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Review

Emerging Functional Divergence of β-Arrestin Isoforms in GPCR Function

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G protein-coupled receptors (GPCRs) are tightly regulated by multifunctional protein β-arrestins. Two isoforms of β-arrestin sharing more than 70% sequence identity and overall very similar 3D structures, β-arrestins 1 and 2, were originally expected to be functionally redundant. However, in recent years multiple lines of emerging evidence suggest they have distinct roles in various aspects of GPCR regulation and signaling. We summarize selected examples of GPCRs where β-arrestin isoforms are discovered to display non-overlapping and sometimes even antagonistic functions. We also discuss potential mechanistic basis for their functional divergence and highlight new frontiers that are likely to form the focal points of research in this area in coming years.

GPCRs and β-Arrestins

GPCRs constitute the largest family of cell surface receptors in the human genome with more than 800 different members [1]. A large array of ligands including small molecules, hormones, peptides, and lipids can bind to different GPCRs and activate downstream signaling cascades [2]. GPCR signaling influences a wide range of physiological processes, including olfaction, behavior, cardiovascular regulation, and the immune response, either directly or indirectly [3]. As a result, aberrant signaling and expression of GPCRs lie at the heart of many pathophysiological conditions such as different types of cancer, allergies, asthma, hypertension, and autoimmune diseases [4]. A large repertoire of currently prescribed medicines exert their effects through binding to GPCRs and by turning them 'on' or 'off' [5,6].

Agonist binding activates the receptor, leading to G protein coupling followed by the generation of second messengers such as cAMP, inositol phosphates, and Ca^{++}, and subsequent downstream signaling. Because sustained signaling is detrimental to cell physiology, a **desensitization** mechanism (see Glossary) is in place that involves phosphorylation of activated GPCRs and subsequent binding of β-arrestin proteins (Box 1) [7,8]. Binding of β-arrestins hinders further G protein coupling and results in termination of G protein signaling. There are four different isoforms of arrestins, two of which referred to as visual arrestins are limited primarily to the visual system. The other two isoforms, β-arrestins 1 and 2, are expressed ubiquitously and they play key roles in the function and regulation of non-visual GPCRs.

Considering the high degree of sequence and structural similarity, it is not surprising that β-arrestins 1 and 2 show significant functional overlap. However, several studies document and establish a clear functional specialization for the two isoforms. There have been three major approaches to dissect isoform-specific roles of β-arrestins. These are depletion of individual β-arrestin isoforms in cultured cell lines using siRNAs [9], the generation of isoform-selective

Trends

GPCR functions are crucially regulated by multifunctional scaffold protein β-arrestins.

Emerging evidence suggests that, for many different GPCRs, the two isoforms of β-arrestin (β-arrestins 1 and 2) play distinct roles in downstream functional outcomes.

Despite highly conserved primary sequence and overall very similar 3D structure, there are conformational differences in β-arrestin isoforms that potentially underlie their functional divergence.

Distinct functional outcomes of β-arrestin isoforms add a new dimension to the functional selectivity of GPCRs and offer novel therapeutic possibilities.

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Box 1. Classical Functions of β-Arrestins and their Expanding Functional Repertoire

β-Arrestin 1 was cloned based on its predicted homology with one of the visual arrestins (referred to as 48 kDa protein at the time) and it was found to be approximately 1000-fold more potent than visual arrestin in desensitizing activated β_2AR [79]. Within two years the second isoform, referred to as β-arrestin 2, was cloned and functionally characterized [80]. The two isoforms exhibit similar expression and localization patterns, and in an *in vitro* reconstituted system appeared to be equally potent in promoting β_2AR desensitization [80]. At this point, the primary function of β-arrestins was conceived to be limited to GPCR desensitization and both isoforms were predicted to play similar roles (Figure IA). However, in subsequent years many new functions of β-arrestins have started to emerge. First, β-arrestins were found to scaffold several key components of the clathrin-mediated endocytosis machinery and promote receptor internalization (Panel B) [13]. Subsequently, β-arrestins were found to scaffold various components of MAP kinase modules (ERK, p38, JNK) ultimately leading to the discovery of the G protein-independent β-arrestin-mediated signaling paradigm (Panel C) [81]. More recently, scaffolding of E3 ubiquitin ligases such as MDM2 (panel D) and targeting them to their potential substrates have emerged as another major function of β-arrestins, further broadening the functional reach of β-arrestins [82].

