Endogenous allosteric modulators of G protein-coupled receptors


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Abbreviations:

2-AG, 2-arachidonoyl glycerol; 5-HT, 5-hydroxytryptamine; AR, adrenoceptor; AT, angiotensin; cAMP, cyclic adenosine monophosphate; \([\text{Ca}^{2+}]_o\), extracellular calcium; CaSR,
calcium sensing receptor; CB, cannabinoid; CCK, cholecystokinin; CCM, cholesterol consensus motif; CSF, cerebrospinal fluid; ERK, extracellular signal-regulated kinase; GABA, \(\gamma\)-amino butyric acid; CP55,940, \((2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol); GPCR, G protein-coupled receptor; glutathione, glutathione (\(\gamma\)-L-glutamyl-L-cysteinyl glycine); GTP, guanosine triphosphate; IgG, immunoglobulin; IP, inositol phosphate; mAChR, muscarinic acetylcholine receptor; MCR, melanocortin receptor; mGluR, metabotropic glutamate receptor; MRAP, melanocortin receptor accessory protein; NAL, neutral allosteric ligand; NAM, negative allosteric modulator; OR, opioid receptor; PTH, parathyroid hormone; RAMP, receptor activity modifying protein; THC, tetrahydrocannabinol; TM, transmembrane domain; VPAC, vasoactive intestinal polypeptide; WIN55212-2, ((R)-(+)\-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]- 1-naphthalenylmethanone)
Abstract

G protein-coupled receptors (GPCRs) are the largest superfamily of receptors encoded by the human genome, and also represent the largest class of current drug targets. Over the last decade and a half, it has become widely accepted that most, if not all, GPCRs possess spatially distinct allosteric sites that can be targeted by exogenous substances to modulate the receptors’ biological state. Although many of these allosteric sites are likely to serve other, (e.g. structural) roles, they nonetheless possess appropriate properties to be serendipitously targeted by synthetic molecules. However, there are also examples of endogenous substances that can act as allosteric modulators of GPCRs. These not only include the obvious example, i.e., the G protein, but also a variety of ions, lipids, amino acids, peptides and accessory proteins that display different degrees of receptor-specific modulatory effects. This also suggests that some GPCRs may possess true ‘orphan’ allosteric sites for hitherto unappreciated endogenous modulators. Of note, the increasing identification of allosteric modulator lipids, inflammatory peptides and GPCR-targeted autoantibodies indicates that disease context plays an important role in the generation of putative endogenous GPCR modulators. If an endogenous allosteric substance can be shown to play a role in disease, this could also serve as an impetus to pursue synthetic neutral allosteric ligands (NALs) as novel therapeutic agents.
Introduction

G protein-coupled receptors (GPCRs) are the largest family of receptor proteins, responding to a vast range of extracellular mediators and widely pursued as drug targets (Bockaert and Pin, 1999; Garland 2013). Traditional drug discovery efforts have largely focused on agonists or antagonists that target the orthosteric site on GPCRs, that is, the binding site(s) utilized by endogenous agonist(s). However, it is now well recognized that most, if not all, GPCRs possess spatially distinct allosteric sites that can also be targeted for therapeutic benefit (Christopoulos and Kenakin, 2002; May et al., 2007; Conn et al., 2009; Christopoulos, 2014; Christopoulos et al., 2014).

The phenomenon of allostery was first described in seminal studies in the field of enzymology (Monod and Jacob, 1961; Monod et al., 1963), but subsequently extended to other classes of proteins (see Changeux and Edelstein, 1998; Changeux, 2013). This highlights the fact that allosteric modulation is a ubiquitous and vital biological process (Fenton, 2008). It is also suggestive that many of the mechanisms underlying allostery, as first formalized in the classic Monod-Wyman-Changeux (Monod et al., 1965) and Koshland-Nemethy-Filmer models (Koshland et al, 1966), are likely applicable to other protein classes, including GPCRs (Canals et al., 2011). Advantages of targeting allosteric GPCR sites include the potential for receptor subtype selectivity, due to either greater sequence divergence in allosteric pockets between receptor subtypes relative to the (necessarily) conserved orthosteric site, or due to subtype-selective cooperativity; the ability to fine tune physiological responses in either a positive or negative direction; a saturability, or ‘ceiling’, to the effect that may lead to greater on-target safety in overdose situations (May et al., 2007; Conn et al., 2014; Christopoulos, 2014). Allosteric modulators can also display distinct pharmacological properties, including the phenomena of ‘probe-dependence’ and ‘biased
agonism/modulation’. Both of these reflect similar conformational mechanisms; probe
dependence describes the situation whereby the magnitude and direction of the allosteric
effect of a given modulator can change depending on the nature of the orthosteric ligand
being used to probe receptor function, whereas biased agonism/modulation describes the
allosteric change in the GPCR’s intracellular signaling preferences (i.e., receptor-transducer
interactions) depending on the nature of the ligand being used to activate or modulate the
receptor (Keov et al., 2011; Christopoulos and Kenakin, 2013).

The theoretical advantages associated with GPCR allosteric modulators have spurred
numerous ongoing research programs in academia and industry over the last decade and a
half (Conn et al., 2014). These studies are not only revealing the extent to which the theory
of allostery can be put to practical use in GPCR drug discovery, but also identifying ongoing
challenges and questions associated with the phenomenon. For example, the prevalence of
probe dependence highlights the need for more broad screening of putative modulator
compounds than previously anticipated, particularly for GPCRs that have more than one
endogenous agonist (Wootten et al., 2013a). Similar considerations apply for biased
agonists/modulators, as in many cases the links between observed cellular drug effects and in
vivo pathophysiology remain unclear (Christopoulos and Kenakin, 2013). Moreover, the
long-term effects of allosteric GPCR ligands on receptor regulation remain largely unknown
(Lane et al., 2013), even though many drug therapies are chronic in nature.

Interestingly, the finding that allosteric GPCR sites are highly prevalent is also leading to a
re-evaluation of the role of many of these sites, and the possibility that they may represent
interaction domains for unappreciated endogenous ligands. This is certainly the case for
other receptor superfamilies. For instance, the function of the nuclear hormone receptors is
characterized by the requirement for interaction with a suite of co-repressor or co-activator molecules, as well as DNA, in a complex network of allosteric interactions (Burris et al., 2013). The γ-amino butyric acid (GABA) family of ligand-gated ion channels is also modulated by endogenous neuroactive steroids (Majewska et al., 1992) and proteins (Christian et al., 2013). The best characterized endogenous allosteric modulator of a GPCR is, of course, the G protein itself (see below) but, increasingly, other endogenous substances are being identified that may act as allosteric GPCR modulators with greater degrees of specificity. This begs the question about the overall extent of endogenous GPCR subtype-specific allosteric modulators. Is it possible that many of the allosteric sites utilized by synthetic small molecule GPCR modulators represent “orphan” allosteric sites for as yet unidentified natural modulators, or are they largely “serendipitous” allosteric sites with no natural ligand (Hardy and Wells, 2004)? In the GPCR field, the latter view has traditionally predominated and remains logical; binding domains associated with orthosteric ligands for one type of GPCR family (e.g., the transmembrane regions for numerous rhodopsin-like Class A GPCRs) can represent serendipitous allosteric sites for other GPCR types (e.g., Class B peptide receptors, or Class C nutrient receptors, where the orthosteric site is largely restricted to extracellular loop or N-terminal domains) (Figure 1A). It is likely that some allosteric sites are cavities that normally arise as a consequence of structural roles (e.g., receptor folding, trafficking) but possess sufficient functional groups so as to be serendipitously exploited by exogenous synthetic compounds (Figure 1B). Nonetheless, it is also possible that some GPCRs may be regulated naturally by endogenous modulators (Figure 1B). This review considers some of the emerging data in support of the latter possibility.

