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Commentary

"Phenotypic" pharmacology: The influence of cellular environment on G protein-coupled receptor antagonist and inverse agonist pharmacology

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ABSTRACT

A central dogma of G protein-coupled receptor (GPCR) pharmacology has been the concept that unlike agonists, antagonist ligands display equivalent affinities for a given receptor, regardless of the cellular environment in which the affinity is assayed. Indeed, the widespread use of antagonist pharmacology in the classification of receptor expression profiles *in vivo* has relied upon this 'antagonist assumption'. However, emerging evidence suggests that the same gene-product may exhibit different antagonist pharmacological profiles, depending upon the cellular context in which it is expressed—so-called 'phenotypic' profiles. In this commentary, we review the evidence relating to some specific examples, focusing on adrenergic and muscarinic acetylcholine receptor systems, where GPCR antagonist/inverse agonist pharmacology has been demonstrated to be cell- or tissue-dependent, before going on to examine some of the ways in which the cellular environment might modulate receptor pharmacology. In the majority of cases, the cellular factors responsible for generating phenotypic profiles are unknown, but there is substantial evidence that factors, including post-transcriptional modifications, receptor oligomerization and constitutive receptor activity, can influence GPCR pharmacology and these concepts are discussed in relation to antagonist phenotypic profiles. A better molecular understanding of the impact of cell background on GPCR antagonist pharmacology is likely to provide previously unrealized opportunities to achieve greater specificity in new drug discovery candidates.

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Abbreviations: *ADP(S, adenosine 5′-O-(2-thiotriphosphate); BPH, benign prostatic hyperplasia; CGP 12177A, (±)-4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazole-2-one; CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; CNS, central nervous system; CRLR, calcitonin receptor-like receptor; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; mACh, muscarinic acetylcholine; MAP kinase, mitogen-activated protein kinase; mRNA, messenger RNA; NECA, 5′-N-ethylcarboxamidoadenosine; OAB, overactive bladder; pFHHSiD, para-fluorohexahydrosiladifenidol; PTx, pertussis toxin; RAMP, receptor activity-modifying proteins; SNP, single nucleotide polymorphism*

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1. Introduction

It has been recognized for many years that the properties of potency and efficacy are dependent not only on the intrinsic properties of the agonist in question, but also on the combined effect of a myriad of cellular factors (including receptor and G protein expression levels and downstream stimulus-response coupling mechanisms), such that observed values of potency and efficacy can vary depending on the cellular environment in which the response is investigated [1,2]. In contrast, the functional affinities of antagonist ligands are usually assumed to be constant for a given receptor subtype, regardless of the cell background in which the receptor is expressed; this has been referred to as the 'antagonist assumption' [3]. As a consequence, differences in antagonist affinity estimates between tissues have been interpreted as representing different receptor populations (e.g. [4]). However, the traditional view that the host cell provides an inert context for GPCR expression has been challenged by the observation of differing pharmacological profiles for the same gene-product in different recombinant cell systems (so-called 'phenotypic' profiles; see [5]).

A number of examples of GPCRs exhibiting 'atypical' pharmacological profiles have been reported, including the α_{1L}-adrenoceptor phenotype [6], where anomalous functional affinity estimates for antagonists in native tissues play a significant role in defining these 'phenotypic' profiles. In addition, there is significant evidence that certain muscarinic acetylcholine (mACh) receptor antagonists used in the management of overactive bladder (OAB), such as darifenacin, tolterodine and oxybutynin, display both *in vitro* [7–9] and *in vivo* [10–12] selectivity for the inhibition of mACh receptor-mediated urinary bladder smooth muscle contraction relative to salivary secretion, effects believed to be mediated by the same M_3 mACh receptor subtype.

A crucial development in our understanding of GPCR function has been an appreciation of their ability to activate cognate G proteins in the absence of agonist binding (termed 'constitutive' receptor activity) [13]. This has been accompanied by the identification of ligands (termed 'inverse agonists'), often previously characterized as competitive antagonists, which can reduce such constitutive receptor activity (i.e. display 'negative efficacy') [13]. Since efficacy can encompass both positive and negative values, it might be anticipated that many of the factors understood to influence the potency and efficacy of agonist (positive efficacy) ligands would also apply to inverse agonists (ligands displaying negative efficacy). Since the majority of ligands previously categorized as antagonists have now been reported to possess some degree of negative efficacy [14], we believe that the assumption that 'antagonist' pharmacology is independent of the cellular environment requires re-examination. This may be of particular importance in the drug discovery process, where the expression of GPCR targets in the appropriate cellular environment may be crucial in correctly identifying candidate antagonist/inverse agonist ligands. In this commentary, we examine the evidence that the pharmacology of GPCR antagonist (or inverse agonist) ligands can be influenced by the cellular milieu, addressing specific examples in the literature where this has been reported, and discuss the potential molecular bases for these phenomena.

2. Atypical β-adrenoceptors

2.1. *The β_4-adrenoceptor as an alternative 'state' of the β_1-adrenoceptor*

The realization that the putative 'β_4-adrenoceptor' is in fact an alternative conformation of the β_1-adrenoceptor gene product has been excellently reviewed elsewhere [15–17] and so will not be re-examined in detail in the present article. Briefly, a third cardiostimulant β-adrenoceptor was originally described by Kaumann [18] and later termed the 'β_4-adrenoceptor' [19]. Activation by 'non-conventional partial agonists' (e.g. CGP 12177A) and resistance to classical β_1 and β_2-adrenoceptor antagonists defined this novel β-adrenoceptor phenotype [18]. The failure to identify any further β-adrenoceptor genes beyond the β_1-, β_2- and β_3-adrenoceptors suggested that one or more of the cloned β-adrenoceptors must generate the putative 'β_4' phenotype. The importance of the β_1-adrenoceptor in the observation of β_4-adrenoceptor pharmacology was suggested by pharmacological studies [20–22], but it was the use of knockout mice lacking combinations of β-adrenoceptor subtypes [22,23] that provided conclusive proof that the 'β_4-adrenoceptor' is an alternative conformational state of the β_1-adrenoceptor.

2.2. *Alternative conformational states of other β-adrenoceptor subtypes?*

Recent evidence suggests that the existence of multiple active conformational states may not be limited to the β_1-adrenoceptor subtype amongst the β-adrenoceptor family. Although the β_2-adrenoceptor does not appear to adopt high and low affinity active states in the way that has been demonstrated for the β_1-adrenoceptor, efficacious agonists have been found to induce changes in antagonist affinity estimates (by up to 10-fold), in an efficacy- and time-dependent manner, via an agonist-mediated phosphorylation of the β_2-adrenoceptor [24]. In addition, ligands have been identified at the β_2-adrenoceptor that are capable of inducing a direct recruitment of arrestin proteins and the downstream activation of the p42/44 MAP kinase pathway, whilst simultaneously acting as inverse agonists through the classical $G_s\alpha$-coupled pathway [25,26].

