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REVIEW

Diversity in arrestin function

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Abstract The termination of heptahelical receptor signaling is a multilevel process coordinated, in large part, by members of the arrestin family of proteins. Arrestin binding to agonist-occupied receptors promotes desensitization by interrupting receptor-G protein coupling, while simultaneously recruiting machinery for receptor endocytosis, vesicular trafficking, and receptor fate determination. By simultaneously binding other proteins, arrestins also act as ligand-regulated scaffolds that recruit protein and lipid kinase, phosphatase, phosphodiesterase, and ubiquitin ligase activity into receptor-based multiprotein 'signalsome' complexes. Arrestin-binding thus 'switches' receptors from a transient G protein-coupled state to a persistent arrestin-coupled state that continues to signal as the receptor transits intracellular compartments. While it is clear that signalsome assembly has profound effects on the duration and spatial characteristics of heptahelical receptor signals, the physiologic functions of this novel signaling mechanism are poorly understood. Growing evidence suggests that signalsomes regulate such diverse processes as endocytosis and exocytosis, cell migration, survival, and contractility.

Keywords G protein-coupled receptor · G protein · Arrestin · Desensitization · Endocytosis · Signal transduction

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Introduction

Heptahelical G protein-coupled receptors (GPCRs) function as ligand-activated guanine nucleotide exchange factors (GEFs) for heterotrimeric G proteins. Agonist binding stabilizes the receptor in an 'active' conformation that enables it to catalyze the exchange of GTP for GDP on heterotrimeric G protein Gα subunits, leading to dissociation of the GTP-bound Gα subunit from the Gβγ subunit heterodimer. Once dissociated, free Gα-GTP and Gβγ subunits regulate the activity of enzymatic effectors, such as adenylate cyclases, phospholipase C isoforms, and ion channels, generating small molecule second messengers that control the activity of key enzymes involved in intermediary metabolism.

G protein-mediated signaling is subject to extensive negative regulation. 'Turning off' the signal involves several steps that collectively limit signal duration and render the cell less responsive to subsequent stimuli. Second messengers are rapidly neutralized by cyclic nucleotide phosphodiesterases, phosphatidylinositol phosphatases, diacylglycerol kinases, and the reuptake and extrusion of cytosolic calcium. G protein activity is limited by the intrinsic GTPase activity of Gα subunits as well as the extrinsic action of regulators of G protein signaling (RGS)

proteins, which function as GTPase activating proteins (GAPs) to return G proteins to their inactive heterotrimeric state [1]. The efficiency of receptor-G protein coupling is controlled by phosphorylation. Heterologous desensitization, so named because it does not require ligand occupancy, results from phosphorylation of GPCR intracellular domains by second messenger-dependent protein kinases like protein kinase (PK)A and PKC. This phosphorylation is sufficient to impair receptor-G protein coupling [2]. In contrast, homologous desensitization is both sensitive to receptor conformation and dependent on the binding of accessory proteins. Specialized G protein-coupled receptor kinases (GRK1-7) phosphorylate agonist-occupied receptors on serine or threonine residues located on the receptor C-terminus or the third intracellular loop [3]. GRK-phosphorylated receptors recruit arrestins, which translocate from the cytosol to the plasma membrane to physically interdict receptor-G protein coupling. The two non-visual arrestins, arrestin 2 (β-arrestin 1) and arrestin 3 (β-arrestin 2), further diminish signaling by acting as adapter proteins that link receptors to the clathrin-dependent endocytic machinery [4]. Arrestin-dependent sequestration, or endocytosis, limits signal duration, removes receptors from the cell surface making it less responsive to subsequent stimuli, and ultimately determines whether receptors are fated to 'resensitize' and recycle to the cell surface or undergo degradation.

While the role of arrestins in GPCR desensitization, sequestration, and intracellular trafficking are well studied, the concept that they function as signal transducers in their own right has only recently gained credence [5, 6]. Arrestins bind numerous other catalytic proteins that redistribute from the cytosol to the plasma membrane as arrestins dock with GRK-phosphorylated receptors. The ability of arrestins to function as ligand-regulated scaffolds enables them to nucleate the formation of multiprotein 'signalsomes' that confer additional signaling capacity on the receptor, leading to the generation of signals, such as activation of the extracellular signal-regulated kinase (ERK)1/2 and *c-jun* N-terminal kinase 3 (JNK3) mitogen-activated protein (MAP) kinase cascades, with spatial and temporal characteristics distinct from signals arising from G protein activation. Emerging evidence suggests that receptor–arrestin signalsomes regulate diverse processes, among them endocytosis and exocytosis, cell migration, survival, and contractility. The further discovery that G protein- and arrestin-mediated signals are not only mechanistically independent but also pharmacologically dissociable has potential implications for the development of novel GPCR-based therapeutics [7, 8]. Here, we review both facets of arrestin function', as signal terminators and transducers, and discuss the implications of arrestin-dependent signaling for heptahelical receptor biology and development of pharmacologic agents with 'biased' efficacy.