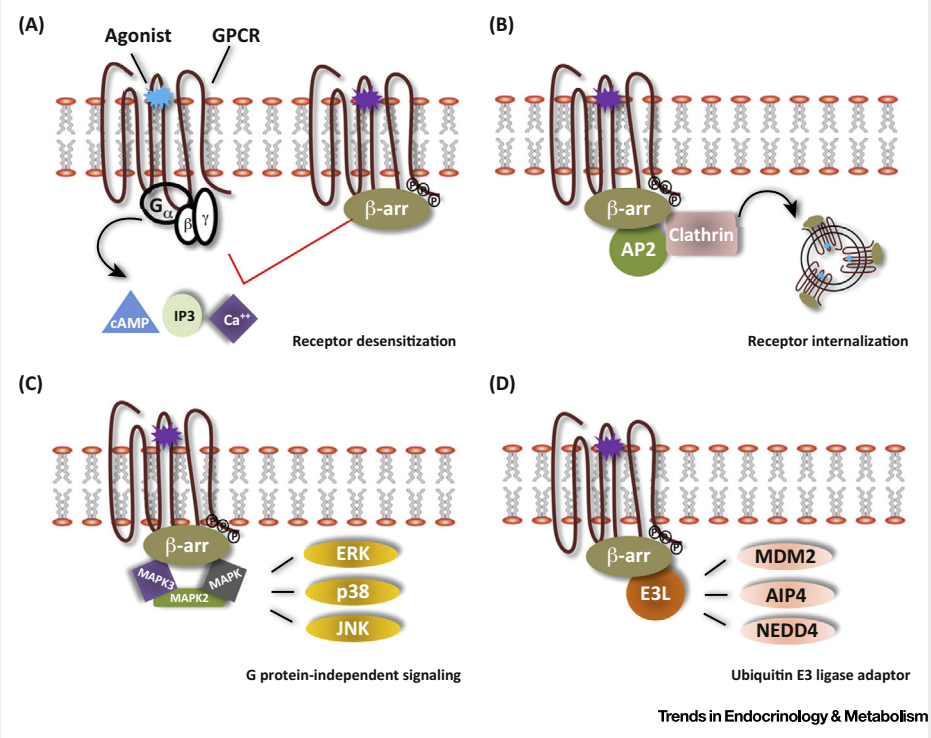

Figure I. Multifunctional Role of β-Arrestins in GPCR Signaling and Regulation. (A) Agonist-induced activation of GPCRs leads to coupling of heterotrimeric G proteins, generation of second messengers, and downstream signaling. Activated receptors are phosphorylated by GRKs, which then leads to recruitment of the cytosolic β-arrestin scaffolding proteins (β-arr 1 and 2). Binding of β-arrestins results in desensitization of the G protein response presumably through a steric hindrance mechanism. Gα, β, γ are three different subunits of the heterotrimeric G proteins. IP3, inositol trisphosphate; cAMP, cyclic adenosine monophosphate. (B) β-Arrestins act as a scaffold protein for various components of clathrin-coated endocytosis machinery, such as clathrin and AP2 (adaptin), and they promote internalization of agonist-activated GPCRs from the cell surface. (C) β-Arrestins also scaffold various components of multiple signaling kinases and phosphatases (e.g., MAP kinase module) to trigger a G protein-independent signaling pathway in the cells. β-Arrestin brings the different kinases of the MAP kinase module in close proximity to facilitate activation of the MAP kinase cascade. MAP kinase, mitogen activated kinase; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase. (D) β-Arrestins can also scaffold multiple ubiquitin E3 ligases (E3L) and bring them into close proximity of GPCRs or other non-GPCR substrates and thereby promote ubiquitination of the target proteins. NEDD4, neural precursor cell expressed developmentally downregulated protein 4; AIP4, atrophin-1-interacting protein 4.

knockout (KO) mouse models [10,11], and embryonic fibroblast cultured cell lines generated from these KO mice [12]. We highlight selected examples where the impact of both β-arrestin isoforms has been assessed in parallel with respect to a given functional readout, for example desensitization, **internalization**, and signaling.

Glossary

Aptamer: specific type of nucleic acid (DNA or RNA) that can adopt secondary and tertiary structures and specifically bind to proteins and other biomacromolecules.

Biased agonism: the ability of agonists to trigger biased signaling, in other words the selective activation of one signaling pathway but not another.

Biased signaling: selective activation of one or other signaling pathway (e.g., G protein- or β-arrestin-dependent) downstream of GPCRs. Ligands that preferentially or selectively trigger one or other signaling pathway are referred to as biased ligands.

Desensitization: the inability of receptors to continue to signal following persistent agonist exposure, resulting from functional uncoupling to G proteins. Typically mediated by a β-arrestin-dependent steric hindrance mechanism.

Internalization: removal of receptors from the cell surface upon agonist stimulation. Also referred to as receptor endocytosis.

Pepducins: these are cell-permeable peptides corresponding to the specific intracellular domains of GPCRs that are utilized to modulate GPCR signaling.

Synthetic antibody fragments: antigen-binding fragments of antibodies that are primarily selected from synthetically designed phage-display antibody fragment library and can specifically bind to different conformations of target proteins.

Selected Examples of Functional Divergence of β-Arrestin Isoforms

β_2-Adrenergic Receptor (β_2AR): Establishing the Paradigm

A key mediator of the 'fight or flight' response, β_2AR, has been one of the best-characterized systems with respect to functional consequences of GPCR–β-arrestin interaction. Early indications of functional specialization of β-arrestin isoforms resulted from multiple studies of agonist-induced β_2AR downregulation (Figure 1A). Selective removal of β-arrestin 2 but not β-arrestin 1 leads to an increase in agonist-induced cAMP response downstream of β_2AR [9]. Because β-arrestins are not known to directly affect cAMP levels in the cells, this finding