G proteins as endogenous allosteric modulators
GPCRs are natural allosteric proteins in the sense that they respond to extracellular stimuli via a distinct interaction domain (the orthosteric site) and transmit the stimulus to a topographically distinct intracellular domain, the G protein-binding site, in order to transduce the signal (Christopoulos and Kenakin 2002). Furthermore, the nature of the interaction is clearly allosteric because of the reciprocal effects that agonists (and inverse agonists) and G proteins exert on one another. For example, Figure 2A illustrates the profound allosteric effect that the guanine nucleotide, guanosine triphosphate (GTP), exerts on the binding of the agonist, carbachol, at the M₂ muscarinic acetylcholine receptor (mACHR). This interaction is characterized by negative cooperativity, because agonist binding favors coupling of the nucleotide-free G protein to form a high affinity “ternary complex”, whereas nucleotide binding uncouples this complex to yield a low affinity, G protein-free state (De Lean et al., 1980; Ehlert, 1985).

Further evidence of the allosteric nature of G protein-GPCR interactions has been provided by studies where the G protein has been overexpressed. For instance, overexpressing Gₛ or G₉ proteins increases the basal constitutive activation of the β₂ adrenoceptor (β₂AR) or the M₁/₅/₇ mAChRs, respectively (Azzi et al., 2001; Burstein et al., 1997). Inverse agonists can decrease this heightened constitutive activity, whereas agonist affinity and potency is generally increased under the same conditions (Burstein et al., 1997; Yan et al., 2008). The tight conformational coupling between the orthosteric and G protein-coupling sites is also highlighted by the fact that the sites show strong patterns of co-evolution (Suel et al., 2003). Direct demonstration of the profound conformational changes that occur in the process of GPCR signal transduction upon association with heterotrimeric G proteins or G protein-mimicking nanobodies has also been revealed in recent breakthrough structural studies (Rasmussen et al., 2011; Ring et al., 2013). Collectively, these studies show that G proteins
can alter the conformational state of the GPCR in an allosteric manner to influence the binding and or signaling properties of agonist and antagonist ligands.

**GPCR – accessory protein interactions**

It is now well accepted that GPCRs can participate in non-canonical signaling networks via interactions with an expanding list of accessory proteins, often in a G protein-independent manner (Couvineau and Laburthe, 2012; Couray et al. 2009; Sato et al. 2006; Brady et al., 2002). Probably the best-characterized example is that of the GPCR-β-arrestin interaction. Although originally identified as playing a vital role in the termination of GPCR signaling via G proteins, β-arrestins are also known to act as scaffolding proteins and novel signal transducers in their own right for certain receptors (Reiter et al. 2012). Interestingly, the interaction of the arrestin with the GPCR can itself modulate the properties of agonists. As shown in Figure 2B, the affinity of isoproterenol for the β2 AR is markedly enhanced in the presence of different arrestin proteins, essentially forming an alternative “ternary complex” to the classic agonist-receptor-G protein paradigm (Gurevich et al., 1997).

The receptor activity modifying proteins (RAMPs) are another family of accessory proteins that play a profound role in modulating the pharmacology of numerous GPCRs. These 3 single transmembrane-spanning proteins were initially identified as essential coupling partners for the Class B calcitonin receptor-like (CLR) GPCR; depending on the heteromer formed between the CLR and a given RAMP, the resultant complex was revealed to be the minimal unit required to yield the pharmacologically defined receptors for CGRP or adrenomedullin (McLatchie et al., 1998; Bühlmann et al., 1999; Christopoulos et al., 1999). These studies were extended to reveal that the calcitonin receptor can also interact with
RAMPs to yield distinct amylin receptors (Poyner 2002; Hay 2004; Udawela 1984; Muff et al., 1999; Zumpe et al., 2000; Hay et al., 2006).

Since these initial breakthroughs, further roles for RAMPs in physiology and pharmacology have been discovered. In addition to the determination of receptor phenotype, outlined above, RAMPs can also modulate the signaling preferences of GPCRs, either in terms of strength of coupling to G protein pathways (e.g., corticotropin-releasing hormone 1 (CRF1) receptor; Wootten et al., 2013b) or even biasing signaling between different intracellular pathways for the vasoactive intestinal polypeptide 1 (VPAC1) receptor (Christopoulos et al., 2003) or calcitonin receptor (Morfis et al., 2008). RAMPs can also play important chaperoning roles for receptor trafficking processes, as noted not only for the canonical interaction with CLR, but also for the Class C extracellular calcium sensing receptor (CaSR) (Bouschet et al., 2005). Generation of genetically engineered mouse models in recent years has unmasked key roles for the different RAMP proteins in the cardiovasacular, renal and respiratory systems, in addition to novel roles in inflammation (Kadmiel et al., 2012; Lenhart et al., 2013; Li et al., 2014). Importantly, the RAMP-receptor interface has emerged as a novel druggable pocket in its own right (Sexton et al., 2009; Sexton et al., 2012; Wootten et al., 2010). Figure 3 shows the recent crystal structure of the N-terminal region of CLR in complex with RAMP1 and the anti-migraine drug, olcegepant (Haar et al., 2010), providing a structural basis for selective anti-CGRP receptor ligands.

The characteristics of the RAMP family are likely to be exemplars of a broader accessory protein paradigm for GPCRs that continues to yield novel findings. For instance, the melanocortin receptor accessory proteins (MRAPs) have recently been identified as single transmembrane-spanning proteins that modulate the expression, trafficking and signaling of
members of the melanocortin receptor (MCR) family with many similarities to RAMPs and their interacting GPCRs (Novoselova et al., 2013). The first MRAP was identified through a genetic screen in patients with familial glucocorticoid deficiency, and found to be most highly expressed in the adrenal gland where it facilitates the cell surface expression of MCR2 and makes the receptor responsive to adrenocortitropic hormone (Metherell et al., 2005). A second homologue, MRAP2, was identified shortly thereafter and shown to be highly conserved in vertebrates (Chan et al., 2009). Like the RAMPs, the MRAPs show a quite wide expression pattern, which suggests that they likely serve other functions that go beyond trafficking and expression. Possibilities include the determination of MCR phenotype and signal coupling preferences (Novoselova et al., 2013). Given that approx. 20% of cases of familial glucocorticoid deficiency are caused my mutations in MRAP (Metherell et al., 2005), this highlights the potential for targeting the MCR-MRAP interaction for therapeutic purposes.

Finally, it is now widely acknowledged that GPCRs may participate in homo- and heterodimeric/oligomeric interactions with one another to yield potentially distinct and targetable pharmacological entities (Milligan et al., 2003; Panetta and Greenwood, 2008; Milligan, 2009). Although beyond the scope of the current review, cooperative interactions within oligomeric receptor arrays clearly highlight that GPCRs have the potential to act as endogenous allosteric modulators of one another.

