Westhuizen et al., 2014). In the original form of the operational analysis method (Kenakin et al., 2012), a parameter referred to as the *transduction coefficient* was formulated, defined as the ratio of $\tau$ and $K_A$. This ratio has since been designated $R$ (usually expressed in the log form; Van Der Westhuizen et al., 2014) and can be determined from traditional concentration–response functional data (Figure 3). $R$ is said to be able to characterise agonism for any given pathway as it combines both affinity and efficacy into a single measure. It is

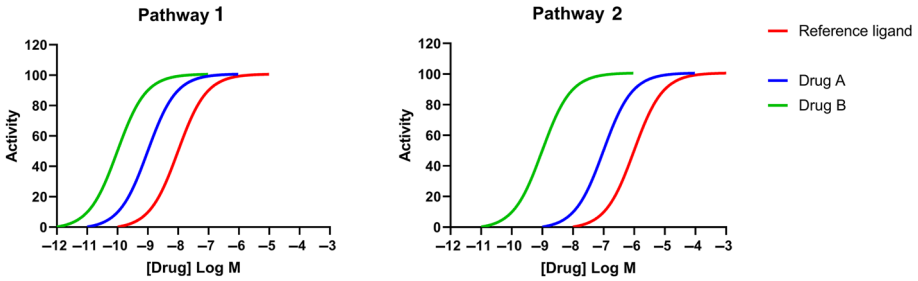


**FIGURE 3** Hypothetical functional data for three different ligands in two pathways, fitted with the "general," Van Der Westhuizen et al. (2014), operational model

therefore a useful composite parameter that allows comparisons between agonists (Kenakin et al., 2012). Applications of operational analysis (for the quantification of functional selectivity) entail normalisation of agonist log*R* values to a reference agonist within each pathway endpoint (giving Δlog*R*). Traditionally (though with exceptions), a further normalisation is then performed whereby each agonist's Δlog*R* values in two pathways of interest are subtracted one from the other (Van Der Westhuizen et al., 2014), giving ΔΔlog*R* (Table 1). Results of this kind of analysis can be graphically presented as a radar plot (or so-called web of bias of $10^{\Delta\Delta\log R}$), with concentric lines (representing the different ligands in the analysis) showing the relative activities at each vertex (pairwise pathway comparison).

In the example given (Figure 3, Table 1), drug responses in Pathway 2 are all lower potency than in Pathway 1—this is classified as a "system" factor (as opposed to a "ligand" factor) and is therefore intentionally excluded from this type of analysis. Compared to the reference ligand (red), the activity of Drug A in Pathway 2 can be predicted by its activity in Pathway 1—its relative potency is preserved and this is reflected by a ΔΔlog*R* of 0.00 (bias factor 1.00), indicating no bias. However, the potency of Drug B is less right shifted in Pathway 2 relative to Pathway 1 than would be predicted by the potency shift of the reference ligand. This is reflected by a ΔΔlog*R* of 1.00 (bias factor 10.00)—which would be interpreted as showing that Drug B is either biased *towards* Pathway 2 or *away* from Pathway 1.

The operational model continues to be developed. Due to its current increasing use, we recently examined the ability of operational analysis parameters to be estimated (their "identifiability"), given either perfect or typical experimental data (Zhu, Finlay, Glass, & Duffull, 2018). This study found that the parameter $\tau$ was identifiable in the case of perfect data ("structurally identifiable"), but the transduction coefficient, log*R* (or $\frac{\tau}{K_A}$), was the minimally estimable parameter given typical experimental data ("deterministically identifiable"). These findings help explain the aforementioned conventions for (empirical) quantification and presentation of biased agonism data.

**TABLE 1** Example operational analysis (data from Figure 3)

| | Pathway 1 | | |
|---|---|---|---|
| | **Ref ligand** | **Drug A** | **Drug B** |
| log*R* | 8.01 | 9.01 | 10.01 |
| Δlog*R* | 0.00 | −1.00 | −2.00 |
| | **Pathway 2** | | |
| | **Ref ligand** | **Drug A** | **Drug B** |
| log*R* | 6.01 | 7.01 | 9.01 |
| Δlog*R* | 0.00 | −1.00 | −3.00 |
| | **Pathway 1–Pathway 2** | | |
| | **Ref ligand** | **Drug A** | **Drug B** |
| ΔΔlog*R* | 0.00 | 0.00 | 1.00 |
| Bias factor | 1.00 | 1.00 | 10.00 |

## 4 | THE FUTURE OF RECEPTOR THEORY: KINETICS

One area of receptor theory that has tended to lag behind other disciplines within experimental pharmacology is that of system kinetics. Indeed, the time course of drug absorption, distribution, metabolism and elimination comprises the entire field of pharmacokinetics (reviewed in Fan & de Lannoy, 2014; Wright, Winter, & Duffull, 2011)—yet the most widely utilised models in receptor theory remain fundamentally equilibrium based, including all of the $E_{MAX}$ model, two-state and other multistate models, ternary complex models and the operational model.