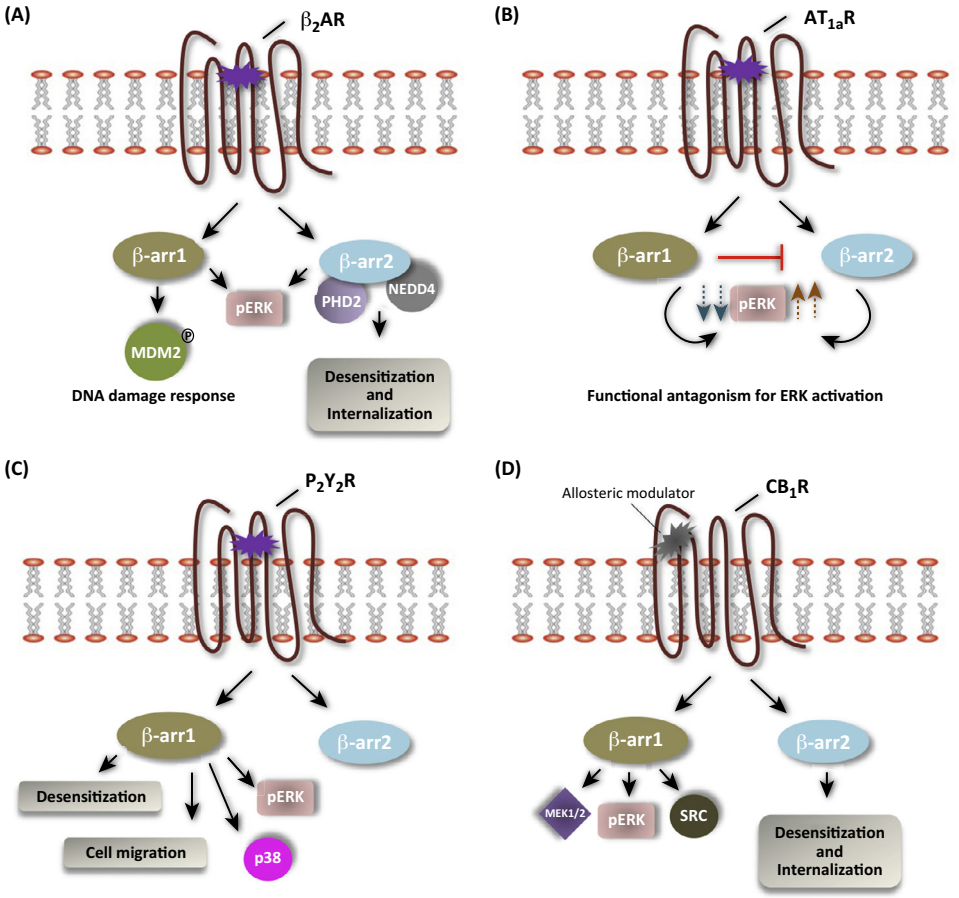

Figure 1. Selected Examples of GPCRs where β-Arrestin Isoforms Display Profound Functional Specialization. (A) Agonist stimulation of the β_2 adrenergic receptor (β_2AR) recruits both isoforms of β-arrestins (β-arr). The desensitization, internalization, and ubiquitination of β_2AR is primarily mediated by β-arrestin 2, whereas β-arrestin 1 is required for MDM2 phosphorylation and a stress-induced DNA damage response pathway. Both isoforms of β-arrestin are essential for ERK activation. (B) β-Arrestins 1 and 2 exhibit functional antagonism for ERK activation downstream of the angiotensin II type 1a receptor ($AT_{1a}R$) in transfected HEK-293 cells. Depletion of β-arrestin 2 ablates ERK activation while β-arrestin 1 deletion augments it. (C) Nucleotide-binding purinergic receptor subtype 2 (P_2Y_2R) recruits both isoforms of β-arrestins in response to agonist stimulation. However, β-arrestin 1 appears to mediate not only desensitization of P_2Y_2R but also downstream signaling cascades of ERK and p38 MAP kinases. β-Arrestin 1 also appears to be the primary isoform mediating agonist-induced migration of arterial smooth muscle cells (ASMCs). (D) Cannabinoid receptor subtype 1 (CB_1R) represents one of the few examples where a β-arrestin biased allosteric ligand has been described. The functional outcomes after activation with the ligand ORG27569 (which engages both isoforms of β-arrestins), are significantly different for the two isoforms. The MAP kinase signaling cascade depends strictly on β-arrestin 1 while endocytosis of the CB_1R is primarily governed by β-arrestin 2. Abbreviations: ERK, extracellular signal regulated kinase; MAP, mitogen activated protein; MDM2, mouse double minute 2 homolog (MDM2 proto-oncogene, E3 ubiquitin protein ligase); MEK1/2, MAP kinase kinase 1/2; NEDD4, neural precursor cell expressed developmentally downregulated protein 4; PHD2, prolyl hydroxylase subtype 2.

suggests that absence of β-arrestin 2 but not β-arrestin 1 delays (and/or slows down) β_2AR desensitization [9]. Agonist-induced endocytosis of many GPCRs, another mechanism for receptor downregulation, occurs via clathrin-coated pit machinery. β-Arrestins act as clathrin adaptors, and β-arrestin 2 interacts relatively strongly with clathrin, compared to β-arrestin 1 [13]. In line with this affinity difference between the β-arrestin isoforms for clathrin, internalization of the β_2AR appears to be largely mediated by β-arrestin 2, and β-arrestin 1 is observed to play a relatively minor role [9,12].

More recently, a novel mechanism of β_2AR internalization has been described that involves prolyl hydroxylase type 2 (PHD2) [14]. PHD2 preferentially interacts with β-arrestin 2 and hydroxylates it at three different proline residues. This interaction retards the recruitment of β-arrestin 2 to the membrane and subsequent internalization of the receptor [14]. This observation further supports the preferential role of β-arrestin 2 in β_2AR internalization. Furthermore, agonist-induced ubiquitination of β_2AR, a process that is linked to receptor internalization, is also predominantly mediated by β-arrestin 2, and this involves preferential interaction of β-arrestin 2 with the E3 ubiquitin ligase NEDD4 (neural precursor cell expressed developmentally downregulated protein 4) [15]. These multiple lines of evidence underscore a more profound role of β-arrestin 2 over β-arrestin 1 in β_2AR endocytosis and downregulation.

Although β-arrestin-dependent activation of the mitogen-activated protein kinase (MAP kinase) ERK (extracellular signal-regulated kinase/MAPK1) downstream of β_2AR appears to involve both isoforms of β-arrestin [16], activation of p38 MAP kinase shows differential dependence on two isoforms [17]. Stimulation of β_2AR with the agonist isoproterenol leads to a biphasic activation (peak time points of 10 min and 90 min) of p38 in a protein kinase A (PKA)-independent manner [17]. β-Arrestin 1 depletion leads to a significant lowering of the early-phase p38 activation without affecting the late-phase response [17]. On the other hand, depletion of β-arrestin 2 augments late-phase p38 activation without significantly altering the early-phase response. Interestingly, only the early phase of p38 activation appears to be responsible for isoproterenol-induced F-actin rearrangement [17] and therefore suggests a functional diversification of β-arrestin isoforms at the level of cellular outcomes.

β_2AR–β-Arrestin Axis: The Disease Connection

Does the functional divergence of β-arrestin isoforms manifest *in vivo*, and is it relevant in pathophysiological contexts? There are examples suggesting this is the case. In a mouse model of myocardial infarction (MI), β-arrestin 1 appears to negatively influence recovery because β-arrestin 1 KO mice exhibit overall increased cardiac function, including lower apoptosis, smaller infarct size, and decreased levels of adverse remodeling compared to wild-type (WT) mice [18]. By contrast, β-arrestin 2 plays a protective role in MI by suppressing the inflammatory response of macrophages in the infarcted area, and β-arrestin 2 KO mice display higher mortality [19]. Another example of functional specialization of β-arrestin isoforms in β_2AR signaling *in vivo* was described recently for stress-induced DNA damage response pathway [20]. Catecholamines (e.g., isoproterenol) induce chronic stress in mice that results in p53 (tumor protein P53, TP53) degradation and accumulation of DNA damage, a response that is mediated by the phosphoinositide 3-kinase (PI3K)/Akt (protein kinase B) signaling pathway [20]. β-Arrestin 1 interacts with both p53 and ubiquitin E3 ligase MDM2 (mouse double minute 2 homolog), and thereby brings them in close proximity to trigger ubiquitination and degradation of p53 [20]. β-Arrestin 1 also mediates phosphorylation of MDM2, a key event that triggers the onset of this pathway and a process that is crucial for β_2AR ubiquitination [20]. Moreover, amyloid β peptide (Aβ), the main component of amyloid plaques formed in Alzheimer's disease, is found to interact with the N-terminus of the β_2AR and mediates its internalization [21,22]. Interestingly, Aβ-induced β_2AR internalization selectively depends on β-arrestin 2 and not on β-arrestin 1, at least in mouse embryonic fibroblasts (MEFs) overexpressing β_2AR [22].

These examples clearly establish differential functional roles of β-arrestin isoforms in β_2AR system and also indicate potential functional specialization in selected pathophysiological contexts. Importantly, many of these paradigms are observed in other GPCR systems, hinting at potentially conserved nature of functional specialization of β-arrestin isoforms.