GRKs and arrestins: the desensitization tag team

Homologous desensitization is a two-step process initiated by GRK phosphorylation of the agonist-occupied receptor. There are seven known GRKs [3]. GRK 1 and 7 have restricted tissue expression similar to visual and cone arrestins and are important for sensory receptor function. GRK 2, 3, 5, and 6 are ubiquitously expressed and are critical for regulation of most heptahelical receptors by non-visual arrestins, while GRK4 is highly expressed in testis [9]. GRK 2 and 3 have C-terminal G$\beta\gamma$ subunit binding domains that recruit them to the plasma membrane and promote GRK activation [10, 11]. GRK 2 and 3 also interact with Gα proteins via N-terminal RGS homology domains that may further aid desensitization by allowing them to bind and hold the receptor, Gα and G$\beta\gamma$ subunits at arms length [12]. GRK 4, 5, and 6 lack these domains and are either constitutively located at the plasma membrane (GRK 4 and 6) or translocate by alternative means (GRK5).

There are four known genes encoding arrestins. Arrestin 1 (visual arrestin) and 4 (cone arrestin) have restricted expression patterns, localizing primarily to visual sensory tissue, where they regulate rhodopsin photoreceptor signaling. Arrestins 2 and 3 are ubiquitously expressed and interact with the vast majority of other G protein-coupled receptors. Visual arrestin and arrestins 2 and 3 are alternatively spliced with two protein isoforms each, and cone arrestin has five splice variants, but the functional significance of these splice variants is poorly understood [4]. All four arrestins share sequence and structural homology. Each has two receptor-interaction domains, an N-terminal domain and a C-terminal domain connected by a polar hinge region (Fig. 1a). In the cytosol, the arrestin C-terminus interacts with the polar core, holding the two domains of the protein in the inactive, or tight, conformation [13].

Homologous desensitization begins on the cue of receptor phosphorylation. GRK-phosphorylated receptors represent high-affinity docking sites for arrestins on the plasma membrane; the affinity of the β2 receptor–arrestin 2 interaction increases 10–30 fold with GRK2 phosphorylation of the receptor [14]. Upon ligand binding, arrestins rapidly move to the receptor, forming a stoichiometric complex. There is evidence that arrestins undergo significant conformational changes upon binding receptors [15]. The arrestin polar core interacts with phosphorylated residues on the receptor tail, displacing the C-terminus, while the concave surface of both the N- and C-terminal domains interact with the intracellular receptor loops [16]. Arrestin-