For the β_3-adrenoceptor, differences in agonist potency and affinity have been observed between intact cell and membrane homogenate assays, leading Arch [27] to propose that the β_3-adrenoceptor may exist in distinct conformations in intact cells compared to membranes. Further to this, Baker [28] investigated the ability of antagonists to inhibit β_3-adrenoceptor-mediated responses to a large number of agonists displaying a wide range of efficacies and concluded that the β_3-adrenoceptor was capable of adopting at least 2 distinct 'active' (i.e. agonist-bound) conformational states. However, Baker [28] found that the choice of agonist dictated the conformational state of the receptor and not whether the receptor is stimulated in intact cells or

membrane homogenates, as proposed by Arch [27]. In contrast to the β_2-adrenoceptor, this was shown to be independent of both agonist efficacy and time of incubation, suggesting that the β_3-adrenoceptor displays a pharmacological profile that is similar to the β_1-adrenoceptor, only with less pronounced differences in antagonist affinity estimates (4–50-fold at the β_3-adrenoceptor, compared with 30–1000-fold at the β_1-adrenoceptor) [28,29]. A full understanding of both the molecular basis and the physiological significance of these intriguing examples of pharmacological pleiotropism remains the challenge for future research in this field.

3. The α_{1L}-adrenoceptor

It is now widely accepted that three subtypes (termed α_{1A}, α_{1B} and α_{1D}) of the α_1-adrenoceptor exist with further heterogeneity resulting from the alternative splicing of the α_{1A}-adrenoceptor (see [30]). Despite the failure to identify additional α_1-adrenoceptor genes, significant evidence has accrued that the α_1-adrenoceptor phenotype observed in numerous tissues in a wide variety of species does not correlate with any of the cloned α_1-adrenoceptors. One pharmacological characteristic common to these 'atypical' phenotypes is an anomalously low affinity for the α_1-selective antagonist prazosin. This led Flavahan and Vanhoutte [31] to propose an alternative classification of α_1-adrenoceptors, dividing them into two groups: those that display high affinity for the α_1-adrenoceptor antagonists prazosin and yohimbine (termed α_{1H}-adrenoceptors) and those that exhibit low affinity for these ligands (termed α_{1L}-adrenoceptors). It has since been proposed that the three cloned α_1- (α_{1A}-, α_{1B}- and α_{1D}-) adrenoceptors may be considered as sub-divisions of the α_{1H} class (as all exhibit high affinity for prazosin) (see [32]). In contrast, α_1-adrenoceptors exhibiting anomalously low affinities for certain antagonists (including prazosin) in functional studies have been observed in a number of tissues (see Table 1), These 'atypical' α_1-adrenoceptor pharmacologies are now classified as α_{1L} phenotypes, but the molecular basis for these receptors has remained elusive as a result of the failure to identify cDNA corresponding to this novel phenotype.

Table 1 – Distribution of α_{1L}-adrenoceptor phenotypes

Species	Tissue	References
Rat	Mesenteric arteries	[33,34]
	Vas deferens	[35]
	Prostate	[36]
Rabbit	Thoracic aorta	[37]
	Iris	[38]
	Liver	[39]
	Mesenteric arteries	[37]
	Carotid artery	[37]
	Prostate	[40]
Guinea-pig	Thoracic aorta	[37]
Dog	Subcutaneous resistance arteries	[41]
Human	Vas deferens	[42]
	Prostate	[43]

3.1. *The α_{1L}-adrenoceptor as an alternative state of the α_{1A}-adrenoceptor*

It has been proposed that the α_{1L}-adrenoceptor phenotype may represent an alternative conformational state of the α_{1A}-adrenoceptor [6]. When recombinantly expressed in CHO cells, the α_{1A}-adrenoceptor displayed a radioligand binding profile consistent with the classically defined α_{1A}-adrenoceptor, including a sub-nanomolar binding affinity for prazosin. However, in [^{3}H]-inositol phosphate accumulation assays, the functional inhibitory profile of a subset of antagonists (including prazosin) closely resembled the pharmacological profile of the putative α_{1L}-adrenoceptor [6]. Crucially, three of the eight antagonists tested displayed similar affinities in both binding and functional assays, indicating that affinity estimates were not simply 'frame-shifted' between assays [6].

Subsequent to this, Hiraoka et al. [36] compared the α_1-adrenoceptor expression profile, investigated by [^{3}H]-tamsulosin binding, with the pharmacological profile of the adrenergic contraction of rat prostatic smooth muscle. They found that while the radioligand binding profile in membrane homogenates was consistent with the predominant expression of the α_{1A}-adrenoceptor subtype, antagonism of the functional response was indicative of a α_{1L}-adrenoceptor mediating contraction [36]. Marti et al. [44] similarly observed that the tail and small mesenteric arteries of rat expressed high levels of α_{1A}-adrenoceptor mRNA, while the functional responses in these arteries most closely resembled the α_{1L}-adrenoceptor phenotype. These studies provide good evidence that the manifestation of the α_{1L}-adrenoceptor phenotype requires the expression of the α_{1A}-adrenoceptor gene. The availability of genetically-modified mice lacking various combinations of α_1-adrenoceptor genes, including the α_{1A}-adrenoceptor (see [45]), together with the recent observation of an α_{1L}-adrenoceptor phenotype in mouse prostate [46], should facilitate the investigation of the role of the α_{1A}-adrenoceptor gene in the generation of the α_{1L}-phenotype, but to date this has not been explored.

So is the α_{1L}-adrenoceptor merely the 'default' functional phenotype of the α_{1A}-adrenoceptor gene-product? Several lines of evidence suggest that it is not quite so simple. First, additional work by Clarke's group indicates that the intact cellular environment is crucial in determining the pharmacological profile of the α_{1A}-adrenoceptor gene product, at least when recombinantly expressed in CHO cells [47]. They found that when radioligand binding assays were performed in intact cells, in physiological buffer solutions at 37 °C (rather than in membrane homogenates at room temperature), antagonist affinity estimates were more in line with the α_{1L}-adrenoceptor profile [47]. In contrast, when expressed in the same CHO cell background, both α_{1B}- and α_{1D}-adrenoceptors exhibited similar pharmacological profiles in membrane homogenates and in intact cells, suggesting that such affinity state pleiotropism is specific to the α_{1A}-adrenoceptor subtype [47]. Although tissue homogenization and membrane preparation has been reported to alter receptor binding properties

[48], the difference in temperature and buffer ionic composition could also be responsible for the discrepancies in receptor binding affinity reported by Williams et al. [47] as the influence of these factors upon affinity estimates derived from radioligand binding studies have been documented previously [49].