Angiotensin II Type 1 Receptor ($AT_{1a}R$): Functional Antagonism of β-Arrestin Isoforms

$AT_{1a}R$, the primary receptor for the vasoconstrictor peptide angiotensin II, has been one of the most widely studied systems to understand β-arrestin signaling [23]. Agonist-induced internalization of $AT_{1a}R$ is affected by the depletion of either β-arrestin isoform; however, similar to β_2AR, the effect of β-arrestin 2 depletion is more profound than that of β-arrestin 1 [9]. Interestingly, however, β-arrestin 2 depletion dramatically reduces agonist-induced ERK activation while short interfering RNA (siRNA) targeting β-arrestin 1 surprisingly leads to a robust increase (Figure 1B) [24]. G protein coupling of $AT_{1a}R$ as assessed by the accumulation of inositol phosphate (IP3) is not significantly altered by depletion of either isoforms of β-arrestins [9,24]. This suggests that the effects observed on ERK signaling do not arise from a difference in desensitization, but rather represent the modulation of G protein-independent ERK pathway. Along the same lines, ERK activation by SII-AngII (a β-arrestin biased ligand for $AT_{1a}R$) is also augmented upon depletion of β-arrestin 1, while it is completely abolished in response to β-arrestin 2 siRNA. These data support that the two isoforms, at least in HEK-293 cells, can display functional antagonism with respect to ERK signaling [9,24]. An interesting twist to this functional specialization is observed for $AT_{1a}R$-dependent ERK activation in rat vascular smooth muscle cells (VSMC) [25]. Unlike HEK-293 cells, depletion of β-arrestin 1 does not alter ERK activation, while β-arrestin 2 significantly diminishes it at late timepoints [25]. These findings suggest that the pattern of isoform functional specialization even for the same receptor–ligand combination can be cell type-dependent.

Functional specialization of β-arrestins along the $AT_{1a}R$–β-arrestin axis is also evident in several physiological and pathophysiological conditions. For example, $AT_{1a}R$ stimulation-dependent development of abdominal aortic aneurism (AAA) in mice appears to selectively involve β-arrestin 2 because β-arrestin 2 KO animals exhibit significantly attenuated AAA [27]. β-Arrestin 2 appears to mediate this effect via the ERK signaling pathway and cyclooxygenase 2 induction [27]. Along the same lines, in a mice model of Marfan syndrome (MFS), abnormal mechano-signaling and increased $AT_{1a}R$ signaling, especially via the ERK MAP kinase pathway, leads to enlarged and dysfunctional heart [28]. Interestingly, selective absence of β-arrestin 2 restores normal mechano-signaling, heart size, and cardiac function [28]. Moreover, β-arrestin biased ligands of $AT_{1a}R$ exert cardioprotective and cytoprotective effects during acute cardiac injury primarily through β-arrestin 2 signaling pathway, and these effects are lost in β-arrestin 2 KO mice [29].

GPR109A and Flushing Response: Functional Specialization *In Vivo*

Additional evidence for the existence of functional differences in β-arrestin isoforms *in vivo* comes from studies on GPR109A [30]. Nicotinic acid (NA) (also referred to as vitamin B3) is a commonly used medicine for decreasing triglyceride level and increasing high-density lipoprotein (HDL) in humans [31]. Most NA-induced physiological effects are thought to be driven primarily by activation of a class A GPCR, GPR109A (also referred to as niacin receptor type 1). A drawback of NA therapy is an adverse side-effect – a flushing response that results in an intense burning and itching sensation in the majority of patients. GPR109A recruits both isoforms of β-arrestins in response to NA stimulation and they are both necessary for ERK activation downstream of this receptor [30] (Figure 2A). NA-induced lipolysis, as assessed by increase in serum-free fatty acids, is primarily mediated by heterotrimeric G protein coupling to GPR109A and it remains unaltered in β-arrestin (either isoform) KO mice [30]. Surprisingly, however, cutaneous flushing is drastically lowered in β-arrestin 1 KO but not in β-arrestin 2 KO mice [30]. Furthermore, phosphorylation of a downstream ERK target, cytosolic phospholipase A2

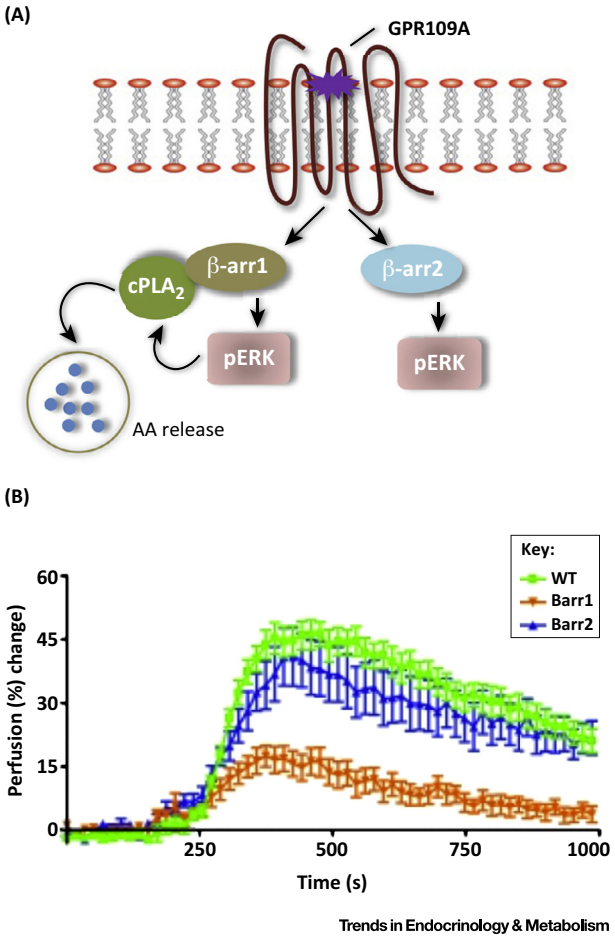

Figure 2. Physiological Manifestation of Functional Divergence of β-Arrestin Isoforms. (A) GPR109A, also referred to as niacin receptor recruits both isoforms of β-arrestins upon agonist stimulation, and receptor-dependent ERK (extracellular signal-regulated kinase) activation is sensitive to the depletion of either of the isoforms in cultured cells. Only β-arrestin 1 binds to the downstream effector cytosolic phospholipase A2 ($cPLA_2$), and binding is required for phosphorylation of $cPLA_2$ and activation of arachidonic acid (AA) release. (B) Nicotinic acid (NA)-induced flushing as measured by perfusion of the ventral mouse ear with laser Doppler perfusion imaging in WT (wild-type) or β-arrestin KO (knockout) mice strictly depends on β-arrestin 1. β-Arrestin 1 KO mice exhibit a significantly reduced flushing response, while β-arrestin 2 KO mice show a similar pattern of NA-induced flushing as the WT mice. Barr1, β-arrestin1 KO mice; Barr2, β-arrestin 2 KO mice. Figure 2B adopted and modified from [30].

(cPLA2), in response to NA and subsequent arachidonic acid (AA) release is restricted to β-arrestin 1 [30] (Figure 2B). Thus, it is tempting to speculate that β-arrestin 1-dependent ERK activation perhaps has a significantly different spatiotemporal profile from activation via β-arrestin 2, which in turn could be responsible for β-arrestin isoform selectivity in $cPLA_2$ activation and AA release.