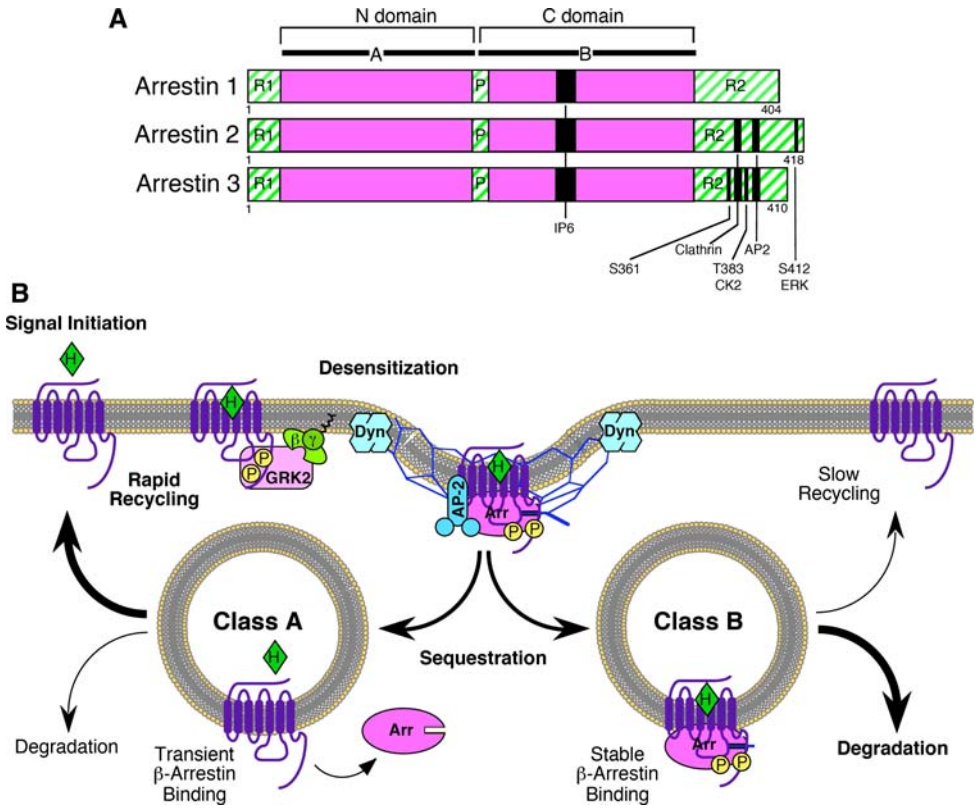

Fig. 1 Role of arrestins in GPCR desensitization and sequestration. **a** Line diagram comparing visual arrestin (arrestin 1) with the non-visual arrestins 2 and 3. The location of the crystallographically defined globular N and C domains are shown in relation to the functionally defined N-terminal (*A*) domain primarily responsible for receptor recognition, the C-terminal (*B*) domain responsible for secondary receptor recognition, the phosphate sensor domain (P), and the N- (R1) and C- (R2) terminal regulatory domains. Also indicated are the locations of binding sites for inositol 6-phosphate (IP6), clathrin and AP2, as well as sites of phosphorylation by casein kinase 2 (CK2) and extracellular signal regulated kinases (ERK). **b** Stability of the receptor–arrestin interaction defines two GPCR classes. Homologous desensitization of GPCRs results from arrestin (Arr) binding to agonist (H)-occupied receptors that have been phosphorylated by GRKs. The two non-visual arrestins direct GPCR sequestration by linking the receptor to clathrin and β2-adaptin (AP-2). Sequestration reflects the dynamin (Dyn)-dependent endocytosis of GPCRs via clathrin-coated pits. Once internalized, GPCRs exhibit two distinct patterns of arrestin interaction. *Class A* receptors dissociate from β-arrestin and are rapidly recycled to the plasma membrane. *Class B* receptors form stable receptor–arrestin complexes. These accumulate in endocytic vesicles and are either targeted for degradation or slowly recycled

bound receptors are sterically precluded from G protein coupling, producing homologous desensitization.

The two non-visual arrestins further attenuate signaling by recruiting endocytic components for clathrin-coated pit formation. Arrestin 2 and 3 directly bind clathrin heavy chain [17] and the β2 adaptin subunit [18] of the AP-2 complex. The clathrin interaction is mediated by an LIEL/F motif found in the C-terminus of arrestins 2 and 3 [19], while β2 adaptin interacts with an R × R motif just downstream of the clathrin binding motif [20]. Clathrin/AP-2 binding causes arrestin-bound receptors to cluster in clathrin-coated pits. The clathrin-coated pit is pinched off the plasma membrane by the motor protein, dynamin, causing the desensitized receptor to enter an endosomal pool (Fig. 1b).

Once off the plasma membrane, the stability of the receptor–arrestin complex is a critical determinant of receptor fate, impacting whether receptors are recycled back to the plasma membrane or targeted for degradation. Most GPCRs can be separated into one of two classes based on their affinity for the two non-visual arrestin isoforms and the longevity of the receptor–arrestin interaction [21]. Class A receptors have higher affinity for arrestin 3 than arrestin 2 and form transient receptor–arrestin complexes that dissociate soon after the receptor internalizes. Such receptors, e.g., β2 adrenergic, are rapidly resensitized and recycled back to the plasma membrane. Class B receptors have equivalent affinities for arrestin 2 and 3, and form stable receptor–arrestin complexes that remain intact as the receptor undergoes endosomal sorting. These receptors, e.g., angiotensin AT1a, are sequestered in endosomes and either recycle slowly or are degraded. Ubiquitination of both receptor and arrestin is important in this post-endocytic sorting [22, 23]. On binding the

receptor, arrestin 3 is ubiquitinated by the E3 ubiquitin ligase, mdm2, to which it binds directly. Arrestin ubiquitination stabilizes the receptor–arrestin complex and is necessary for internalization. When bound to class A receptors, arrestin is de-ubiquitinated once the complex internalizes, and de-ubiquitination appears to be required for arrestin release from the receptor. With class B receptors, the arrestin remains ubiquitinated and the complex stays intact throughout sorting. Receptor ubiquitination is also arrestin-dependent. While not essential for endocytosis, it plays a key role in selecting receptors for downregulation versus recycling [22, 24]. Ubiquitination of the β2 adrenergic receptor C-terminus or V2 vasopressin receptor third intracellular loop promotes proteosomal degradation. Different E3 ligases account for receptor ubiquitination; the β2 adrenergic receptor is ubiquitinated by the E3 ligase Nedd4 [25], while the chemokine receptor CXCR4 is ubiquitinated by the E3 ligase atrophin-interacting protein 4 (AIP4) [26].