**Ions as allosteric modulators**

**Sodium:**

The functionality of many GPCRs is sensitive to modulation by various ions. One of the best-studied examples involves the effects of sodium on GPCR sensitivity to agonists and
inverse agonists. A seminal study by Pert et al. (1973) was the first to suggest that Na⁺ could act as a negative allosteric modulator of opioid receptor agonist binding while having relatively little effect on antagonists. Subsequent studies suggested that the effect of Na⁺ likely involves modulation of the conformational state of the receptor (Simon and Groth, 1975) and that the binding of certain antagonists can actually be potentiated by the ion (Appelmans et al., 1986); one of the key characteristics exploited in the biochemical identification and validation of inverse agonism (Costa and Herz, 1989). These observations were soon extended to numerous other Class A GPCRs, including those for biogenic amines, nucleosides, peptides and lipids (Eriksen et al., 2009; Liu et al., 2012; Katrich et al., 2013); Figure 4A shows an example of opposing effects of Na⁺ on the binding of an agonist (quinpirole) and inverse agonist (epitepride) at the dopamine D₂ receptor (Neve, 1991), which is consistent with the expectations of behavior within a two-state receptor model (Christopoulos, 2014). However, the affinities of other known inverse agonists of the D₂ receptor are not necessarily sensitive to sodium (e.g., Vivo et al., 2006), suggesting that the phenomenon is also dependent on ligand structure and remains an active area of research. Importantly, many of the allosteric effects of sodium occur at or near physiological concentrations (e.g., Costa and Herz, 1989) and thus suggest that this plays a key general role in GPCR biology.

Mutagenesis studies identified a highly conserved aspartic acid residue, D².S0, in the second transmembrane domain of Class A GPCRs as being key to the allosteric actions of sodium (Fraser et al., 1990; Horstman et al., 1990; Neve et al., 1991; Strader et al., 1994). However, the structural basis of this interaction has only recently begun to be delineated as a consequence of high resolution GPCR crystal structures revealing a coordinated Na⁺/water cluster in the transmembrane bundle of inactive-state GPCRs, including the adenosine A₂A
(Liu et al., 2012), protease-activated receptor 1 (Zhang et al., 2012), β₁ AR (Miller-Gallacher et al., 2014) and δ opioid (Fenalti et al., 2014) receptors. The latter structure is shown in Figure 5A, highlighting the network of amino acid side chains (green) and coordinated waters (red) connecting the ligand binding pocket to the sodium ion (blue). This sodium site appears to collapse in active-state structures, suggesting that it plays a key role in constraining GPCRs in an inactive state (Wootten et al., 2013b; Katrich et al., 2014). Interestingly, the sodium site has also been recently implicated in the regulation of biased agonism by the δ opioid receptor (Fenalti et al., 2014), and in the actions of synthetic small molecule allosteric ligands for the μ opioid receptor (Livingston and Traynor, 2014). It should be noted, however, that a subset of Class A GPCRs do not possess the requisite acidic residue at the 2.50 position. These GPCRs include the visual opsins, which do not possess a diffusible endogenous activator, and a number of other atypical receptors whose functionality and/or ligands are not currently determined (Katrich et al., 2014).

**Zinc:**
Zinc is an important ion in the body that regulates the activity of many different proteins, and has been suggested to act as an allosteric modulator of a number of GPCRs, including the dopamine, melanocortin, adrenergic and opioid receptors (Schetz and Sibley, 1997; 2001; Holst et al., 2002; Lagerstrom et al., 2003; Swaminath et al., 2003; Tejwani and Hanissian, 1990; Rodriguez et al., 1992; Stengaard-Pedersen et al., 1981). Zinc inhibits orthosteric ligand binding at D₁, D₂L and D₄ dopamine receptors, MC1 and MC4 melanocortin receptors, β₂ and α₁A ARs, and μ, κ, and δ opioid receptors (OR) (Schetz and Sibley, 1997; 2001; Holst et al., 2002; Lagerstrom et al., 2003; Swaminath et al., 2002; Tejwani and Hanissian, 1990; Rodriguez et al., 1992; Stengaard-Pedersen et al., 1981). It increases the dissociation rate of antagonists from the D₁ and D₂L receptors (Schetz and Sibley, 1997; 2001), whereas it
decreases the dissociation rates of antagonists from the $\beta_2$- and $\alpha_{1A}$-ARs (Swaminath et al., 2002; Ciolek et al., 2011). Functionally, zinc potentiates the effects of agonists at the MC1, MC4 and $\beta_2$-AR toward cAMP accumulation, however, it negatively modulates the effects of agonists at the $\alpha_{1A}$-AR towards calcium signaling (Holst et al., 2002; Swaminath et al., 2002; Ciolek et al., 2011). Together these results show that zinc can act as an allosteric modulator of GPCRs, with positive or negative effects toward orthosteric ligand binding and function.

**Magnesium:**

Magnesium is another ion that has been implicated as an allosteric modulator at some GPCRs. Magnesium increases the specific binding of opioid receptor agonists and antagonists in a concentration-dependent manner (Pasternak et al., 1975; Rodriguez et al., 1992). Some differences are seen between the different opioid receptor subtypes, where for the $\mu$-OR and $\kappa$-OR, magnesium increases the affinity of agonist binding without changing the number of binding sites, respectively, whereas for the $\delta$-OR, the affinity for the agonist, DPDPE, was reduced with an increase in the Bmax (Rodriguez et al., 1992). For antagonists, the number of binding sites doubled in the presence of magnesium, without changing the affinity of the ligands for the ORs (Rodriguez et al., 1992). Agonist binding affinity for $\beta$-ARs (Williams et al., 1978) or dopamine D$_2$ receptors (Sibley and Creese, 1983) increases in the presence of magnesium, whereas no effect is observed for antagonist binding. It should be noted, however, that magnesium also plays a role in the receptor-G protein interaction, because it can also interact directly with the G protein itself (Birnbaumer and Zurita, 2010). Thus, some of the allosteric effects ascribed to magnesium in classical radioligand binding assays may be reflecting an indirect effect manifested at the level of the receptor-G protein interaction, rather than the receptor-ligand interaction. Nonetheless, a clear demonstration of
magnesium acting as an allosteric modulator via a well-defined allosteric site on the M₂ mAChR has been documented (Burgmer et al., 1998).

**Other ions:**

Several additional ions have been suggested to act as allosteric modulators of GPCRs, however, the evidence of a true allosteric modulation is limited. Binding of the μ-opioid receptor agonist, [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin, to guinea pig brain homogenates, and the β-adrenergic receptor agonist, hydroxybenzylisoproterenol, to frog erythrocyte membranes, increased in the presence of calcium or manganese (Williams et al., 1978; Rodriguez et al., 1992). Increasing the calcium concentration also affected the affinity and efficacy of orthosteric and allosteric ligands at the metabotropic glutamate 1a receptor (mGluR1a) (Jiang et al., 2014). Copper inhibits the binding of the antagonist, prazosin, in COS7 cells expressing human α₁A-ARs without altering the dissociation of prazosin from the receptor, and has complex effects on the binding and signaling of the endogenous agonist, epinephrine (Ciolek et al., 2011). Cobalt has also been reported to decrease the affinity of agonist binding to μ-ORs in guinea pig brain homogenates (Rodriguez et al., 1992).