Following the Pattern: Other Examples

Several other GPCRs also display functional specialization of β-arrestin isoforms (Table 1). For example, melanocortin 1 receptor (MC_1R), a $G\alpha_s$-coupled family A GPCR primarily responsible for proliferation and differentiation of melanocytes, primarily utilizes β-arrestin 2 for desensitization and internalization [32]. Interestingly, β-arrestin 1 competes with β-arrestin 2 for receptor binding and exerts an inhibitory effect on β-arrestin 2-mediated internalization and desensitization [32]. Similarly, stimulation of a nucleotide-binding GPCR, P_2Y_2R, with its natural agonist UTP leads to comparable recruitment of both β-arrestin isoforms [33]; however, β-arrestin 1 plays a dominant role in its desensitization [34,35] (Figure 1C). Interestingly, depletion of β-arrestin 2 does not alter UTP-stimulated ERK and p38 activation, whereas β-arrestin 1 knockdown significantly augments these responses [34]. Furthermore, β-arrestin 1 is required for agonist-induced migration of arterial smooth muscle cells (ASMCs), while β-arrestin 2 is dispensable. In stark contrast, for the endothelin receptor subtype A (ET_AR), endothelin 1 (ET1)-induced ERK and p38 activation in ASMCs is almost entirely dependent on β-arrestin 2, although both isoforms appear to be necessary for ET1-induced migration of ASMCs [34].

Table 1. Selected Examples of Functional Specialization of β-Arrestin Isoforms[a,b]

GPCR	Function of β-arr 1/2	Experimental approach[c]	Refs
β_2AR	β-Arr 2 plays a dominant role in desensitization, endocytosis, and ubiquitination. β-Arr 1 is implicated in chronic stress-induced DNA damage response	siRNA, OE, KO	[9,20]
$AT_{1a}R$	β-Arr 2 mediates G protein-independent ERK signaling and positively regulates chemotaxis. β-Arr 1 inhibits ERK activation and mediates stress-fiber formation	siRNA, OE, KO	[24,100–102]
GPR109A	β-Arr 1 is required for NA-induced $cPLA_2$ phosphorylation, AA release, and cutaneous flushing	siRNA, OE, KO	[30]
MC_1R	β-Arr 2 but not β-arr 1 mediates internalization and desensitization	siRNA	[32]
CB_1R	The allosteric ligand ORG27569 mediates β-arr 1-induced activation of ERK1/2, MEK1/2, and SRC. β-Arr 2 is crucial for receptor internalization	siRNA, OE	[37]
MOR	β-Arr 1 but not β-arr 2 is required for agonist (DAMGO)-induced receptor ubiquitination	KO	[86]
DOR	β-Arr 1 but not β-arr 2 mediates p27 and FOS acetylation and transcription	OE, siRNA	[58]
PAR_2	β-Arr 1 mediates the early phase of receptor endocytosis while β-arr 2 is crucial for the late phase. β-Arr 1 but not β-arr 2 is likely to have a role in lysosomal receptor degradation	KO	[87]
FZD	β-Arr 2 but not β-arr 1 is recruited to the receptor and mediates its endocytosis as well as agonist-induced tumorigenesis	siRNA	[88]
C3aR	β-Arr 2 mediates desensitization and internalization, and also inhibits NF-μ2 activation and CCL4 generation. β-Arr 1 promotes mast cell degranulation	siRNA	[89,90]
CXCR4	β-Arr 2 but not β-arr 1 predominantly mediates ERK activation as well as receptor internalization	siRNA	[91]
CXCR2	β-Arr 2 but not β-arr 1 is required for β2-integrin regulation, Rap1 activation, and adhesion strengthening	siRNA	[92]
CXCR7	β-Arr 2 but not β-arr 1 is required for CCL12 accumulation	siRNA, KO	[93]
B_2R	β-Arr 1 suppresses myometrial cell movement while β-arr 2 is inhibitory to p38 activation	siRNA	[94]
GPR43	β-Arr 2 but not β-arr 1 preferentially interacts with the receptor and mediates its endocytosis	siRNA	[95]
GPR54	β-Arr 2 but not β-arr 1 mediates G protein-independent ERK signaling	siRNA	[96]
LPAR	β-Arr 2 but not β-arr 1 regulates NF-κB activation and IL-6 expression	KO	[97]
P2Y2	β-Arr 1 but not β-arr 2 plays a more dominant role in desensitization and cellular migration	siRNA	[33,34]
TA_2R	β-Arr 2 but not β-arr 1 mediates actin remodeling-dependent receptor endocytosis	OE	[98]
D_2R	β-Arr 2 but not β-arr 1 scaffolds a multiprotein complex involving PP2A and Akt that mediates dopaminergic neurotransmission and behavior	OE, KO	[99]

[a]There may be further examples of β-arrestin isoform selectivity in the literature; this table is not exhaustive.
[b]Abbreviations: B_2R, bradykinin receptor subtype 2; $C_{3a}R$, complement type 3 receptor; CXCR, chemokine (C-X-C motif) receptor; DOR, δ-opioid receptor; D_2R, dopamine receptor subtype 2; FZD, frizzled receptor; LDLR, low density lipoprotein receptor; LPAR, lysophosphatidic acid receptor; MOR, μ-opioid receptor; PAR_2, protease activated receptor subtype 2; TA_2R, thromboxane receptor subtype 2; TNFR, tumor necrosis factor receptor.
[c]Experimental procedures: KO, β-arr knockout in mice or mouse embryonic fibroblast cell lines from knockout mice; OE, transfection-based cellular overexpression of β-arr isoforms; siRNA, selective depletion of a β-arr isoforms.

In addition to orthosteric ligands, allosteric modulators of GPCRs have emerged as promising therapeutic tools. Cannabinoid receptor subtype 1 (CB_1R) belongs to the class A subfamily of GPCRs and, in response to conventional agonists, it recruits both isoforms of β-arrestins [36,37]. Interestingly, however, an allosteric ligand (ORG27569) that acts as a β-arrestin biased ligand induces activation of SRC (SRC proto-oncogene, non-receptor tyrosine kinase), MEK1/2 (MAP kinase kinase 1/2, MAP2K1/2), and ERK1/2 in a strictly β-arrestin 1-dependent fashion, while it triggers internalization of the receptor in a β-arrestin 2-dependent manner (Figure 1D) [37]. This finding presents an intriguing example of functional dichotomy where different isoforms of β-arrestins govern different functional outcomes induced by a biased ligand.