A colorful palette of arrestin-mediated signals

The role of arrestins in GPCR desensitization, sequestration, and trafficking highlights their function as adapter proteins, linking desensitized receptors to the endocytic machinery and chaperoning them through the intracellular environment. The first clue that arrestin binding is a signaling mechanism in its own right was the discovery that arrestins bind Src family nonreceptor tyrosine kinases and recruit them to agonist-occupied receptors [27]. Subsequent yeast two-hybrid and proteomic screens have revealed that arrestins can bind a broad range of catalytically-active proteins and recruit them into receptor-based 'signalsome' complexes [28]. While many of these putative signaling roles for arrestins have yet to be validated in native systems or assigned a clear physiologic function, there is sufficient evidence to conclude that arrestin binding does not mark the end of heptahelical signaling, but rather the beginning of a 'second wave' of signaling that adds new dimensions to receptor function (Fig. 2).

Src family nonreceptor tyrosine kinases

Arrestins bind Src family kinases and recruit them to activated GPCRs. The N-terminus of arrestin 2 is proline-rich and contains three PXXP motifs that interact with the Src Homology (SH)3 domain of c-Src [27]. Additional contacts involving the c-Src SH1 (catalytic) domain confer added binding affinity [29]. Visual arrestin binding to c-Src is different. Arrestin 1 has only a single PXXP motif, and binding appears to involve contact with the c-Src SH2 domain [30]. Internalization of receptor–arrestin complexes is not required for arrestin-dependent c-Src recruitment, since β2 adrenergic receptor–arrestin complexes containing active endogenous Src family kinases can be visualized on the plasma membrane after isoproterenol stimulation [27], but phosphorylation of Ser412 in the

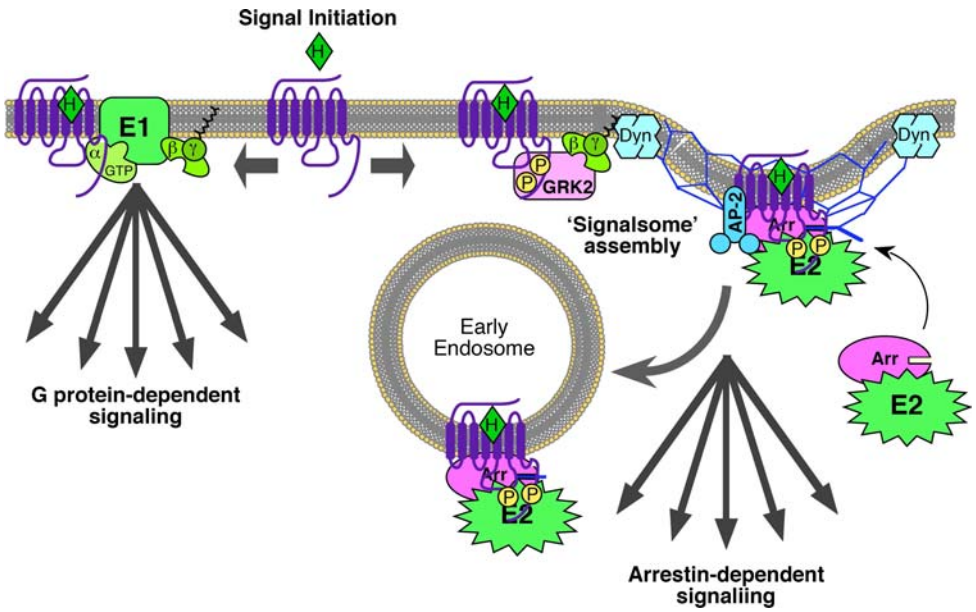

Fig. 2 GPCRs adopt both G protein-coupled and arrestin-coupled signaling states. Upon agonist (H) binding, GPCRs engage heterotrimeric G proteins, rapidly activating G protein-regulated effectors (E1) at the plasma membrane. Concurrently, GRK phosphorylation of the receptor creates high affinity arrestin binding sites. Arrestin binding uncouples the receptor from heterotrimeric G proteins while targeting it for endocytosis. As arrestins translocate from the cytosol to the receptor on the membrane, they recruit additional catalytically-active proteins (E2), that transmit a distinct set of signals as the receptor internalizes and transits the intracellular compartment

C-terminus of arrestin 2, which destabilizes the receptor–arrestin complex, does appear to disrupt the arrestin-Src interaction [27, 31].