Collectively, the aforementioned studies suggest that numerous ions may influence GPCRs allosterically. However, further work is required to conclusively establish whether or not ion-mediated changes in GPCR ligand affinity, apparent number of receptor binding sites and/or function are truly allosteric interactions and not due to perturbations in the osmolarity of the buffers used. Moreover, there remains a paucity of studies establishing that the concentrations of many of these ions required to mediate allosteric effects on GPCRs are routinely attained under physiological or pathophysiological conditions.
Lipids as allosteric modulators

Cholesterol:

Given that GPCRs are integral membrane proteins, it is not surprising that the lipid bilayer environment can markedly influence their behavior. In general, there are three ways by which this can occur. The first is through changes in the physical properties of the bilayer, for instance in terms of membrane fluidity, curvature or stress, such that the conformational landscape of a given GPCR is indirectly modulated. This has been best demonstrated in studies of rhodopsin, where the transition between the metarhodopsin I and II states can be substantially influenced by the physical properties of the membrane (Mitchell et al., 1990; Botelho et al., 2002; Soubias et al., 2010). The second mechanism by which lipids influence GPCR activity is via their ability to contribute to the subcellular compartmentalization of the receptor and associated effector molecules in highly ordered domains such as caveolae and lipid rafts (Chini and Parenti, 2004; Ostrom and Insel, 2004; Patel et al., 2008). The third mechanism is via a direct interaction of different lipid types with specific binding domains on the GPCR itself. In all cases, probably the best-studied example of a GPCR-modulating lipid is cholesterol (Paila and Chattopadhyay, 2010).

Cholesterol is an important constituent of lipid rafts, dense platforms that are found in lipid bilayers. The affinities of ligands binding to the oxytocin receptor, cholecystokinin receptor 1 (CCK1), mGluR1a and the 5-HT\textsubscript{1A} receptor are increased when these receptors are present in cholesterol-rich rafts compared to when they are expressed in cholesterol-depleted rafts, (Gimpl et al., 1997; Eroglu et al., 2003; Potter et al., 2012; Prasad et al., 2009). Restoring the cholesterol content of cholesterol-depleted membranes increased the number of receptors in the lipid rafts and restored the binding affinity of the ligands to the receptors, in the case of oxytocin and mGluR1a receptors (Gimpl et al., 1997; Eroglu et al., 2003). These results
suggest that the ligand binding properties of GPCRs can depend on the sterols and the lipid environment of the membrane. Cholesterol depletion has also been shown to affect receptor signaling, where decreases in calcium mobilization for the CCK1 receptor and extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylation and c-Fos activation for the gonadotrophin-releasing hormone receptor are lost, but can be restored upon replenishment of the cholesterol (Potter et al., 2012; Navratil et al., 2003). Similarly, depletion of membrane cholesterol content reduced μ OR signaling, whereas δ OR signaling was insensitive to the same manipulation (Levitt et al., 2009). Of note, the increases in agonist affinity for GPCRs with increased cholesterol content of membranes is not always observed; while the CCK1 receptor is sensitive to cholesterol content, the related CCK2 receptor is not (Potter et al., 2012). This suggests that cholesterol has some selectivity for different GPCRs.

Substantial interest in the potential for cholesterol to directly interact with GPCRs gained further impetus upon the solution of a 2.8Å structure of the β2 AR simultaneously bound to timolol and two molecules of cholesterol, and the identification that this binding site may contain a cholesterol a consensus motif (CCM) (Hanson et al., 2008). As shown in Figure 5B, this motif comprises three residues from transmembrane domain (TM) 4 and 1 aromatic residue from TM2. The most significant interaction is proposed to come from the highly conserved aromatic residue at position 4.50, which is a tryptophan in the majority of cases, and the other residues contribute additional interactions. At least 96 different GPCRs have been proposed to contain a CCM (Hanson et al., 2008), although it remains to be determined to what extent this is likely to truly constitute such a site for modulation by cholesterol. For example, both the CCK1 and CCK 2 receptors contain the CCM, yet only the CCK1 receptor is sensitive to cholesterol depletion (Potter et al., 2012). Furthermore, some GPCRs that
contain the CCM are able to be expressed and display binding and function in membranes that lack cholesterol, such as those of *E. Coli.* (Oates and Watts, 2011).

Given that cholesterol levels can vary dramatically in, and contribute directly, to disease (Ricker, 2014; Vance, 2012), their ability to influence GPCR biology may play role in pathophysiology. However, a definitive link between pathophysiological variations in membrane cholesterol and GPCR signaling remains to be determined.

**Endocannabinoids:**

Anandamide and 2-arachidonoylglycerol (2-AG) are the principal endogenous cannabinoid (CB) ligands found in the brain and periphery (Castillo et al. 2012). Although their cognate receptors are the CB₁ and CB₂ GPCRs, potential receptor-specific interactions have also been reported between these substances and other classes of GPCR. For example, anandamide has been shown to inhibit binding of ligands to central 5-HT receptors (Kimura et al., 1998) and mAChRs (Lagalwar et al., 1999). Effects on mAChRs were also evident in recombinant cell lines expressing the human M₁ (Christopoulos and Wilson, 2001; Lanzafame et al., 2004) or M₄ mAChR (Christopoulos and Wilson, 2001). More detailed studies indicated that the effects on the M₁ mAChR, at least, reflected the ability of anandamide to inhibit antagonist binding in a non competitive manner by decreasing the apparent number of mAChR binding sites without affecting antagonist affinity (Lanzafame et al., 2004). However, anandamide did not change the competition binding profiles of classical mAChR orthosteric agonists or allosteric modulators, but did affect agonist function in a manner correlated with agonist efficacy. This suggests that its actions are either via a novel allosteric site on the M₁ mAChR, or through a membrane-perturbing effect that is sensitive to receptor conformation (Lanzafame et al., 2004).
In a similar fashion, 2-AG has also been shown to modulate the binding of ligands to a non-cannabinoid GPCR. Specifically, the binding of both agonists and antagonists to the adenosine A3 receptor was inhibited by 2-AG in a non-competitive manner (Lane et al., 2010). Figure 4B illustrates the effects of increasing concentrations of 2-AG on the binding of the radiolabeled A3 agonist, [125I]AB-MECA, where it can be seen that most of the inhibition occurs over a very narrow concentration range that is not consistent with competition for a common binding site. Importantly, these effects of 2-AG were not observed at the related A1 or A2A adenosine receptors, suggesting a certain degree of specificity to the effect. Given that adenosine A3 and mAChRs are co-localized in similar brain regions as cannabinoid receptors, it is possible that elevated local levels of endocannabinoids may mediate hitherto unappreciated roles as endogenous negative allosteric modulators. The mechanism underlying this effect, however, remains unclear. For instance, it may reflect an interaction with a specific allosteric site on each receptor, or it may reflect cooperativity between orthosteric sites within an adenosine-mAChR heterodimeric complex.