Hiraizumi-Hiraoka et al. [50] took this concept a stage further by comparing functional affinity estimates with binding affinities derived from radioligand binding assays in both membrane homogenates and intact tissue segments from rabbit ear artery. Functional affinity estimates were consistent with a α_{1L}-adrenoceptor-mediated contractile response, while in membrane homogenates only α_{1A}-adrenoceptors could be identified with a range of α_{1A}/α_{1L}-selective ligands [50]. In contrast, in ear artery segments both α_{1A}- and α_{1L}-adrenoceptor subtypes could be detected. These data confirm an important role for the intact cellular/tissue environment for the α_{1L}-adrenoceptor phenotype, but also illustrate that both α_{1A}- and α_{1L}-adrenoceptor subtypes can co-exist within the same tissue, providing persuasive evidence that the intact cell environment may be necessary, but is not sufficient to generate the α_{1L}-adrenoceptor phenotype *in vivo*.

This is supported by studies detailing functional responses in intact tissue of rat tail artery [51] and perfused rat kidney [52], where the pharmacological profiles of these responses were most consistent with the α_{1A}-adrenoceptor. It therefore seems that not only can both α_{1A}- and α_{1L}-adrenoceptors co-exist within the same tissue, but that the α_{1A}-adrenoceptor can in some cases mediate functional responses. Indeed, Amobi et al. [42] have reported that in the human vas deferens, while the α_{1L}-adrenoceptor mediates contraction of the longitudinal smooth muscle, contraction of the circular muscle occurs via α_{1A}-adrenoceptor activation. Taken together, these data suggest that the pharmacological profile of the functional α_1-adrenoceptor population in a given background is sensitive to the influence of the cellular environment through an as yet poorly defined mechanism. While there is significant evidence that these influences may be lost upon tissue homogenization and membrane preparation (see above), the intact cell environment alone does not generate the α_{1L}-adrenoceptor phenotype *per se* and α_{1L}-adrenoceptor sites can be detected alongside α_{1A}-adrenoceptors using [^{3}H]-prazosin in membrane homogenates of vascular smooth muscle [53]. The functional α_1-adrenoceptor phenotype may therefore represent the concerted influence of a number of factors expressed in a cell-dependent manner.

3.2. *α_1-adrenoceptor antagonists in the treatment of benign prostatic hyperplasia*

Benign prostatic hyperplasia (BPH) is characterized by a nodular enlargement of the prostate gland. This leads to an obstruction of the urethra, which is exacerbated by a dynamic α-adrenoceptor-mediated constrictor tone in the prostate [54]. A number of competitive α-adrenoceptor antagonists (e.g. prazosin, alfuzosin, terazosin) can provide effective symptomatic relief in BPH patients, but their use is limited by often severe cardiovascular side-effects [54]. However, a number of so-called 'uro-selective' α-adrenoceptor antagonists have been developed, which display higher affinity for the antagonism of adrenergic contractile responses in the lower urinary tract than for similar responses in vascular smooth muscle [55–58].

Despite the identification of the α_{1A}-adrenoceptor as the predominant α-adrenoceptor subtype expressed and functionally active in prostate stroma [59,60], there is not always a good correlation between selectivity for the α_{1A}-adrenoceptor subtype and selectivity for the lower urinary tract versus the cardiovascular system [54,56,58,61]. Since most vascular tissues express multiple α-adrenoceptor subtypes, it is not entirely clear which subtype(s) mediates the cardiovascular side-effects of many α_1-adrenoceptor antagonists. However, it is now appreciated that the α_{1A}-adrenoceptor contributes significantly to the regulation of vascular tone (see [30,45]) and could therefore explain the lack of correlation between uro-selectivity and α_1-adrenoceptor subtype selectivity. Instead, it has been proposed that the uro-selectivity of some α_1-adrenoceptor antagonists may be related to their selectivity for the α_{1L}-adrenoceptor subtype over α_{1B}- and α_{1D}-adrenoceptor subtypes [56]. Indeed, there is significant evidence that the functional profile of α-adrenoceptors expressed in human [43], rabbit [56] and rat [36] prostate is most consistent with the α_{1L} phenotype. Leonardi et al. [56] therefore proposed that if prostatic smooth muscle contraction is mediated by the α_{1L}-adrenoceptor, antagonist functional affinity in this tissue might correlate with α_{1A}-adrenoceptor affinity for the many ligands which display similar affinities for α_{1A}- and α_{1L}-adrenoceptors [62], while those ligands with sufficiently discriminatory affinities for the two subtypes would fail to display such a correlation.

However, the situation is further complicated by reports of the involvement of the α_{1L}-adrenoceptor subtype in small resistance arteries of a number of animal model species (see [30]), suggesting that a α_{1L}-adrenoceptor subtype-selective ligand might not necessarily display uro-selectivity. It remains to be established whether this is true for human resistance arteries, but clearly a fuller understanding of the molecular basis for the α_{1L}-adrenoceptor phenotype would facilitate clarification of the sites at which this subtype predominates. This in turn might allow more efficient exploitation of this fascinating example of pharmacological pleiotropism in the drug discovery process and in the clinic.

4. Tissue-dependent pharmacology of muscarinic acetylcholine receptors

To date, five mACh receptor genes have been cloned and thorough pharmacological characterizations of at least four (M_1–M_4) subtypes have been reported [63]. These receptor subtypes are widely expressed throughout central and peripheral tissues and agonists or antagonists at the mACh receptors could provide effective pharmacological treatments for number of debilitating disorders, including Alzheimer's disease, schizophrenia, irritable bowel syndrome, asthma and OAB [64]. However, the therapeutic potential of mACh receptor ligands is limited by a paucity of subtype-selective ligands, such that many drugs targeting mACh receptors can cause

severe side-effects, affecting the CNS, cardiovascular system, salivary glands and gastrointestinal and genito-urinary systems. There has therefore been considerable interest in the observation that certain antagonists at the mACh receptors display 'functional selectivity' in a tissue-dependent manner.

An early example of such 'functional selectivity' came from the observation that the M_3-mACh receptor-selective antagonist *para*-fluorohexahydrosiladifenidol (pFHHSiD) exhibited significantly lower pA_2 values in tracheal smooth muscle than in ileal smooth muscle of the same species [65]. However, although Ehlert et al. [66] recently confirmed the ileal selectivity of pFHHSiD relative to tracheal smooth muscle, they concluded that the higher apparent affinity in the ileum was mediated by a more potent, non-muscarinic target, present in ileal, but absent from tracheal smooth muscle. Nonetheless, the mechanisms underlying other examples of tissue-dependent mACh receptor pharmacological profiles remain to be resolved, including the observation that the affinity of zamifenacin at tracheal M_3 receptors was lower than at either ileal or oesophageal M_3 mACh receptors [67].

The nature of the mACh receptors involved in the contraction of uterine smooth muscle is also the subject of some controversy. For instance, although Boxall et al. [68] observed a radioligand binding profile consistent with a homogeneous M_2 receptor population in guinea-pig uterine smooth muscle, functional studies with a range of subtype-selective antagonists suggested a dual role for M_2 and M_3 receptors in the contractile response. Overall, the pharmacological profile was not typical of the classically defined mACh receptor subtypes and suggested that functionally, the M_2 receptor population detected in radioligand binding studies behaved atypically [68].