Arrestin-dependent recruitment of Src family kinases appears to be important for clathrin-dependent endocytosis of receptors. Arrestin-scaffolded c-Src phosphorylates GRK2, providing negative feedback on receptor desensitization by destabilizing GRK2 and promoting its entry into the proteosome pathway where it is rapidly degraded [32]. Arrestin-Src binding is required for phosphorylation of Tyr597 of dynamin 1, which regulates dynamin self-assembly [29, 33, 34]. Expression of a Y597F mutant of dynamin I impairs the internalization of both the β2 adrenergic [33] and the M2 muscarinic acetylcholine receptor [35]. The β2 adaptin subunit of AP-2 is another endocytic protein whose regulation by Src is arrestin-dependent [36–38]. c-Src stabilizes the constitutive association between arrestin 3 and β2 adaptin independent of its kinase activity. Src-mediated phosphorylation of β2 adaptin Tyr737 occurs in clathrin-coated pits on the plasma membrane in response to AT1a, β2 adrenergic, V2 vasopressin, or B2 bradykinin receptor activation, leading to dissociation of AP-2 from the complex. If β2 adaptin phosphorylation is blocked, receptor–arrestin complexes are retained at the membrane in clathrin-coated pits [38].

Some evidence suggests that arrestin-Src complexes also regulate heptahelical receptor-mediated exocytosis. Arrestin-dependent activation of the Src family kinases c-Hck and c-Fgr by the interleukin 8 receptor (CXCR1) appears to be important for granule release, since expression of P91G/P121G arrestin 2 with mutations in the PXXP motifs antagonizes CXCR1-induced exocytosis in granulocyte cells [39]. Similarly, the endothelin type A receptor assembles an arrestin 2-dependent complex with c-Yes that positively regulates endothelin-1-stimulated translocation of the glucose transporter Glut4 to the plasma membrane [40].

Beyond vesicle trafficking, arrestin-dependent Src signaling affects the activity of several downstream signaling cascades. Src recruitment to β2 adrenergic receptors contributes to activation of the ERK1/2 MAP kinase cascade [27]. The neurokinin (NK)1 receptor forms a receptor–arrestin-c-Src signalsome in response to substance P stimulation [41]. Gel filtration of the complex from stimulated cells reveals a larger-than-expected size, suggesting the incorporation of other proteins (stokes radius of 6 nM; $\sim$300 kDa). Signalsome assembly has been implicated in NK1 receptor-mediated ERK1/2 activation, cell survival, and proliferation. The luteinizing hormone (LH) receptor activates c-Fyn in an arrestin 3-dependent manner [42]. Downregulating arrestin expression by RNA interference reduces the rate of internalization of hCG by 50% and inhibits LH receptor-mediated activation of c-Fyn, phosphorylation of the anti-apoptotic focal adhesion kinase (FAK), and the release of EGF-like growth factors. D2 dopamine receptor activation of the nuclear factor (NF)-κB pathway requires c-Src as well as Gαi proteins, and this response is potentiated by overexpression of arrestin 2 [43]. c-Src is also recruited to rhodopsin-arrestin 1 complexes, which concentrate in the rod outer segment compartment upon light exposure [30]. It is hypothesized that c-Src binding to arrestin 1 promotes the formation of high affinity phospho-Tyr binding sites in the rod outer segment that lead to translocation of other SH2-containing proteins.

Extracellular signal-regulated kinases 1 and 2

The best-studied arrestin-dependent signal is activation of the ERK1/2 cascade. ERK1/2 are the terminal kinases in a three-component MAP cascade consisting of the MAP kinase kinase kinases (MAPKKK), c-Raf1 and B-Raf, the MAP kinase kinases (MAPKK), MEK1/2, and the MAP kinases, ERK1/2. ERK1/2 activity is required for G0–G1 cell cycle transition and the passage of cells through mitosis or meiosis [44]. Heptahelical receptors employ multiple mechanisms to activate ERK1/2, often simultaneously, from PKA- and PKC-dependent signals, to transactivation of epidermal growth factor (EGF) receptors induced by shedding of preformed EGF-family growth factors, to signals transmitted via arrestins [45, 46]. Considerable data indicate that both arrestin 2 and 3 can simultaneously engage all three component kinases and function as ligand-regulated scaffolds; increasing signal fidelity by co-localizing pathway components, increasing the efficiency of the sequential Raf-MEK-ERK phosphorylation, and imposing spatial constraint on the resulting signal.