**Lipoxin-A4:**

The arachidonic acid derivative, lipoxin A4, has recently been shown to induce cannabinoid-like responses in mouse brain that are antagonized by CB1 receptor antagonists, suggesting that lipoxin A4 acts at the CB1 receptor (Pamplona et al., 2012). The traditional biological target of lipoxin A4 is the formyl peptide receptor FPR1 (Chiang et al., 2006), leading to the possibility that the non-classic eicosanoid may be acting allosterically at the CB1 receptor. In agreement with the latter possibility, the same study found that lipoxina A4 can enhance the affinity of the agonists, anandamide, [3H]-CP55940 and [3H]-WIN55212-2, while partially
displacing antagonist binding (Pamplona et al., 2012). Allosteric effects of lipoxin A4 were also seen functionally, where co-injection of sub-effective doses of anadamide or 2-AG into mice brain, with lipoxin A4 potentiated the cataleptic effects of the endocannabinoids (Pamplona et al., 2012).

**Pregnenolone:**

A very recent study found that administration of Δ9-tetrahydrocannabinol (THC), the main active component of marijuana, to rats resulted in an increase in the levels of pregnenolone, which has traditionally been viewed as the inactive precursor to the generation of neurosteroids (Valle et al., 2014). Surprisingly, however, pregnenolone appeared to exert negative modulatory effects on the cannabinoid CB1 receptor through a potential allosteric mechanism. Although pregnenolone had no effect on agonist affinity, it inhibited CB1-mediated ERK1/2 phosphorylation and THC-induced changes in cellular and mitochondrial respiration. It also inhibited key in vivo effects on locomotion, temperature, catalepsy and analgesia commonly attributed to THC (Valle et al., 2014).

Taken together, many of the aforementioned studies indicate an unappreciated complexity in the cannabinoid system either as a source of GPCR lipidic modulators and/or a target for novel endogenous modulators. It is possible the overactivation of cannabinoid receptors, in particular, needs to be tightly regulated, thus providing a rationale for the identification of different putative endogenous negative allosteric modulators (NAMs). However, it should also be noted that further experimental studies are required to validate and extend these intriguing results, but may yield rich opportunities for novel therapeutic interventions.
**Progesterone:**

Similar to observations with anandamide at the mAChRs, the steroid hormone, progesterone, inhibited agonist and antagonist binding at the recombinantly expressed rat oxytocin receptor cells by reducing the maximal binding capacity without changing apparent ligand affinity (Grazzini et al., 1998). Functionally, progesterone inhibited oxytocin-promoted inositol phosphate accumulation and calcium responses at the rat oxytocin receptor in a concentration-dependent manner (Grazzini et al., 1998). The effects of progesterone appear to be species-specific, since progesterone did not compete for [3H]-oxytocin binding to the human oxytocin receptor (Grazzini et al., 1998). However, the progesterone metabolite 5β-dihydroxyprogesterone, did inhibit agonist binding to the human oxytocin receptor (Grazzini et al., 1998). Likewise, in functional assays, inositol phosphate accumulation was inhibited only by 5β-dihydroxyprogesterone and not progesterone at the human oxytocin receptor (Grazzini et al., 1998)

**Oleamide:**

Oleamide is an endogenous fatty acid amide found in cerebrospinal fluid that plays a role in sleep regulation. Its mechanism of action remains unclear, but is likely to involve multiple neurotransmitter systems, in particular agonistic activity at CB receptors and inhibition of fatty acid amide hydrolase (Leggett et al., 2004). However, allosteric effects of the substance have also been reported at various 5-HT receptor subtypes. For instance, at the 5-HT2A receptor, oleamide potentiated the effects of agonist-stimulated IP turnover in rat P11 cells without having an effect if applied alone (Thomas et al., 1997; 1998). In HeLa cells transfected with 5-HT7 receptors, oleamide or 5-HT each increased cAMP accumulation,
however, when they were co-applied, a reduction was observed in 5-HT-promoted cAMP responses, suggesting an allosteric interaction (Thomas et al., 1997). Direct negative cooperativity between oleamide and 5-HT was subsequently demonstrated in radioligand binding assays at the 5-HT$_7$ receptor (Hedlund et al., 1999). In Xenopus oocytes expressing 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors, oleamide potentiated 5-HT-promoted chloride channel currents (Huidobro-Toro and Harris, 1996).

**Amino acids and peptides as allosteric modulators**

**Amino acids:**

In addition to their obligate roles in the synthesis of proteins or other major biomolecules, some of the 20 eukaryotic amino acids have also been identified as putative endogenous allosteric modulators of specific GPCRs. Arguably the best-validated example is that of aromatic amino acids, such as L-Phe, L-Trp and L-Tyr, that allosterically modulate the actions of extracellular calcium ([Ca$^{2+}$]$_o$) at the CaSR (Conigrave et al., 2000). These amino acids bind in the venus flytrap domain, near the orthosteric site, to potentiate the actions of [Ca$^{2+}$]$_o$ at a variety of intracellular pathways (Conigrave et al., 2000; Mun et al., 2004; Zhang et al., 2002), in addition to enhancing [Ca$^{2+}$]$_o$-dependent suppression of parathyroid hormone (PTH) secretion in parathyroid cells (Lee et al., 2007). Figure 4C illustrates an example of the effects of L-Phe on intracellular calcium mobilization in response to the endogenous agonist. Although the potentiating effects of amino acids on CaSR signaling are relatively subtle, they are nonetheless likely to be physiologically relevant, as small degrees of positive allosteric modulation of the CaSR by synthetic modulators acting in the receptor’s transmembrane domain (e.g., Davey et al., 2012) are known to be clinically relevant (Block et al., 2004; Saidak et al., 2009).
The aromatic amino acid, L-Phe, as well as the aliphatic L-Leu and L-Ile, have also been suggested to allosterically modulate the properties of baclofen at the GABA$_B$ receptor (Kerr et al., 2003), although the significance of this effect remains unclear since no modulation by these amino acids of the endogenous agonist, GABA, could be detected in either native or recombinant systems (Urwyler et al., 2004).

**Amino acid metabolites:**

Some of the best-characterized orthosteric neurotransmitters and hormones, such as serotonin, dopamine, nor/epinephrine, GABA and nucleotides, are biosynthesized from amino acids. However, other amino acid metabolites have been suggested to act as allosteric modulators. For example, the methionine metabolite, homocysteine, has been proposed to interact with the third extracellular loop of the dopamine D$_2$ receptor to negatively modulate the binding of dopamine, whilst exhibiting neutral allosteric properties against the antagonist raclopride (Agnati et al., 2006). It is acknowledged that increased plasma levels of homocysteine are strong indicators of neurological disorders and could therefore play a detrimental role in various diseases including, dementia, Alzheimer’s, Huntington’s, and Parkinson’s diseases (Andrich et al., 2004; Seshari et al., 2002; Morris et al., 2003; Muller et al., 2008). Another notable amino acid metabolite, agmatine (decarboxylated arginine) positively modulates norepinephrine-mediated inhibition of norepinephrine release at the $\alpha_2$-adrenergic receptor, although multiple modes of interaction involving both the orthosteric and an allosteric site on this receptor have also been proposed (Molderings et al., 2000). Biosynthesis of agmatine, and subsequent activation of $\alpha_2$-adrenergic receptor, may have cardiovascular protective effects, as well as anti–convulsant, –neurotoxic, and –depressant actions (Piletz et al., 2013).
**Small peptides:**