4.1. *Evidence for functionally selective mACh receptor antagonists: OAB therapies*

Activation of the cholinergic system is the major pathway by which micturition is initiated in humans [69]. mACh receptor antagonists have therefore been a mainstay in the clinical management of OAB for many years now [70]. Two mACh receptor subtypes (M_2 and M_3) have been identified in bladder smooth muscle by a variety of techniques, with the M_2 subtype making up approx. 80% and the M_3 subtype 20% of the total mACh receptor population (see [71]). However, as with the majority of smooth muscle types, pharmacological characterizations (see [71]) and gene 'knockout' studies [72,73] agree on the predominant involvement of the minority M_3 mACh receptor population in mediating detrusor smooth muscle contraction in a number of species, including human [74].

These data suggest that an M_3 mACh receptor-selective antagonist should be an effective OAB therapy and indeed current therapies for OAB, including oxybutynin and tolterodine, display high affinity for the M_3 mACh receptor [10,75], while the M_3 mACh receptor-selective antagonists darifenacin and solifenacin have shown promise in clinical trials [76,77]. However, the involvement of the M_3 mACh receptor in a variety of other physiological processes (e.g. salivation, gastrointestinal motility and pupillary constriction), can lead to significant side-effects in anti-muscarinic OAB therapy. At least 50% of patients taking oxybutynin experience severe dry mouth, which is the most common reason why at least 25% of patients discontinue medication [78]. Evidence from both pharmacological (e.g. [79]) and gene knockout [72,80] studies indicate the primary involvement of the M_3 mACh receptor in the regulation of salivary flow, although some controversy remains over the potential role of 'non-M_3' mACh receptors in the cholinergic modulation of salivation [81–83]. The significant overlap in the functional mACh receptor expression profiles of the detrusor smooth muscle and the salivary glands has prompted the search for pharmacological agents with selectivity for the inhibition of detrusor smooth muscle contraction versus salivary secretion. The observation that some mACh receptor antagonists display tissue-dependent pharmacology therefore provides a potentially exciting means of developing such selective muscarinic therapeutics.

4.2. *In vivo evidence*

The best characterized examples of mACh receptor antagonists displaying 'atypical' pharmacological profiles are for a number of current and candidate OAB therapies, including oxybutynin, tolterodine and darifenacin. Initial observations of the tissue-selectivity of these compounds came from *in vivo* studies, where tolterodine was found to be approx. 2.5-fold selective for the inhibition of ACh-induced bladder contractions versus electrically evoked salivation in anaesthetized cat [10]. In support of this, Gillberg et al. [75] reported that tolterodine was 2.5–3.3-fold selective for bladder (relative to salivary gland) in anaesthetized cat, while in anaesthetized dog, Gupta et al. [12] found that tolterodine was 4-5-fold selective for the inhibition of pelvic nerve-stimulated bladder contractions versus salivary secretion.

Darifenacin displays high affinity and selectivity for the M_3 mACh receptor and has been demonstrated to dose-dependently inhibit micturition pressure and increase micturition volume and interval at doses lower than those required to inhibit salivary secretion in rat [11]. Darifenacin also displayed 10-fold selectivity for inhibition of pelvic nerve-stimulated bladder contractions versus salivary secretion in anaesthetized dog [12]. Importantly, in all of these studies, one or more compound (e.g. atropine) failed to display selectivity between bladder and salivary gland responses, suggesting that the selectivity observed for tolterodine and darifenacin represents a specific discrimination and not a generalized frame-shifting of affinities between responses in the two tissues.

However, not all studies are in agreement with the bladder-selectivity of these mACh receptor antagonists. For instance, Gillberg et al. [75] reported that darifenacin displayed slightly higher affinity for the inhibition of salivary responses than for inhibition of bladder contractions, while in the case of oxybutynin, there is also a lack of agreement between studies (e.g. [10,12]). However, given that the major first pass metabolite of oxybutynin, *N*-desethyl-oxybutynin, may significantly contribute to the pharmacological actions of oxybutynin [84], it is conceivable that this could contribute to the complex species- and tissue-dependence of *in vivo* pharmacology of oxybutynin. Oxybutynin has also been shown to exert significant 'non-muscarinic' effects (e.g.

blockade of Ca^{2+} channels [85]), which may further complicate the analysis of its actions *in vivo*.

4.3. *In vitro evidence*

The evidence for mACh receptor antagonists displaying tissue-dependent pharmacologies extends beyond *in vivo* studies, to *in vitro* organ bath and second messenger assays, suggesting that this functional selectivity occurs at the ligand-receptor level and/or at the interface between receptor and proximal components of downstream signaling pathways. For instance, comparison of the inhibition of carbachol-stimulated ^{86}Rb efflux from submandibular salivary glands with the inhibition of ACh-mediated bladder contractions in guinea-pig indicated that darifenacin (six-fold) and tolterodine (two-fold) both displayed higher affinity for the inhibition of bladder contractions than for the inhibition of salivary gland responses [7]. However, controversy over the tissue-selectivity of oxybutynin persists for *in vitro* studies, with evidence that oxybutynin is bladder-selective [8,9 (in Ca^{2+} mobilization assays)] and also reports that it is either non-selective [7,9 (in contraction/salivation studies)] or modestly selective for salivary gland responses [86].

In our own work we have examined the pharmacology of oxybutynin, tolterodine and darifenacin (in comparison with atropine) in the inhibition of mACh receptor-stimulated phospholipase C (PLC) activity in guinea-pig urinary bladder and submandibular salivary gland slices [8]. It was found that all three test ligands (oxybutynin, darifenacin and tolterodine) exhibited between 7.4- and 9.3-fold selectivity for the bladder responses, relative to those in the submandibular gland slices, while atropine was non-selective (see Fig. 1). This study also addressed the issue of potential 'non-M_3' PLC-coupled mACh receptors being involved in the mACh receptor-PLC response in the submanibular gland. Using radioligand binding techniques together with the most selective ligands available, we found no evidence for a significant population of 'non-M_3' mACh receptors in this tissue, suggesting that the observed functional selectivity of a subset of mACh receptor antagonists could not be explained simply in terms of the involvement of other classically defined mACh receptor subtypes in the salivary gland responses [8]. Taken together, these studies highlight the importance of choosing the appropriate cell background (i.e. detrusor smooth muscle in the case of OAB therapies) in which to investigate GPCR-targeted therapeutic candidates.