Potential Areas of Possible Functional Divergence

In addition to the above-mentioned examples, there are further key areas that might also harbor β-arrestin isoform-dependent functional specialization, although these remain to be experimentally documented. For example, TRV130, a G protein biased agonist of the μ-opioid receptor (μ-OR), shows significantly different efficacies of β-arrestin 2 recruitment to the human, mouse, rat, and dog receptors [38], suggesting some degree of species-dependence. Along the same lines, ligand-dependent MAPK activation, especially of the p38 pathway, appears to exhibit different patterns between the human and rat μ-opioid receptors [39]. It would be interesting to investigate whether the same receptor from different species might have a differential preference for the two β-arrestin isoforms. Another interesting avenue that remains somewhat underexplored is the potential functional specialization of β-arrestin isoforms in different cell types [40]. It is plausible that different cell types might express different isoforms (and/or levels) of different G protein-coupled receptor kinases (GRKs), and therefore a given receptor might exhibit a differential phosphorylation pattern resulting in preferential recruitment of one or the other β-arrestin isoforms. Furthermore, the onset and termination of recently discovered post-G protein signaling (or sustained signaling after receptor internalization) demonstrated for multiple GPCRs [41–43] might also involve some extent of functional diversification between the two β-arrestin isoforms, and this warrants further investigation.

The examples described above, taken together with other instances highlighted in Table 1, establish that the functional divergence of β-arrestin isoforms in the context of GPCR signaling and regulation is not limited to a given receptor system, but appears instead to be a conserved phenomenon in the GPCR family. Although multiple lines of evidence suggest functional divergence *in vivo* as well, many of the examples described in cell lines need to be experimentally validated under physiological conditions to fully appreciate the potential therapeutic implications of this paradigm. In addition, growing evidence points to roles for β-arrestins that extend beyond GPCRs, and in several of those cases functional divergence of β-arrestins has also been reported (Box 2).

Emerging Mechanistic Basis of Functional Divergence

What is the mechanistic basis of functional divergence in β-arrestin isoforms? Although a clear-cut mechanistic framework remains to be established and rigorously tested, multiple clues have begun to emerge from a series of structural, biophysical, and proteomic studies.

Hints from Sequence Analysis

β-Arrestins 1 and 2 display more than 70% sequence identity, and the crystal structures of both isoforms are highly similar and consist primarily of anti-parallel β-sheets (Figure 3A,B). The major divergence in their sequences occurs in the C-terminus that harbors a clathrin binding site. Although the sequence of clathrin box is highly conserved between the two isoforms [44,45], and they are both known to bind clathrin, it is plausible that neighboring residues induce a conformational change that might be responsible for a difference in affinity and temporal binding. This might explain the differential impact of the two isoforms on the internalization of several

Box 2. β-Arrestin Promiscuity: Going Beyond GPCRs

There is substantial evidence that, in addition to GPCRs, β-arrestins also bind to and regulate other membrane proteins including ion channels, transporters, and tyrosine kinase receptors [83]. For these non-GPCR systems, the roles of β-arrestins primarily appear to be in mediating their downregulation by promoting endocytosis or ubiquitination. Functional differences of the β-arrestin isoforms are also evident for these non-GPCR targets. For example, $AT_{1a}R$, β-arrestin, and transient receptor potential channel type 4 (TRPV4, a non-selective Ca^{++} channel), form a multiprotein complex. Agonist stimulation of $AT_{1a}R$ in this complex leads to ubiquitination of TRPV4 in a β-arrestin-dependent fashion [60]. Knocking down β-arrestin 1 inhibits TRPV4 ubiquitination; however, removal of β-arrestin 2 results in increased ubiquitination of TRPV4. These findings mirror the functional antagonism of the two isoforms observed for ERK signaling downstream of $AT_{1a}R$ as discussed earlier [24]. Although this specific requirement of β-arrestin 1 is likely to emerge from its scaffolding of AIP4 (an E3 ubiquitin ligase crucial for TRPV4 ubiquitination), it is also possible that β-arrestin 2 maintains a check on TRPV4 ubiquitination by bringing a DUB (deubiquitinating enzyme) into the proximity of TRPV4. Another example of functional specificity between the β-arrestin isoforms in a potentially non-GPCR but therapeutically promising system is observed in the mouse model of chronic myelogenous leukemia (CML) [84]. Here, absence of β-arrestin 2 but not β-arrestin 1 leads to significant reduction of self-renewal of hematopoietic stem cells (HSC) [84]. Furthermore, β-arrestin 2 KO mice exhibit dramatically higher survival rates compared to β-arrestin 1 KO mice when they were both transplanted with HSCs infected with tyrosine kinase BCR–ABL virus [84]. Similarly to β_2AR system, β-arrestin 2 appears to play a more prominent role in endocystosis of the low-density lipoprotein receptor (LDLR) compared to β-arrestin 1 [85]. These examples highlight that functional divergence of β-arrestins extends beyond GPCRs.

different GPCRs. Furthermore, although the sequences of the major loops involved in interactions with activated GPCRs are mostly conserved, they display structural and orientation differences that might be crucial for determining receptor specificity and affinity. The crystal structure of β-arrestin 1 bound to the phosphopeptide corresponding to the C-terminus of V2 vasopressin receptor (V_2R, referred to as V_2Rpp) [46,47] identifies the Lys and Arg residues that interact with the phosphate groups of V_2Rpp, and these residues are highly conserved in β-arrestin 2. This observation supports a potentially conserved interaction mechanism between the two isoforms and the phosphorylated C-terminus of the GPCRs. Even so, it may not be ruled out that different sets of Lys and Arg in β-arrestin 1 versus β-arrestin 2 engage the phosphate groups, and thereby fine-tune the functional outcome.

Conformational Differences

It is possible that, despite overall structural similarity, there might exist local conformational differences between the two β-arrestin isoforms that are responsible for some of the functional divergences. Supporting evidence for this notion arises from *in vitro* studies that revealed fine conformational differences between activated β-arrestin 1 and 2, especially in the inter-domain hinge region [48,49]. More recently, hydrogen–deuterium exchange approach coupled with mass spectroscopy (HDX-MS), a biophysical method that reports local conformational changes in proteins, has revealed that the β-strands II, III, and IV are more dynamic in β-arrestins 2, while the middle loop of β-arrestin 1 shows a relatively higher flexibility in solution [50,51]. Although a direct link between these conformational differences and distinct functional outcomes remains to be directly demonstrated, these findings provide a hypothesis that can now be tested through directed mutagenesis experiments. It must be underlined, however, that a limitation of these studies is using either phosphopeptide-based activation of β-arrestins or partially constitutively activated β-arrestin mutants. Therefore, the quest to delineate the exact nature of activated GPCR-bound conformations of the two β-arrestin isoforms at high resolution and to couple them to functional specialization remains open.