Gel purification of activated protease-activated receptor (PAR)-2 revealed that c-Raf1 and ERK1/2 are components of a large multi-protein signalsome complex that assembles around the arrestin-bound receptor [47]. Similar results have been obtained with another Class B receptor, the angiotensin AT1a receptor, where confocal microscopy revealed that, after several minutes, activated ERK1/2 co-localizes with the receptor and arrestin 3 in early endosomes [48]. Arrestin 2 and 3 bind directly to c-Raf1 and ERK1/2. MEK1/2 do not appear to bind directly, but overexpression of either c-Raf1 or ERK1/2 increases their co-precipitation with arrestins [47]. In overexpression systems, the arrestin–Raf–MEK–ERK complex exists preformed in the cytosol with the ERK1/2 in an inactive state. In this setting, overexpression of c-Raf1 dramatically increases phosphorylation of arrestin-bound ERK1/2, suggesting that arrestin binding promotes ERK1/2 activation by increasing the efficiency of the sequential phosphorylation. Upon receptor activation, the cytosolic

arrestin–Raf–MEK–ERK complex translocates to the membrane, resulting in localized ERK1/2 activation [49]. Because the Class B receptor–arrestin complex is stable, active ERK1/2 remains associated with the receptor, trafficking with it into endosomal vesicles (Fig. 3a).

It is still unclear how arrestin binding to a heptahelical receptor activates ERK1/2. Data generated using G protein-uncoupled AT1a and β2 receptor mutants, as well as 'biased' ligands that promote arrestin recruitment without G protein activation, indicate that G protein-mediated