Could a non-muscarinic target account for all of these examples of functional selectivity amongst mACh receptor antagonists? As described previously, oxybutynin has demonstrated non-muscarinic inhibitory actions in smooth muscle [85,87]. However, both tolterodine [87] and darifenacin [88] have been demonstrated to mediate their inhibitory effects upon bladder contraction only via mACh receptor inhibition and not by the blockade of Ca^{2+} channels or inhibition of

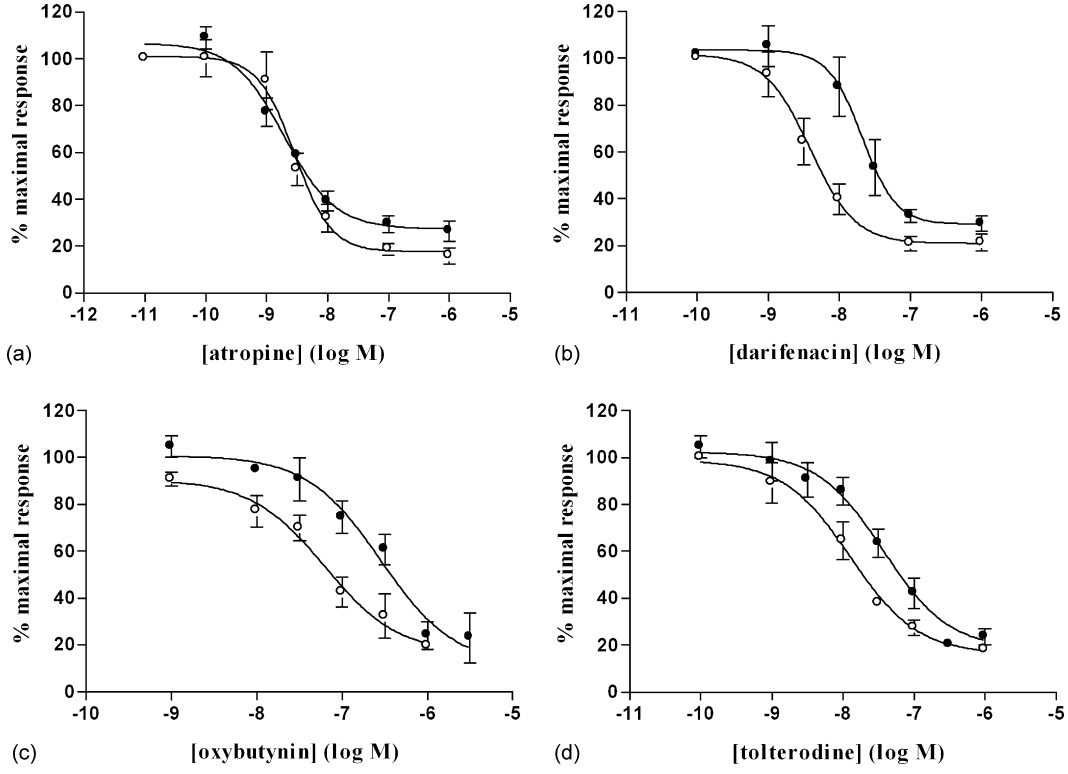

Fig. 1 – Functional selectivity of antagonists at the M_3 mACh receptor. Inhibition of CCh (50 μM)-mediated functional responses in guinea-pig bladder ([^{3}H]-inositol phosphate accumulation; open symbols) and submandibular gland (Ins(1,4,5)P_3 mass; closed symbols) slices by atropine (a), darifenacin (b), oxybutynin (c) and tolterodine (d). Data are expressed as percent maximum response (in the absence of antagonist). Results are expressed as mean ± S.E.M, $n \geq 3$. Figure reproduced with permission from Nelson et al. [8].

atropine-resistant contractions. Ehlert et al. [66] investigated the affinity of pFHHSiD for the inhibition of mACh receptor-mediated [^{3}H]-inositol phosphate accumulation in ileal and tracheal smooth muscle tissue and found that it failed to display any selectivity in this assay, consistent with its high affinity inhibitory effect in the ileum being mediated by a non-muscarinic mechanism. In contrast, we have reported that oxybutynin, tolterodine and darifenacin display functional selectivity for detrusor smooth muscle at the level of second messenger generation [8], while the M_3-selective antagonist solifenacin has been demonstrated to exhibit bladder selectivity in both *in vitro* and *in vivo* assays [89]. Taken together, these data suggest that any non-muscarinic, non-competitive effects of these ligands cannot account for their functional selectivity *in vivo*.

Alternative pharmacokinetic explanations for functional mACh receptor selectivity have been proposed, including the partitioning of lipophilic ligands into tissues, giving rise to high local drug concentrations in detrusor smooth muscle (see [70]). The influence of such localized concentrations of ligand is further complicated by the presence of mACh receptors on the urothelium that have been demonstrated to mediate the release of a diffusible factor that inhibits detrusor contractility [90]. However, the correlation between *in vitro* and *in vivo* studies with respect to functional selectivity of mACh receptor antagonists suggests that pharmacokinetic mechanisms (as with potential 'non-muscarinic' targets) cannot fully account for observed tissue-dependent pharmacologies. The precise molecular basis for this, therefore, remains to be resolved.

5. Potential mechanisms for generating tissue-dependent pharmacological profiles

Fig. 2 summarizes the processes involved in the life cycle and regulation of a GPCR, highlighting the points at which the cellular environment could influence receptor pharmacology. In the following sections we will briefly discuss the evidence that GPCR pharmacology may be shaped by each of these processes, with particular reference to the examples of phenotypic pharmacology discussed in the preceding sections. Here, we have chosen to highlight a number of key processes in GPCR regulation, but there are of course numerous other potential mechanisms by which novel