The antioxidant glutathione (\(\gamma\)-L-glutamyl-l-cysteinyl glycine), synthesized predominantly by the liver, is the most abundant thiol present in mammalian cells (up to 10 mM; Meister, 1988). Glutathione has recently also been identified as an endogenous allosteric modulator of the CaSR, acting in a similar location and manner as the aromatic amino acids to positively modulate the functional effects of \([\text{Ca}^{2+}]_o\) (Wang et al., 2006; Broadhead et al., 2011). Glutathione could potentially be of value in the physiological suppression of high PTH levels in various forms of hyperparathyroidism. Another brain-derived tri–peptide of interest is LPG (Leu–Pro–Gly), which has also been referred to melanotrophin release inhibiting factor or melanocyte-stimulating hormone release-inhibiting factor-1 (Horvath and Kastin, 1990). LPG appears to be a positive allosteric modulator of the binding and function of agonists at the dopamine D\(_2\) receptor (Bhargava et al., 1983; Johnson et al., 1986; Ott et al., 1996; Mishra et al., 1999). Because enhanced sensitivity of dopamine receptors to endogenously released dopamine may effectively compensate for partially degenerating dopaminergic terminals in Parkinson’s disease, the pursuit of positive allosteric modulators of these receptors remains of significant interest. A third endogenous peptide with potential neurological benefits is 5-HT–moduline (Leu–Ser–Ala–Leu). 5-HT–moduline is released in various parts of the brain, particularly under conditions of stress (Fillion, 2000). Interestingly, it is a NAM of serotonin binding (Rouselle et al., 1996), and of agonist–induced synaptosomal activity (Massot et al., 1996) at the 5-HT\(_{1B/1D}\) receptors, acting therefore as a potential protective agent against depression (Fillion et al., 2000).

**Large peptides and proteins:**

Figure 4D shows the effects of pepcan-12, a 12 amino acid neuropeptide that is highly expressed in mouse brain (Gomes et al., 2009), on the binding of the orthosteric radioligand
agonist, $[^3]H$WIN55212-2 ($[^3]H$ ((R)-(+)\-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone), at the cannabinoid CB$_1$ receptor. In contrast to the complete inhibition of radioligand binding mediated by the orthosteric agonist, CP55,940 (2-\([(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)\] cyclohexyl\]-5-(2-methyloctan-2-yl)phenol), pepcan-12 only partially inhibited specific radioligand binding at saturating concentrations, consistent with limited negative cooperativity (Bauer et al., 2012). This negative allosteric effect on CB$_1$ agonist affinity is also manifested in functional studies of receptor signalling (Bauer et al., 2012). Negative allosteric modulation of the CB$_1$ receptor may have beneficial effects in pathological states, such as obesity and type 2 diabetes, while avoiding on-target depression-related side effects often attributed to classic CB$_1$ antagonists (de Kloet et al., 2009).

The M$_2$ mAChR is the first GPCR for which synthetic allosteric modulators were identified (Lullman et al., 1969; Clark and Mitchelson, 1976). Structural studies have established a key role for positively charged modulators to interact with a well-defined allosteric site in the extracellular vestibule of these receptors (Dror et al., 2013). It is interesting, therefore, that endogenous, arginine-rich, peptides were previously speculated to act as natural negative allosteric modulators of this receptor. For example, protamine negatively modulates the binding of radiolabeled orthosteric antagonists at cardiac M$_2$ mAChRs with many of the hallmarks associated with extracellular small molecule allosteric modulators (Hu et al., 1992). However, given that this protein is not secreted into the extracellular space, the specific modulatory effect at the M$_2$ mAChR may not be physiologically relevant (Balhorn, 2007). In contrast, dynorphin-A (1-13) and myelin basic protein display similar allosteric actions to protamine at this receptor (Hu et al., 1993), and they can be found extracellularly. Dynorphin-A, an endogenous opioid peptide, is widely expressed in the CNS, and myelin
basic protein is a major component of nerve myelin (Gupta, 1987). High expression of these two proteins may induce a dampening effect of central M2 mACHRs thus modifying the negative feedback effect that these auto-receptors normally play in the process of controlling neuronal ACh release.

The M2 mACHR is also highly expressed in the periphery, particularly in the post-ganglionic nerves in the airways where its autoreceptor activity plays a central role in various airway diseases (Barnes et al., 1988). A hallmark of asthma is the infiltration of eosinophils to the site of inflammation (Gleich et al., 1995) and their degranulation to release major basic protein, a highly basic, arginine-rich protein, with a primary role in activating mast cells and neutrophils (Rosenberg et al., 2013). It has been proposed that in asthmatics, neuronal M2 mACHRs are dysfunctional predominantly due to the presence of major basic protein in the neuromuscular junction (Jacoby et al., 1998). This pro-inflammatory protein is able to bind with micromolar affinity to the M2 mACHR and allosterically modifies the binding properties of the receptor (Jacoby et al., 1993). This is of particular relevance, as high micromolar concentrations of major basic protein are found in the sputum of asthmatics, with possibly even higher concentrations in surrounding tissues (Frigas et al., 1981; Fryer et al., 1992).

**Allosteric autoantibodies**

Over the last two decades, a number of chronic diseases have been associated with the presence of circulating autoantibodies, either as initiators or amplifiers of the disease. A common feature is that most GPCR–directed autoantibodies bind to parts of extracellular domains of the receptors, often in an allosteric mode. Whilst most autoantibodies have been characterized for their ability to induce agonist-like activity at their target GPCR, a small number have also been classified as antagonists, inhibiting the GPCR signaling processes
(Table 1). Interestingly, some autoantibodies show very specific, non-canonical GPCR activity, ranging from permanent agonist-like activity, such as for the thyroid-stimulating hormone receptor (TSHR) in hyperthyroidism from Grave’s disease (Kendall-Taylor et al., 1975), to a complete inability to desensitize their target receptors, as for the β1-AR, M2 mAChR or even angiotensin 1 (AT1) receptors in myocarditis, and dilated cardiomyopathy (Wallukat et al., 1991a and b, Wallukat et al., 1999; Xia et al., 2013). It should be noted, however, that the majority of these studies to date have not delved into the precise allosteric mechanisms mediating the GPCR-autoantibody interaction.

**Autoantibodies targeting the central & peripheral nervous system:**

It is now becoming evident that immune responses affecting neurons of the central or peripheral nervous system can result in a broad spectrum of neurological syndromes ranging from encephalitis to Alzheimer’s disease and schizophrenia (van Coevorden-Hameete et al., 2014). The last few years have seen the emergence of several lines of evidence suggesting that GPCR–directed autoantibodies are indeed involved in an ever increasing number of neurological disorders (Figure 6). For instance, the sera or the cerebrospinal fluid (CSF) of Hodgkin’s lymphoma, lung cancer, ovarian cancer, and breast cancer patients with paraneoplastic cerebellar ataxia contain antibodies that react with mGluR1 (Sillevis Smitt et al., 2000; Coesmans et al. 2003). The CSF of patients with limbic encephalitis contains autoantibodies against GABA B receptors (Lancaster et al. 2010; Boronat et al., 2011), whilst sera from patients with basal ganglia encephalitis with dominant movements and psychiatric disorders contains autoantibodies against the dopamine D2 receptor (Dale et al., 2012). The pathogenesis of debilitating disorders such as schizophrenia may in some instances involve a repertoire of autoantibodies specifically targeting both M1 and M2 mAChRs (Borda et al. 2004; Jones et al., 2014). Most of these GPCR–directed autoantibodies have recently been
isolated from patients but remain to be fully characterized in terms of binding epitope and pharmacological activity.