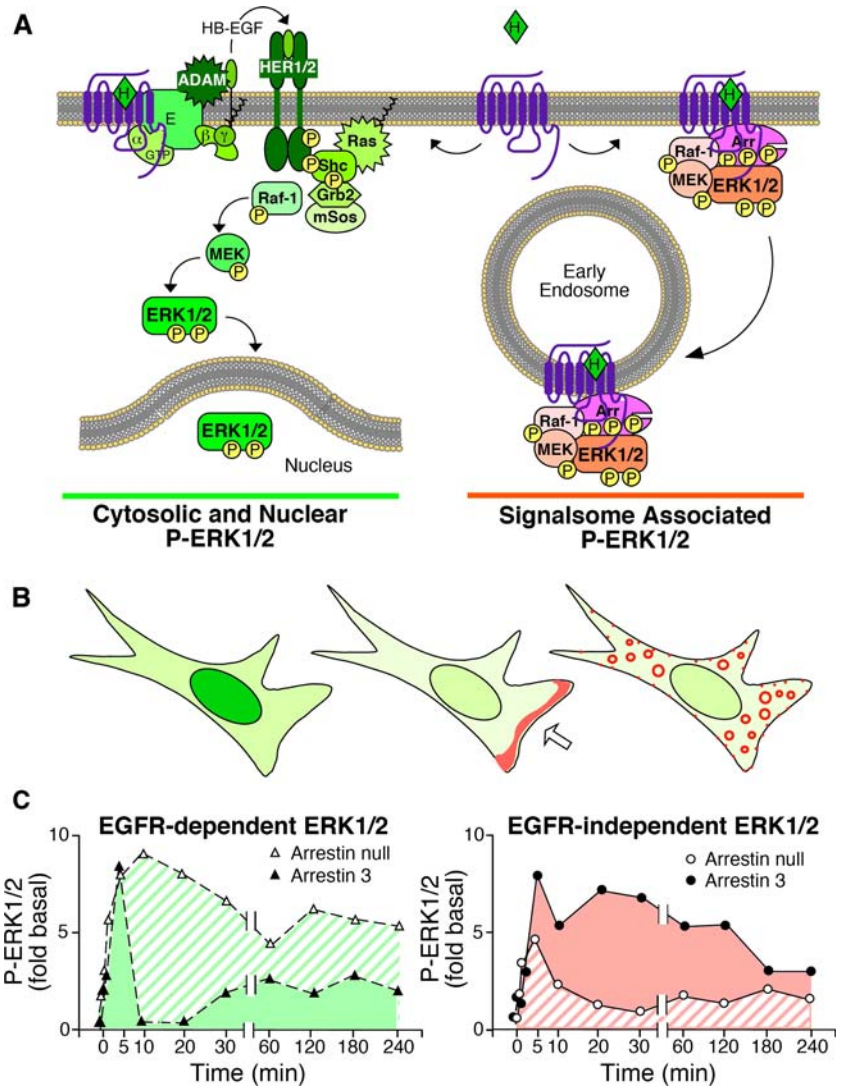

Fig. 3 G proteins and arrestins regulate spatially, temporally and functionally distinct pools of ERK1/2. **a** G protein-dependent and arrestin-dependent mechanisms of ERK1/2 activation. Transactivation of EGF receptors (HER1/2) results from GPCR-stimulated shedding of preformed EGF-family ligands, such as heparin-binding (HB)-EGF, by membrane-anchored ADAM family matrix metalloproteases. Activated HER1/2 recruits a Ras activation complex containing the adapter proteins Shc and Grb2, and the Ras-GEF, mSos, leading to Ras-dependent activation of the Raf-1-MEK-ERK1/2 cascade. By acting as scaffolds, arrestins (Arr) promote the assembly and membrane targeting of a GPCR-based Raf-1-MEK-ERK1/2 signalsome on the plasma membrane. This process does not require prior G protein activation [50]. In the case of GPCRs that form stable receptor–arrestin complexes, the signalsome complex traffics to early endosomes. **b** ERK1/2 activated by G protein-dependent mechanisms is distributed diffusely throughout the cytosol and accumulates in the nucleus (*green shading*) to elicit a transcriptional response. Active ERK1/2 in GPCR-arrestin signalsomes (*red shading*) is spatially constrained, accumulating at the leading edge of cells in a chemoattractant gradient (*arrow*) or in endosomal vesicles off the plasma membrane. **c** Arrestins limit the duration of G protein-dependent ERK1/2 while conferring long-lasting arrestin-dependent ERK1/2 activity. EGF receptor transactivation is the dominant mechanism of LPA receptor-mediated ERK1/2 activation in arrestin 2/3 null MEFs [63]. In the absence of arrestins, LPA stimulates prolonged EGF receptor-dependent ERK1/2 activation (*green stripes*). When arrestin 3 is reintroduced, the duration of transactivation-dependent signaling is markedly shortened (*solid green*), reflecting arrestin-dependent desensitization of receptor-G protein coupling. Conversely, EGF receptor-independent ERK1/2 activation (*red stripes*) is a minor component of ERK1/2 activity in the null background, while restoration of arrestin expression confers long-lasting G protein-independent ERK1/2 activity (*solid red*). Figure adapted from [63]

signals are not required [50–52]. Expression of a G protein-uncoupled NK1-arrestin 1 chimera leads to constitutive activation of a pool of ERK1/2 that remains bound, along with c-Raf1 and MEK1/2, to the internalized receptor–arrestin chimera [53]. Since membrane targeting of c-Raf1 is itself sufficient to activate ERK1/2 [54], one possibility is that the arrestin simply functions as a passive scaffold, moving cytosolic c-Raf1 to the plasma membrane in response to heptahelical receptor activation. The finding that plasma membrane recruitment of arrestin 3 independent of receptor binding is sufficient to activate ERK1/2 is consistent with this model [55]. ERK1/2 bound to the signalsome complex is also relatively protected from dephosphorylation by MAP kinase phosphatases, suggesting that a slower rate of inactivation also promotes sustained activity [53]. Alternatively, Raf activation may involve the recruitment of additional signalsome components. Another putative arrestin binding partner, protein phosphatase (PP)2A, is known to promote ERK1/2 activation by acting on c-Raf1 Ser259, an inhibitory site that must be dephosphorylated for Raf activation [56]. Since PP2A both positively regulates c-Raf [56, 57] and negatively regulates ERK1/2 [58–60], its presence in the signalsome may play a role in arrestin-dependent ERK1/2 regulation.

Another area of uncertainty surrounds reported isoform-specific differences in arrestin-dependent ERK1/2 activation. For AT1a receptors ectopically-expressed in HEK293 cells, arrestin 2 and 3 appear to perform opposing functions [61, 62]. Whereas downregulating endogenous arrestin 3 expression by RNA interference inhibits wild type AT1a receptor ERK1/2 activation by about 50% and abrogates activation by a G protein-uncoupled DRY-AAY mutant or in response to the arrestin pathway-selective ligand [Sar^1-Ile^4-Ile^8]-AngII, silencing arrestin 2 expression paradoxically enhances the signal. This has led to the hypothesis that, with respect to ERK1/2 activation, arrestin 3 is the 'signaling' arrestin isoform, while arrestin 2 functions only in desensitization. Arrestin 3 is certainly the key isoform for Class A receptors like the β2 adrenergic and lysophosphatidic acid (LPA) receptors, which do not bind arrestin 2 [51, 63], but the dichotomy of isoform-specific arrestin function observed with the AT1a receptor does not appear to hold for all Class B receptors. Arrestin-dependent ERK1/2 activation by the type 1 parathyroid hormone (PTH1) receptor, for example, is inhibited when either isoform is downregulated, suggesting that both are required to assemble functional signalsomes [64].