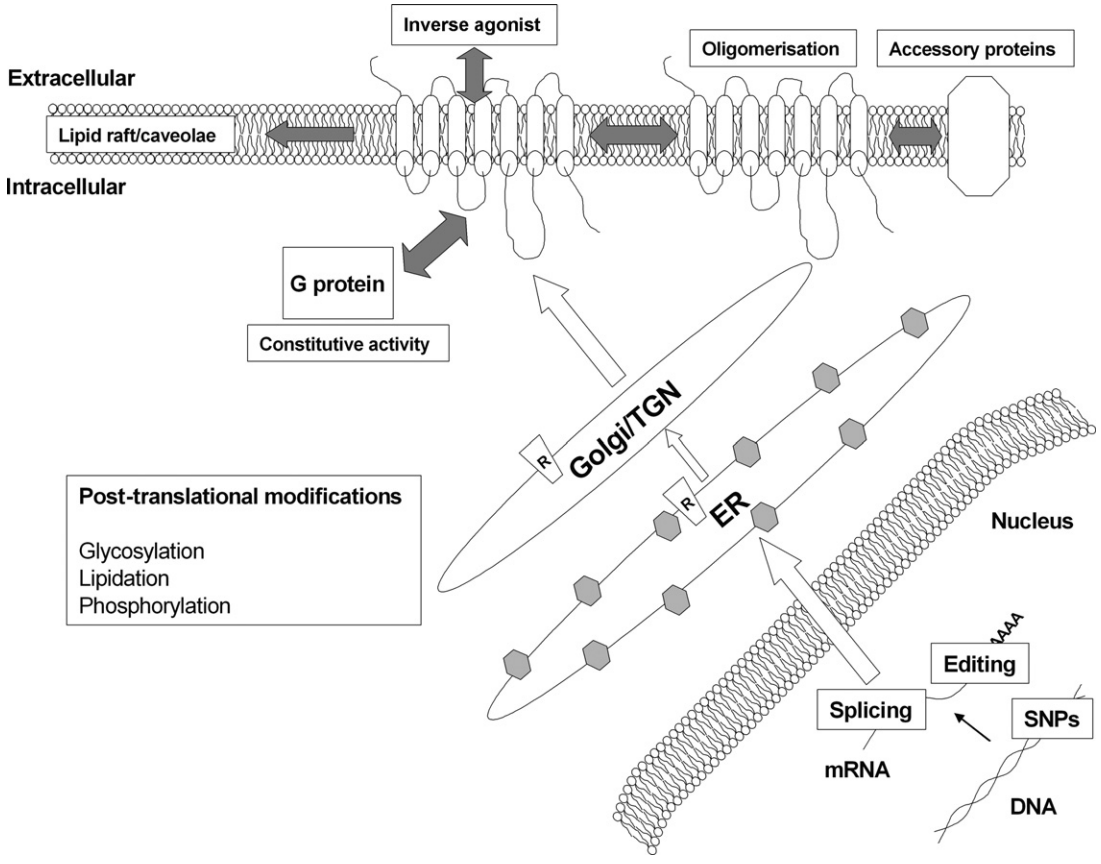

Fig. 2 – Possible mechanisms for generating phenotypic pharmacological profiles of GPCRs. The phenotypic expression of a GPCR may be determined by variation in DNA sequence resulting from single nucleotide polymorphisms (SNPs), post-transcriptional modifications (including RNA editing and alternative splicing), post-translational changes (glycosylation, lipidation, phosphorylation), localization of the receptor protein into lipid/protein-enriched plasma membrane compartments, physical association of receptor protein with accessory proteins and the prevailing level of constitutive interaction between receptor and hetero-trimeric G protein (constitutive activity).

pharmacological phenotypes could be generated (e.g. ligands acting at allosteric GPCR binding sites (see [91] for review)) which cannot be further discussed here.

5.1. *Single nucleotide polymorphisms (SNPs)*

Variations in DNA sequence occurring with an allelic frequency of >1% in the population are termed polymorphisms, the most common form of which are single nucleotide substitutions, known as single nucleotide polymorphisms (SNPs). 'Non-synonymous' SNPs (those leading to a single amino acid substitution) occurring in coding sequences of DNA can introduce allelic variation in amino acid sequence. Such non-synonymous SNPs have been identified in a wide variety of GPCRs and in some cases have been reported to influence receptor function (e.g. [92]). SNPs may also be associated with altered pharmacology. For instance, the Cys23Ser variant of the 5-HT_{2C} receptor displays lower affinity for agonist than the wild-type receptor [93] and schizophrenic patients possessing Ser at position 23 have been reported to respond less well to clozapine [94]. In contrast, the Asn40Asp SNP in the μ-opioid receptor displays higher binding affinity for the endogenous agonist β-endorphin [95]. So could non-synonymous SNPs play a role in generating phenotypic pharmacologies such as those discussed earlier? A recent study by Lei et al. [96] identified nine naturally occurring SNPs in the coding region of the α_{1A}-adrenoceptor, seven of which were non-synonymous. Interestingly, two of these polymorphic variants displayed altered antagonist binding affinities relative to wild-type: Ile200Ser displayed significantly lower affinity than wild-type for phentolamine (but equivalent affinity for prazosin and 5-methylurapidil), while Val311Ile exhibited higher affinity than wild-type for 5-methylurapidil (and equivalent affinity for prazosin and phentolamine) [96].

Amongst the mACh receptors, the coding sequences of both M_2 and M_3 subtypes are highly conserved, with little or no evidence of polymorphic variation within the coding sequences [97,98]. In contrast, Lucas et al. [99] identified nine polymorphisms within the coding region of the M_1 mACh receptor, one of which is non-synonymous, encoding an amino acid change of Cys to Arg at position 417. Savarese et al. [100] have previously characterized a Cys417Ser mutation in the rat M_1 mACh receptor as having an enhanced agonist affinity and potency. Of particular interest, the Cys417Ser mutant also displayed lower affinity than the wild-type receptor for the antagonists pirenzepine, atropine and AF-DX 116 [100]. These studies therefore suggest that, at least in principle, naturally occurring polymorphisms can influence antagonist pharmacology. Given that the behavior of some SNPs has been reported to vary in a cell background-dependent manner [101], the potential for tissue-dependent expression of SNPs to influence GPCR antagonist pharmacology certainly merits further investigation.

5.2. *RNA editing*

The process of RNA editing involves the conversion of adenosine to inosine by adenosine deaminases that act on RNA (ADARs), leading to insertion/deletion of single nucleotides, thus altering the sequence of primary gene transcripts. The 5-HT_{2C} receptor is known to undergo RNA editing events at up to 5 distinct sites and the extent to which this occurs has been shown to influence constitutive receptor activity and agonist potency [102]. RNA editing has also been implicated in the generation of agonist-directed trafficking of signaling at the 5-HT_{2C} receptor [103], suggesting that specific RNA editing sites are located in regions crucial in determining the pharmacological profile of the receptor. RNA editing of the 5-HT_{2C} receptor also appears to be a regulated process, with evidence that it provides a mechanism for fine-tuning the responsiveness of the receptor system in response to changes in synaptic input in the CNS [104]. However, to date the 5-HT_{2C} receptor is the only GPCR reported to undergo RNA editing, and it is not yet clear if this is a more common mechanism for the generation of pharmacological diversity amongst GPCRs.