**Autoantibodies targeting the cardiovascular system:**

Arguably, GPCR-directed autoantibodies have been better studied with regards to their contributions to cardiovascular diseases (Wallukat et al., 2014; Unal et al., 2012). One of the major GPCR targets for autoantibodies in vascular diseases is the AT$_1$ receptor. Antibodies against the AT$_1$ receptor have been found in patients with renal transplant rejections associated with vascular pathology (Dragun et al., 2005; Dragun et al., 2007), in pre-eclamptic women (Wallukat et al., 1999; Xia et al., 2013; Herse et al. 2013), in hypertensives (Fu et al., 2000; Xia et al., 2013), as well as in systemic sclerotic patients (Riemekasten et al., 2011). In all cases, these autoantibodies were able to induce agonist-like activity at the AT$_1$ receptor, promoting chronic activation. Some cardiovascular diseases generate a highly heterogeneous antibody repertoire, such as hypertension, and systemic sclerosis. For example, some patients with hypertension have also shown high levels of antibodies targeting the $\alpha_1$-AR (Fu et al., 1994; Luther et al., 1997; Wenzel et al., 2008), whilst patients suffering systemic sclerosis may be positive for antibodies against the endothelin ET$_A$ receptor (Kill et al., 2014; Becker et al., 2014). Additionally, 5-HT$_4$ receptor autoantibodies were found in the sera of some patients with systemic lupus erythematosus (Eftekharis et al., 2000). This subtype of serotonin receptor plays a critical role in regulating atrial arrhythmias in the developing heart, explaining why children of women with 5-HT$_4$ receptor autoantibodies develop neonatal lupus-induced congenital heart block. Finally, among the cardiovascular diseases associated with GPCR-directed autoantibodies, cardiomyopathy is a common pathology (Wallukat et al., 2014). Antibodies for two specific cardiac GPCRs, the $\beta_1$ AR and the M$_2$ mAChR are a common feature. Immunoglobulins
(IgGs) targeting and activating either or even both receptors simultaneously (Elies et al., 1998) have been isolated in patients with idiopathic cardiomyopathy (Wallukat et al., 1987; Fu et al., 1993), peripartum cardiomyopathy (Warraich et al., 2005; Stavrakis et al., 2009; Stavrakis et al., 2011; Liu et al., 2014), and in Chagas-induced cardiomyopathy (Sterin-Broda et al., 1976; Borda et al., 1984; Elies et al., 1996; Wallukat et al., 2010; Munoz Saravia et al., 2010).

**Autoantibodies in other systemic disorders:**

In addition to nervous and cardiovascular system disorders, autoimmune thyroid disease is another established example involving GPCR-directed autoantibodies (Rapoport et al., 1998). In Grave’s disease, the autoimmune pathogenesis is well characterized and attributed to autoantibodies able to bind the ectodomain of the TSHR, and either chronically activating it to cause hyperthyroidism (the most common form of Grave’s disease; Orgiazzi et al., 1976), or inactivating it, causing hypothyroidism (Endo et al., 1978). Recently, neutral TSHR-directed antibodies have also been identified (Morshed et al., 2010). Some patients can swing between hyper and hypothyroidism depending on the levels of stimulating or blocking TSHR autoantibodies (McLachlan et al., 2013). In the sera of patients suffering from Sjögren’s syndrome, a systemic autoimmune disease characterized by the severe impairment of salivary and lacrimal secretions, M₃ mAChR autoantibodies have been identified (Bacman et al., 1998). Interestingly, these antibodies, which are directed toward the ECL3 domain of the M₃ mAChR (Koo et al., 2008), exhibit antagonist-like activity (Jin et al., 2011). In the sera of complex regional pain syndrome patients, the presence of autoantibodies with agonist-like activity against the β₂-AR and/or the M₂ mAChR has also been identified (Kohr et al., 2011). Upon targeting the ECL2 of these receptors, the autoantibodies can stimulate the release of intracellular calcium. Recently, a blocking
autoantibody against the CaSR has been isolated and reported to cause acquired hypocalciuric hypercalcemia (AHH) (Kifor et al., 2003). The autoantibody itself is able to inhibit the calcium-mediated signaling pathways of the CaSR, reducing IP accumulation as well as ERK1/2 phosphorylation. In contrast, a different autoantibody was identified in a patient suffering from AHH that displayed biased allosteric modulation, namely a potentiation of Gq-mediated IP accumulation but inhibition of Gi-mediated ERK1/2 phosphorylation (Makita et al., 2007). Patients suffering autoimmune polyendocrine syndrome type 1 have also shown high levels of CaSR-directed autoantibodies that activate, rather than inhibit, the receptor (Gavalas et al., 2007; Kemp et al., 2009). In patients with allergic asthma, autoantibodies showing antagonist-like activity at the β2-adrenoceptor have been isolated (Turki et al., 1995). Finally, in Chagas’ disease, digestive manifestations sometimes occur leading to a syndrome called megaviscera (Haberland et al., 2013). Autoantibodies isolated from Chagasic patients with megacolon are able to bind and activate the M2 mAChR, increasing the basal tone of the colon, as well as inhibiting intracellular cAMP signaling (Sterin-Borda et al., 2001).

Concluding remarks

The study of endogenous allosteric modulators of GPCRs represents a burgeoning field with some provocative findings that, in many instances, require further validation. Nonetheless, the concept is important and raises a number of issues. The first, as alluded to in the Introduction, is whether there should be an expectation that most GPCR allosteric sites represent orphan sites for hitherto unidentified modulators. This is probably not the case except in the broadest sense, i.e., GPCRs are structurally similar, yet highly dynamic in the types of substances that they interact with; as highlighted in Figure 1A, an orthosteric domain on one type of GPCR can be serendipitously exploited as an allosteric site on another type of
GPCR. However, as noted for other receptor superfamilies, there are examples of endogenous substances that indeed appear to play key roles in allosteric modulation of some GPCRs. These types of observations raise a second important issue for future studies, namely, the validation and classification of endogenous allosteric substances. Short of direct structure determination, there are a number of key experimental criteria required to demonstrate an allosteric effect, including validation of potential saturability in effect, probe dependence and, ideally, thermodynamic reciprocity (Christopoulos et al., 2014); in many of the studies performed to date, these properties have not been exhaustively considered for putative endogenous GPCR allosteric modulators. Even if experimentally well-validated, consideration is required with respect to terminology. If an endogenous substance meets the classic criteria for an allosteric modulator, it may be classified as such, but if it also is shown to demonstrate agonism or inverse agonism in its own right, it is possible that the substance should be re-classified as another orthosteric ligand of the same receptor. As outlined recently (Christopoulos et al., 2014), there is no reason why multiple orthosteric ligands cannot interact allosterically at a given receptor. In this instance, the ligands are orthosteric, but the interaction is allosteric.