Phosphorylation Bar Code and the Affinity of the GPCR–β-Arrestin Interaction

The 'bar-code' hypothesis, that differential phosphorylation patterns of the GPCR C-terminus by different GRK isoforms results in different conformations of recruited β-arrestins and distinct sets of functional outcomes (e.g., desensitization vs signaling), also offers potential mechanistic insights into functional divergence [52,53]. Considering that the extent and spatial pattern of receptor phosphorylation sites are key to β-arrestin recruitment, it is tempting to speculate that phosphorylation 'bar-coding' might also be responsible for selectively engaging distinct isoforms

(A)

```
β-arr1          MGDK-GTRVFKKASPNGKLTVYLGKRDFVDHIDLVEPVDGVVLVDPEYLKERRVYVTLTC
β-arr2          MGEKPGTRVFKKSSPNCKLTVYLGKRDFVDHLDKVDPVDGVVLVDPDYLKDRKVFVTLTC
                **:* *******:*** ***************:* *:***********:***:*:*:*****

β-arr1          AFRYGREDLDVLGLTFRKDLFVANVQSFPPAPEDKKPLTRLQERLIKKLGEHAYPFTFEI
β-arr2          AFRYGREDLDVLGLSFRKDLFIANYQAFPPTPNPPRPPTRLQERLLKKLGQHAHPFFFTI
                ***************:******:** *:***:*:   :* *******::***:**:** * *

β-arr1          PPNLPCSVTLQPGPEDTGKACGVDYEVKAFCAENLEEKIHKRNSVRLVIRKVQYAPERPG
β-arr2          PQNLPCSVTLQPGPEDTGKACGVDFEIRAFCAKSLEEKSHKRNSVRLVIRKVQFAPEKPG
                * ***********************:*::****:.**** ***************:***:**

β-arr1          PQPTAETTRQFLMSDKPLHLEASLDKEIYYHGEPISVNVHVTNNTNKTVKKIKISVRQYA
β-arr2          PQPSAETTRHFLMSDRSLHLEASLDKELYYHGEPLNVNVHVTNNSTKTVKKIKVSVRQYA
                ***:*****:*****:.**********:******:.********:.*******:******

β-arr1          DICLFNTAQYKCPVAMEEADDTVAPSSTFCKVYTLTPFLANNREKRGLALDGKIKHEDTN
β-arr2          DICLFSTAQYKCPVAQVEQDDQVSPSSTFCKVYTITPLLSNNREKRGLALDGKIKHEDTN
                *****.*********  * ** *:**********:**:*:*********************

β-arr1          LASSTLLREGANREILGIIVSYKVKVKLVVSRGGLLGDLASSDVAVELPFTLMHPKPKEE
β-arr2          LASSTIVKEGANKEVLGILVSYRVKVKLVVSRGG--------DVSVELPFVLMHPKPHDH
                *****:::****:*:***:***:***********        **:*****.******::.

β-arr1          PPHR---------------EVPEHETPVDTNLIELDTN---DDDIVFEDFARQRLKGMKD
β-arr2          IALPRPQSAPTHPPTLLPSAVPETDAPVDTNLIEFETNYATDDDIVFEDFARLRLKGLKD
                  .                 *** ::*********:**   *********** **** :**

β-arr1          -DKEEEEDGTGSPRLNDR
β-arr2          EDYDDQFC----------
                 * ::
```

(B)

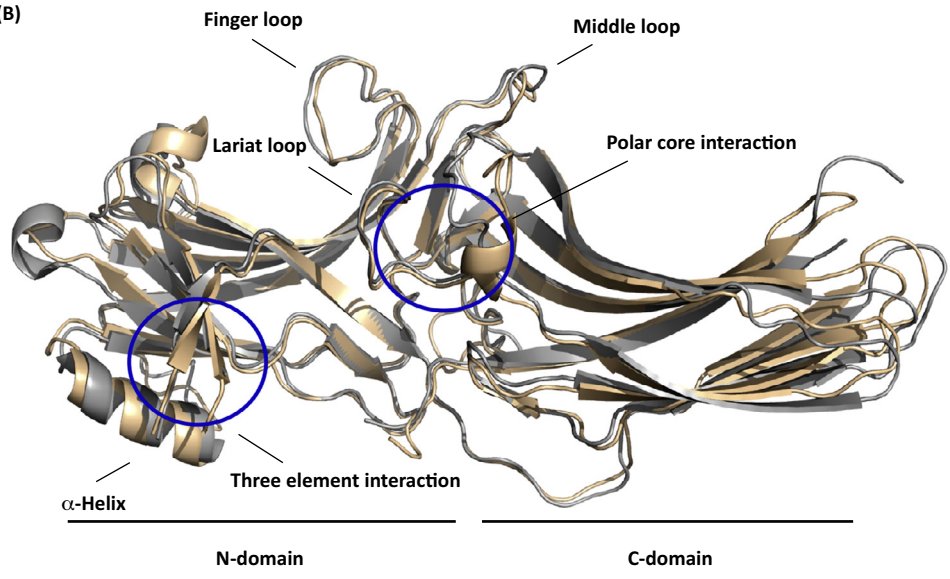

Figure 3. Overall Sequence Similarity and Structural Homology of β-Arrestin Isoforms. (A) Sequence alignment of bovine β-arrestins 1 and 2 (β-arr1/2) was carried out using the web-based multiple sequence alignment tool CLUSTALW. β-Arrestin 1 sequence is presented in grey while β-arrestin 2 sequence is shown in orange. The primary sequence of the two isoforms is highly identical for the most part except at the C-terminus (highlighted in blue box). The clathrin binding site, referred to as the clathrin box, is highlighted by the red box. Lys (K) and Arg (R), which are within interacting distance of the phosphate groups of the C-terminus of the arginine vasopressin subtype 2 receptor (V_2R) as revealed by the crystal structure of V_2Rpp-bound β-arrestin 1, are highlighted by blue boxes. These residues are highly conserved between β-arrestin 1 and 2. The regions which display significant sequence divergence between two isoforms are highlighted in dashed boxes. (B) Superimposition of β-arrestin 1 (shown in grey) and 2 (shown in gold) crystal structures in basal conformations reveals an overall very similar 3D structure. The two sets of interactions referred to as 'three element interaction' and the 'polar core interaction', that maintain β-arrestins in their basal conformation, are highlighted. The three different loop regions, referred to as the finger loop, middle loop, and lariat loop, that are thought to be crucial for interaction with GPCRs are also highlighted.

of β-arrestins to the activated receptor. This differential and preferential recruitment might then dictate the functional outcome downstream of the receptor. Furthermore, it is also possible that different cell types express different GRK isoforms that in turn can result in different phosphorylation bar codes on GPCRs. Such a scenario might be responsible for cell type-specific functional actions of β-arrestin isoforms as observed for ERK activation downstream of $AT_{1a}R$ in HEK-293 versus vascular smooth muscle cells [25]. Along the same lines, the broad classification of GPCRs into two major classes (classes A and B) with respect to β-arrestin interaction also provides some hints [54]. Class A GPCRs such as β_2AR interact with β-arrestins transiently, while class B GPCRs such as $AT_{1a}R$ exhibit a more robust interaction. Class A receptors show preferential binding to β-arrestin 2 over β-arrestin 1, while class B receptors bind to both isoforms indiscriminately. Although a systematic study to evaluate the connection between functional distinction of the β-arrestin isoforms and class A versus B recruitment pattern is still lacking, one can speculate that differential affinity between a GPCR and β-arrestin isoforms might also direct their functional specialization.