5.3. *Alternative splicing*

Alternative splicing refers to the process by which coding sequences (exons) within pre-mRNA are spliced together and non-coding sequences (introns) are selectively excised from the translated mRNA species. This process is highly regulated, by factors such as developmental stage, activity and type of cell in which the gene is transcribed and thus provides another means by which cells may further expand proteomic diversity. The regulation of alternative splicing is still relatively poorly understood, but the discovery of the Nova protein family provides the first evidence of mammalian tissue-specific splicing factors [105]. There is evidence that alternative splicing of GPCR genes can generate novel pharmacological profiles. For instance, Cadet et al. [106] reported that an alternatively spliced variant of the mu-opioid receptor exhibited opioid peptide insensitivity and opiate alkaloid selectivity, characteristic of the putative mu_3 receptor subtype, for which a molecular definition was lacking until that point. It is therefore possible that a fuller understanding of the alternative splicing patterns of other GPCRs might reveal a molecular basis for other 'atypical' receptor phenotypes. However, an unusually large proportion of mammalian GPCRs (including the mACh receptors) is intronless and therefore cannot undergo alternative splicing. Gentles and Karlin [107] surveyed mammalian GPCR sequences in GenBank and found that more than 90% were intronless in their open reading frame, compared with the overall proportion of intronless human genes, which is believed to be no more than 5%. Nonetheless, four distinct splice variants of the human α_{1A}-adrenoceptor ($\alpha_{1A\text{-}1}$ to $\alpha_{1A\text{-}4}$) have been identified [108,109]. This led Ford and co-workers to investigate the radioligand binding and functional profiles of each splice variant expressed recombinantly in CHO [110]. Antagonist affinities for the products of the $\alpha_{1A\text{-}1}$-$\alpha_{1A\text{-}4}$ splice variants were consistent with the classical α_{1A}-adrenoceptor profile in radioligand binding assays, but exhibited an α_{1L}-adrenoceptor phenotype in functional inositol phosphate accumulation assays [110], as was previously reported for the unspliced α_{1A}-adrenoceptor expressed in CHO cells [6]. Thus, the α_{1L}-adrenoceptor phenotype does not appear to represent the functional phenotype of any individual splice variant of the α_{1A}-adrenoceptor gene.

5.4. *Post-translational modifications*

GPCRs are subject to a number of permanent and transient covalent modifications during their normal life cycle. Co-translational modifications (e.g. glycosylation) are generally considered to be stable and to be important for the correct trafficking of the nascent receptor to the plasma membrane and to its conformation and stability [111,112]. The consequences of interfering with normal GPCR glycosylation have been fairly extensively studied, but to date little evidence has accrued to suggest that this modification can result is tissue-dependent differences in receptor pharmacology. In contrast, evidence emerging from both genetic and biochemical studies indicates that receptor palmitoylation is more dynamically regulated [113] and might be important in maintaining the fidelity of G protein coupling at a variety of GPCRs, including β-adrenoceptors and mACh receptors (see [111]).

Other receptor modifications can occur much more dynamically. By far the most studied of these is receptor phosphorylation, which occurs primarily within the intracellular loops and C-terminal domain of GPCRs, and can regulate many aspects of receptor activity including desensitization, internalization and selection of G protein coupling partners [114,115]. In addition, there is some evidence that phosphorylation patterns generated in response to receptor activation can vary in a cell background-dependent manner: for example, Ozcelebi et al. [116] have reported differences in the phosphorylation profile of cholecystokinin receptors in pancreatic acinar versus CHO cell backgrounds. Furthermore, the observation that phosphorylation status of the β_2-adrenoceptor significantly alters its affinity for antagonist ligands [24] suggests that such post-translational receptor modifications can contribute to GPCR phenotypic pharmacological variation.

5.5. *Receptor oligomerization*

GPCR oligomerization, has been the subject of a number of excellent recent reviews [117–119] and a thorough discussion of oligomerization *per se* is beyond the scope of this Commentary. However, of relevance to our discussion, there are now a number of studies reporting that hetero-oligomeric GPCR assembly can generate novel pharmacological profiles (see [119]). For example, heterodimeric association of adenosine A_1 ($G_{i/o}$-coupled) and adenine nucleotide $P2Y_1$ ($G_{q/11}$-coupled) receptors produces hybrid pharmacology with the $P2Y_1$ receptor agonist ADP(S displacing A_1 adenosine receptor agonist ([^{3}H]-NECA) binding [120]. In addition, in cells co-expressing A_1 and $P2Y_1$ receptors, ADP(S could cause a marked reduction in forskolin-stimulated cyclic AMP accumulation via a mechanism sensitive to PTx and to the A_1 receptor antagonist DPCPX, suggesting that ADP(S can act via the $G_{i/o}$-coupled A_1 receptor within the hetero-multimer [120]. Recent data from Javitch and colleagues suggest that a conformational change at the D_2 dopamine receptor dimer interface (in the fourth transmembrane domain) might be important in the receptor activation process, as agonist binding was found to accelerate (and inverse agonist binding to decelerate) cross-linking of residues shown to be important in 'locking' the receptor in the active state [121]. Such data raise the issue of whether receptor hetero-oligomerization could be responsible for generating any of the phenotypic pharmacologies described earlier.

With respect to the α_{1L}-adrenoceptors, there is some evidence that hetero-oligomerization of α-adrenoceptor subtypes can significantly alter receptor pharmacology [122], but not in a manner that generates an α_{1L}-adrenoceptor-like phenotype. Ramsay et al. [123] considered the potential for the co-expression of α_1-adrenoceptor splice variants to generate novel pharmacological profiles by co-expressing the $\alpha_{1A\text{-}1}$, $\alpha_{1A\text{-}2}$ and $\alpha_{1A\text{-}3}$ splice variants in HEK293 cells. This group demonstrated that all combinations of the α_{1A} isoforms can form oligomeric assemblies, however, none of these species exhibited a binding affinity profile consistent with the α_{1L}-adrenoceptor, suggesting that the hetero-oligomeric association of a combination of these three splice variants is not responsible for the α_{1L}-adrenoceptor phenotype observed *in vivo* [123]. It should be noted, however, that the $\alpha_{1A\text{-}4}$-adrenoceptor splice variant was not investigated by Ramsay et al. [123], and since this isoform is known to be highly expressed in tissues where an α_{1L}-adrenoceptor pharmacology has been observed, such as human prostate [109], it remains possible that hetero-oligomers containing the $\alpha_{1A\text{-}4}$ splice variant might account for α_{1L}-adrenoceptor pharmacology. However, it should be remembered that the clearest example of α_{1L}-adrenoceptor pharmacology has been reported in CHO cells recombinantly expressing the α_{1A}-adrenoceptor [6]. CHO cells are generally considered to express low levels of endogenous GPCRs and therefore it is unlikely that oligomerization of recombinant and endogenous species could account for the α_{1L}-adrenoceptor phenotype.

With respect to mACh receptors, Maggio and co-workers have provided some evidence for hetero-oligomerization, using M_2 and M_3 mACh receptor truncations split at the third intracellular loop to form either M_2- or M_3- 'trunc' (TM I–V) or 'tail' (TM VI and VII) fragments [124]. Co-expression of wild-type M_2 receptors with M_3-tail fragments (which do not themselves bind muscarinic ligands) yielded biphasic displacement curves for the M_1-selective antagonist pirenzepine, in radioligand binding studies, with binding affinities consistent with those observed for pirenzepine at the wild-type M_2 receptor and at the chimeric receptor species resulting from co-expression of M_2 trunc and M_3-tail fragments. These data suggest a physical association between the M_2 receptor and C-terminal tail of the M_3 receptor, forming a viable structure capable of antagonist binding [124]. Interestingly, pirenzepine was able to distinguish two sites in these co-expressing cells due to its significantly higher affinity for the M_2/M_3 chimera than for either wild-type M_2 or M_3 receptors, suggesting that if similar interactions were to occur in the formation of an M_2/M_3 mACh receptor dimer, the resulting species might display a unique pharmacology, at least with respect to pirenzepine.