The incongruence of the equilibrium assumption with the nature of GPCR activity has been identified by some researchers to date. A notable example of this includes a study examining biased agonism at $D_2$ dopamine receptors. In order to more closely examine the impact of receptor activity kinetics, operational analysis was performed at repeated intervals over an extended time course of assay detection in several pathways (Klein Herenbrink et al., 2016). Interestingly, both agonist potency rank order and bias conclusions for the ligands changed (sometimes even reversing) time-dependently for the pathways included. This was put down to different agonist association/dissociation kinetics—agonists that dissociated slowly from their receptors were more active at later time points (relative to other ligands) and fast-dissociating agonists were similarly favoured at earlier time points (Klein Herenbrink et al., 2016). Despite such approaches having useful diagnostic utility, this approach is undermined by the fact that operational analysis remains an equilibrium-based model (and therefore this "cross-sectional" analysis approach is somewhat simplistic) and hence does not provide evidence to quantify the magnitude of agonists' biases. True kinetic models are therefore required.

As derived above, a ligand's equilibrium dissociation constant, $K_D$, is also defined as a ratio of two kinetic parameters, the dissociation and association rate constants ($\frac{k_{off}}{k_{on}}$). Clearly, it is possible for different ligands to differ hugely in these parameters, which can be reflected in different signalling time courses even if their equilibrium dissociation constants are identical. Recent reports have started to examine ways to directly estimate these rate constants, to help explain different signalling pathway kinetics (reviewed in Sykes, Stoddart, Kilpatrick, & Hill, 2019). The intrinsically differing kinetics of different activity pathways will interact and confound the overall response time course. However, to date, very few models exist that directly and specifically account for both different ligand *binding* kinetics and different *signalling pathway* kinetics. Such an integrated model framework of receptor activity dynamics will be required, in order to explain all components (particularly different effect/activity time courses) of drug responses. We and others are pursuing research in this area currently (e.g. Bridge, Mead, Frattini, Winfield, & Ladds, 2018; Hoare, Pierre, Moya, & Larson, 2018; Zhu, Finlay, Glass, & Duffull, 2019). Further complexity originates from the contribution of receptor desensitisation and internalisation—these are themselves time- and agonist-dependent effects (Zhu et al., 2019) but inherently assert a type of "negative feedback" effect on the activity of other pathways. This type of regulatory effect has received little attention in GPCR functional modelling to date.

Given the hitherto pre-eminence of equilibrium models of receptor function, it may be surprising that thoughts about the kinetics of receptor pharmacology are not entirely new. Once again, AV Hill was the first to put forward a theoretical justification for non-instantaneous equilibrium in his work on the nicotine concentration–effect relationship in muscle (Hill, 1909). In this work, Hill derived $\lambda$, a parameter that is independent of the nicotine concentration. This new parameter implicitly explains an observed delay in nicotine-induced contractile responses between two different response compartments (intra- and extra-muscular) and thus added a kinetic dimension to Hill's otherwise equilibrium-based concentration–effect model (Hill, 1909). These concepts were discovered again in the 1970s and incorporated into the theory of in vivo drug concentration kinetics—the so-called biophase delay or hypothetical effect compartment (Sheiner, Stanski, Vozeh, Miller, & Ham, 1979; reviewed in Wright et al., 2011).

Progress in the area of receptor theory now depends on close collaboration between empirical pharmacologists and pharmacometricians, the former to produce data that can inform model development and inference by the latter and in turn the latter to produce utilitarian tools that can be applied by the former. It seems likely that the reason that the method for analysing functional selectivity using the operational model (Van Der Westhuizen et al., 2014) has become so widespread is that it was published as a drop-in method of analysis in GraphPad Prism. As research proceeds, and as more of the elements of receptor function are incorporated quantitatively into complex mechanistic models, it is to be hoped the "two-pronged," co-dependent evolution of this important subject can be as productive in the future as it has been in the past.

## 4.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Kelly et al., 2019).

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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