Differences in Nuclear Localization

β-Arrestin isoforms are primarily cytoplasmic; however, emerging data reveal nucleocytoplasmic shuttling that might contribute to their functional divergence [55–58]. β-Arrestin 1 contains a classical nuclear localization sequence (NLS) motif in its N-terminal domain and it can be imported into the nucleus [55]. β-Arrestin 2 also contains the conserved NLS motif; however, it also harbors a leucine-rich nuclear export signal (NES) in its C-terminal domain [57]. Multiple studies suggest that both isoforms can be imported into the nucleus, but perhaps β-arrestin 1 is retained there longer while β-arrestin 2 is rapidly exported back to the cytoplasm [58]. Such a scenario could explain the isoform specificity with respect to nuclear function – for example strict dependence of MDM2 phosphorylation and p53 degradation on β-arrestin 1 in the stress-induced DNA damage response pathway [20].

Non-Overlapping Interactome of β-Arrestin Isoforms

A key property of β-arrestins is the scaffolding of different proteins involved in diverse cellular functions [8]. An interesting clue in to their functional divergence comes from a global proteomic analysis of their interaction partners upon stimulation of $AT_{1a}R$ [59]. This analysis identified significantly different interactomes for the two isoforms, not only under basal conditions but also in response to agonist stimulation [59]. The number of proteins interacting with β-arrestin 2 is higher than for β-arrestin 1, and it is therefore tempting to speculate that β-arrestin 2 has a broader functional outreach. Although this difference might simply reflect the relative expression levels of the two isoforms, in most commonly used cell types β-arrestin 1 is typically expressed at higher levels than β-arrestin 2. The non-overlapping interaction partners belong to different functional categories, and many of these interactions have been validated and studied in detail, adding confidence to proteomics-based predictions [59–61]. Although this analysis used $AT_{1a}R$ as a model system, it is safe to assume that other receptors might also manifest an overall similar pattern and, therefore, this non-overlapping set of interaction partners provides a potential basis for the specific functional roles of the two β-arrestin isoforms.

Disease Connection and Therapeutic Implications

GPCRs are prominent drug targets, and conventionally the primary focus has been on designing antagonists or agonists to turn the receptors 'on' or 'off' depending on the given pathophysiological condition [5,62]. More recently, the concept of **biased agonism** – in other words, the ability of some ligands (referred to as biased ligands) to selectively trigger one or other signaling pathways – is beginning to significantly impact on the GPCR drug discovery landscape [63–65]. However, an area that remains relatively unexplored but harbors significant therapeutic potential is selective targeting and functional inhibition of individual β-arrestin isoforms. There are many emerging instances where β-arrestins appear to play a central role in disease onset.

For example, β-arrestin 2 physically interacts with the γ-secretase complex, increases its catalytic activity, and promotes the generation of amyloid-β peptide in a transgenic mouse model of Alzheimer's disease [26]. Furthermore, there is evidence to suggest that β-arrestin 2 contributes to insulin sensitivity and is substantially downregulated in a diabetic mouse model [66]. Absence of β-arrestin 2 decreases AKT, FOXO (forkhead box O), and GSK3β (glycogen synthase kinase 3β) phosphorylation in mouse pancreatic islets, and β-arrestin 2 KO mice display impaired glucose tolerance and insulin secretion [67,68]. Moreover, β-arrestin 1 appears to suppress diet-induced obesity in mice and influence whole-body insulin sensitivity [69]. In addition, lack of β-arrestin 1 also promotes production of anti-inflammatory cytokines (e.g., interleukins IL-10 and IL-22) while suppressing proinflammatory IL-6, thereby attenuating gut inflammation and leading to protective effects in a mouse model of colitis [70].

Does targeting β-arrestin isoforms have therapeutic promise? In fact, selective disruption of the β-arrestin 2–ERK2 interaction using an RNA **aptamer** was found to inhibit leukemic cell growth *in vitro* [71]. Along the same lines, one can envisage designing small-molecule cell-permeable compounds or **pepducins** as inhibitors of β-arrestin functions, or a **synthetic antibody fragment** that can selectively target functional domains on β-arrestin isoforms [72–74]. It would also be interesting to explore whether it is possible to add one more layer of specificity in biased GPCR ligand design through identifying ligands that can selectively engage one or other isoform of β-arrestin. This could be particularly helpful in scenarios where the two β-arrestin isoforms display functional antagonism.

Outstanding Questions

How broad is the impact of β-arrestin isoform-selective functions on the cellular and physiological consequences of GPCR signaling?

How relevant is the functional divergence of β-arrestin isoforms when designing GPCR biased ligands for potential therapeutic use?

What is the detailed mechanistic basis of the functional divergence of the two β-arrestin isoforms?

How different will the structures of two β-arrestin isoforms be in activated (i.e., GPCR bound) conformations? Will they show an overall similar two-step binding mechanism?

Can small-molecule inhibitors be designed to selectively target β-arrestin isoforms with therapeutic potential?

Concluding Remarks and Future Perspectives

In conclusion, the functional divergence of β-arrestin isoforms in GPCR regulation and signaling adds another layer of fine-tuning in GPCR function. Considering the rapidly-expanding repertoire of β-arrestin functions, we are likely to witness many more examples of isoform-specific functional specialization in the coming years. Rapid progress in the area of GPCR structural biology has set the stage for direct visualization of structural determinants that govern the functional divergence in β-arrestin isoforms [75–77]. A key focus area going forward is likely to be structure determination of GPCR–β-arrestin complexes, and the recent structure of the rhodopsin–visual arrestin complex represents a start in this direction [78]. Furthermore, complementary dynamic studies in solution to decipher mechanistic basis of this intriguing functional specialization will also be essential, although stable preparation of the GPCR–β-arrestin complex remains a hurdle to be surmounted. Furthermore, this emerging framework should be factored in so as to fully appreciate the depth of GPCR **biased signaling**, and should also be considered while designing GPCR biased ligands, especially for therapeutic purposes. Exploring the full repertoire of physiological roles of β-arrestin isoforms remains a major challenge and will require the development of focused animal models – for example, tissue-specific KO of β-arrestin isoforms instead of the global KO mice utilized currently. Finally, direct modulation and/or inhibition of β-arrestin function in pathophysiological conditions harbors significant therapeutic potential and offers a unique possibility for novel drug design involving the GPCR system. However, considering the high degrees of sequence and structural similarity, targeting individual isoforms is likely to be very challenging.

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