There is emerging evidence that hetero-dimeric GPCR species may provide novel and viable therapeutic targets. For instance, Whistler and colleagues [125] reported that 6′-guanidinonaltrindole not only selectively targets opioid receptor hetero-dimers (and not homo-dimers), but is also able to selectively induce analgesia when administered into

the spinal cord, but not in the brain. The authors concluded that kappa-opioid/delta-opioid receptor hetero-dimers are present (and mediate analgesia) in the spinal cord, but not in the brain [125], providing *in vivo* evidence for tissue-dependent hetero-dimeric assemblies. It is also becoming clear that the large number of orphan GPCRs may represent hetero-dimeric binding partners for other receptors, leading to the formation of novel pharmacological entities, as has been shown very recently for GPR50 and the melatonin MT_1 receptor [126]. It is therefore conceivable that tissue-dependent phenotypes may represent the product of hetero-dimeric interactions between well-characterized receptor subtypes and orphan GPCRs.

5.6. *Compartmentalization of GPCRs and interactions with accessory proteins*

Spatial organization of GPCRs and other signaling components into specific membrane domains and scaffolding networks has also been shown to influence signal transduction [127]. A wide variety of proteins have been reported to form stable or transient associations with GPCRs, including scaffolding proteins (e.g. AKAP79/150, Homer, spinophilin), cytoskeletal proteins and receptor activity-modifying proteins (RAMPs) [127,128]. In many cases these interactions promote efficient signal transduction and may facilitate crosstalk, by concentrating components of cellular signaling cascades within microdomains and, in specific cases, changes in GPCR pharmacology have been observed as a result of protein–protein interactions.

One of the most striking examples of accessory proteins altering the pharmacology of their associated GPCR is that of the RAMPs. Interaction between RAMPs and the calcitonin receptor-like receptor (CRLR) determine the ligand binding of the receptor, as receptors associated with RAMP1 specifically bind calcitonin gene-related peptide (CGRP) while those interacting with RAMP2 or RAMP3 bind adrenomedullin [129]. Co-expression of RAMP3 with the calcitonin receptor has been shown not only to influence agonist potency ranking orders, but also antagonist affinity estimates [130].

The localization of GPCRs and their downstream signaling partners to specific membrane lipid compartments (so-called 'lipid rafts' and in particular the caveolin-containing subpopulation of lipid rafts known as caveolae) perhaps provide a more widely applicable paradigm for the regulation of GPCR signaling [131]. An interesting property of caveolae is that they selectively accumulate a variety of signaling molecules, including GPCRs, G proteins and second messenger-regulated kinases, prompting the proposal that they might regulate signal transduction [132]. Given that the β_2-adrenoceptor [133] and M_2 mACh receptor [134] have been demonstrated to translocate out of and into caveolae, respectively, upon receptor activation, it is clear that the activation status of the receptor may be tightly linked to its localization in lipid microdomains. It remains to be established whether or not the influence of caveolar localization could be extended to GPCR antagonist pharmacology, but there is evidence for tissue-specific differences in the expression of GPCR signaling components in caveolae/lipid rafts [131], perhaps providing another mechanism for the cell background to influence GPCR pharmacology.

5.7. *Constitutive receptor activation*

It is now widely appreciated that GPCRs are able to bind to and activate their cognate G proteins in the absence of agonist-stimulation, generating 'constitutive' receptor activity [13]. The extent to which GPCRs are constitutively active is dependent on the level of receptor and G protein expression levels [14] and may also depend upon a variety of other factors including the compartmentalization of the receptor and its interactions with other cellular proteins (e.g. Homer proteins) (for review, see [135]). GPCR constitutive activity is, therefore, a phenotypic receptor behavior in itself (i.e. it is dependent on the cellular context in which the receptor is expressed). Perhaps understandably, much of the research in this field to date has relied heavily on recombinant model systems, where high levels of receptor and/or G protein expression, often coupled with specific constitutively-activating point mutations in the receptor, has generated sufficient levels of constitutive activity to allow clear demonstration of inverse agonism. As a result, the extent to which the phenomenon of constitutive receptor activity and inverse agonism are of physiological relevance still remains to be established. However, there are some (albeit relatively few) excellent studies detailing constitutive activity/inverse agonism at GPCRs expressed *in vivo* in unmodified native systems (e.g. [136,137]), indicating that the potential for tissue-dependent differences in constitutive receptor activity should be considered in relation to native pharmacological profiles.

According to the extended ternary complex model, any perturbation of the system in favor of the active receptor state (R_a) and/or the G protein-coupled active state (R_aG) will reduce the apparent affinity of an inverse agonist for the receptor population [138]. Given that approx. 85% of ligands originally classified as 'antagonists' are in fact inverse agonists [14], it might be anticipated that the observed affinities of the majority of 'antagonist' ligands (such as those crucial in the definition of phenotypic pharmacologies) would be highly dependent on the prevailing level of constitutive activity in the experimental system. However, theoretical estimates based upon the extended ternary complex model predict that only if a large fraction of the total receptor population (>90%) is present in the active state (R_a) would a large shift in inverse agonist affinity be observed [139]. Indeed, in several cases where highly constitutively active systems have been engineered by the introduction of constitutively activating mutations into GPCRs, significant reductions in inverse agonist affinity have not been observed [140]. It is therefore unlikely that cell background-dependent differences in constitutive receptor activity could account for significant differences in antagonist/inverse agonist affinity estimates between systems.

6. Concluding remarks

As our understanding of the complexity and diversity of GPCR signaling grows, it becomes clear that the cellular environment possesses an almost unlimited repertoire of potential protein–protein (and protein–lipid) interactions, which can act

in concert to shape the pharmacological profile of GPCRs expressed within it. Given this degree of complexity and the emerging view that GPCRs are capable of translating multiple extracellular signals into distinct intracellular responses and not merely acting as 'on/off' switches for a single linear downstream signaling cascade, it should not be at all surprising that antagonist (and in particular inverse agonist) pharmacology can be influenced by the cellular milieu. However, progress in elucidating the precise molecular mechanisms responsible for generating specific examples of such phenotypic pharmacology has been slow. The numerous potential mechanisms, only some of which have been touched upon in this Commentary, may in part be responsible for this, and we anticipate that there is much more to be discovered about this fascinating aspect of GPCR pharmacology in the future. Notwithstanding the lack of progress in defining precise molecular mechanisms giving rise to phenotypic pharmacologies, it is clear that the potential impact of cell background needs to be taken into account in the drug discovery process. An approach utilizing functional assessments of antagonist activities in cells/tissues that represent sites of desired (and undesired) drug action, may reveal subsets of drugs exhibiting desirable tissue selectivities that are ''phenotypic'' in origin.

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