Going forward, it will also be important to understand the role of validated endogenous allosteric GPCR modulators in both physiology and disease. The presence of receptor-specific endogenous modulators likely points to a need for particularly tight regulation of certain GPCRs, as exemplified, for instance, by the CaSR. The current high incidence of lipidic substances as putative endogenous GPCR modulators adds to the growing body of evidence for lipids as under-appreciated bioactive molecules in GPCR biology. Similarly, changes in the extracellular milieu as a consequence of inflammatory processes likely introduce a plethora of substances (peptides, lipids and others) that could otherwise go...
undetected as potential allosteric modulators. It is possible that the study of inflammation represents fertile ground for further breakthroughs in GPCR allostery, given its association with many diseases including neurological, cardiovascular, respiratory, metabolic and oncogenic. Indeed, the increasing identification of GPCR-directed allosteric autoantibodies is in line with this notion. Although most current drug discovery programs are pursuing positive or negative allosteric modulators as therapeutic agents, it is possible that NALs can find a niche if it can be demonstrated that a given pathophysiological state is mediated by aberrant endogenous allosteric modulation; in this case, the NAL could be used to block the binding of the endogenous allosteric modulator while sparing the binding and signaling of the endogenous orthosteric agonist. It is acknowledged that many of these considerations currently remain theoretical but, as has been proven repeatedly over the last decade and a half, the study of allosteric modulation of GPCRs continues to yield new insights and exciting opportunities for improving human health.
Authorship Contributions

Wrote or contributed to the writing of the manuscript:

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Footnotes

*These authors contributed equally to this work.

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Figure Legends

Figure 1  (A) General modes of ligand binding to GPCRs. A domain associated with binding of an orthosteric ligand at one type of GPCR may be exploited as an allosteric site at another type of GPCR  (B) GPCR allosteric sites may arise ‘serendipitously’ as a consequence of favorable structural features upon folding and membrane insertion, or may represent ‘orphan’ sites for hitherto unappreciated endogenous modulators.

Figure 2  Allosteric effects on agonist binding due to GPCR-protein ternary complex formation.  (A) Receptor-G protein interaction. Effects of increasing concentrations of GTP on the affinity of the agonist, carbachol, at atrial M2 mAChRs. Data replotted from (Ehlert, 1985).  (B) Effects of different arrestin proteins on the binding of the agonist, isoproterenol, to the β2 adrenoceptor. Data replotted from Gurevich et al., (1997).

Figure 3  Crystal structure of the N-terminal region of CLR in complex with the accessory protein, RAMP1, and the anti-migraine drug, olcegepant. PDB: 3N7S.

Figure 4  Endogenous allosteric GPCR modulators.  (A) Effects of sodium on the binding of an agonist (epitepride) and antagonist (quinpirole) at the dopamine D2 receptor. Replotted from Neve (1991).  (B) Inhibition of the binding of the orthosteric agonist, $[^{125}\text{I}]$AB-MECA, by the endocannaboid, 2-arachidonyl glycerol (2-AG), at the adenosine A3 receptor. Replotted from Lane et al. (2009).  (C) Potentiation of the effects of calcium by the amino acid, L-Phe, at the CaSR. Replotted from Lee et al., (2007).  (D) Effects on the binding of the cannabinoid agonist, $[^{3}\text{H}]$WIN55,212-2 by an exogenous orthosteric agonist,
CP55,940, or an endogenous allosteric peptide, pepcan-12, at the cannabinoid CB₁ receptor. Replotted from Bauer et al. (2012).

Figure 5  Structural insights into endogenous GPCR modulators.  (A) High resolution crystal structure of the δ opioid receptor (δ OR) in complex with the antagonist, naltrindole. The sodium ion is shown in blue, the network of coordinated water molecules connecting the orthosteric site and the ion are indicated as CPK, and the key amino acid side chains are indicated in green ball and stick. PDB: 4N6H.  (B) Crystal structure of the β₂ adrenoceptor in complex with the inverse agonist, timolol, and two cholesterol molecules (PDB: 3D4S). A putative cholesterol consensus motif that is conserved across a range of GPCRs is highlighted in orange.

Figure 6  Examples of central nervous system and cardiovascular disorders mediated by endogenous autoantibodies targeting various GPCRs.
### Table 1  Selected examples of putative endogenous GPCR allosteric modulators

<table>
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<tr>
<th>Modulator</th>
<th>Targets</th>
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<tr>
<td><strong>Ions</strong></td>
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<tr>
<td>Na⁺</td>
<td>Various</td>
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<tr>
<td>Zn²⁺</td>
<td>Various</td>
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<tr>
<td>Mg²⁺</td>
<td>(\mu)-OR, (\kappa)-OR, (\beta_1)-AR</td>
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<tr>
<td>Mn²⁺</td>
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<td>CaSR</td>
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<td>(\alpha_{2D})-AR</td>
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<td>Major Basic Protein</td>
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<td>Receptors/Receptor Sites</td>
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<tr>
<td>IgG</td>
<td>5-HT&lt;sub&gt;4&lt;/sub&gt;R, α&lt;sub&gt;1&lt;/sub&gt;-AR, AT&lt;sub&gt;1&lt;/sub&gt;R, β&lt;sub&gt;1&lt;/sub&gt;-AR, β&lt;sub&gt;2&lt;/sub&gt;-AR, CaSR, D&lt;sub&gt;2&lt;/sub&gt;R, ET&lt;sub&gt;3&lt;/sub&gt;R, GABA&lt;sub&gt;B&lt;/sub&gt;R, M&lt;sub&gt;1&lt;/sub&gt; mAChR, M&lt;sub&gt;2&lt;/sub&gt; mAChR, M&lt;sub&gt;3&lt;/sub&gt; mAChR, mGluR&lt;sub&gt;1&lt;/sub&gt;, mGluR&lt;sub&gt;5&lt;/sub&gt;, TSH-R</td>
</tr>
</tbody>
</table>
**FIGURE 2**

A. 

![Graph showing the effect of GTP concentration on [3H]NMS binding](image1)

- [GTP] (μM):
  - 0
  - 0.1
  - 0.3
  - 1
  - 10
  - 100

- Log [Carbachol]

B. 

![Graph showing the effect of Arrestin concentration on [125I]Pindolol binding](image2)

- Control
- Arrestin 1 (1 μM)
- Arrestin 2 (1 μM)
- Arrestin 3 (300 nM)

- Log [ISO]
**Fig. 4**

**A**
- Graph showing the binding of different inhibitors as a function of log [Inhibitor] (M).
- Symbols: ○ Epinephrine, □ Quinpirole, ■ + Na^+.

**B**
- Graph showing the % specific binding of [125I]AB-MECA as a function of log [2-AG] (M).

**C**
- Graph showing the free Ca^2+ concentration in the cytoplasmic space as a function of extracellular Ca^2+ concentration (mM).
- L-Phe (10 mM) and Vehicle conditions.

**D**
- Graph showing the % of [3H]WIN55,212-2 binding as a function of log [Compound] (M).
- Symbols: ○ CP55,940, □ Pepcan-12.