Signalsome formation has profound effects on the spatial, temporal and functional characteristics of ERK1/2 (Fig. 3b). When recruited to a Class B receptor, active ERK1/2 accumulates in early endosomes, failing to translocate to the cell nucleus. Class A receptors, like the β2 adrenergic and LPA receptors, also appear to use arrestin scaffolds to activate ERK1/2 [51, 63], but the transient nature of the receptor–arrestin interaction does not support endosomal targeting. This difference in targeting affects both the location and function of ERK1/2. Wild-type PAR-2 receptors predominantly utilize the arrestin-dependent pathway to activate ERK1/2 [47]. As a result, the active ERK1/2 is excluded from the nucleus and does not stimulate proliferation. In contrast, a C-terminal phosphorylation site mutant PAR-2, that does not bind arrestins or internalize, activates ERK1/2 via a G protein-dependent pathway that promotes its nuclear translocation and stimulates proliferation. The AT1a and V2 vasopressin receptors behave similarly. The wild-type AT1a receptor activates ERK1/2 using both G protein-mediated and arrestin-scaffolded pathways, increasing both cytosolic and nuclear ERK1/2 [65, 66], whereas the G protein-uncoupled DRY-AAY mutant, which utilizes only the arrestin pathway, only activates cytosolic ERK1/2 and fails to elicit a detectable transcriptional response [62]. Native V2 receptors also engage both pathways [67]. Exchanging the V2 receptor C-terminus for that of the Class A β2 adrenergic receptor, which converts the receptor from stable to transient arrestin binding, increases the proportion of ERK1/2 that enters the cell nucleus and permits the chimeric receptor to stimulate cell proliferation. The opposite effect is obtained when the V2 receptor tail is appended to the β2 receptor.

For Class B receptors, the spatial constraint imposed by arrestin binding appears to dictate ERK1/2 function by preferentially targeting it to membrane or cytosolic substrates. ERK1/2 phosphorylates Ser412 in the C-terminus of arrestin 2, limiting its ability to bind clathrin [68]. Arrestin 2 in the cytosol is almost stoichiometrically phosphorylated on Ser412 and must be dephosphorylated upon receptor binding to promote receptor internalization and ERK1/2 activation. Re-phosphorylation by ERK1/2 in the signalsome complex probably provides either negative feedback regulation of receptor endocytosis or facilitates receptor internalization by promoting dissociation of arrestin and clathrin, allowing the receptor to exit clathrin-coated vesicles. Arrestin-dependent targeting of ERK1/2 to the plasma membrane also appears to play a role in chemotaxis. During PAR-2-induced chemotaxis, PAR-2 receptor–arrestin-ERK1/2 complexes localize to the leading edge of the cell where ERK1/2 activity is required for actin cytoskeletal reorganization [69]. Cytosolic ERK1/2 substrates include arrestin 2 [68] and the ribosomal S6 kinase, p90RSK [70]. ERK1/2 phosphorylation of p90RSK is activated by a mutant AT1a receptor with a deletion in its second intracellular loop that inhibits G protein coupling [71]. Using RNA interference to downregulate arrestin 3, it has been possible to show that arrestin-dependent ERK1/2 activation

by the AT1a receptor mediates phosphorylation of Mnk1 and eIF4E, increasing rates of mRNA translation [72].

The fact that arrestins perform dual roles; limiting G protein signaling by promoting receptor desensitization while at the same time activating arrestin-mediated pathways, effects the duration, as well as the location, of GPCR-stimulated ERK1/2 activity (Fig. 3c). For example, in arrestin 2/3 null murine embryo fibroblasts (MEFs), ERK1/2 activation by endogenous LPA receptors primarily results from regulated metalloprotease-dependent shedding of EGF family growth factors that transactivate EGF receptors [63]. Since LPA receptors do not desensitize normally in the absence of arrestins, LPA-stimulated ectodomain shedding is persistent and EGF receptor-dependent ERK1/2 activation lasts for several hours in the continued presence of LPA. When LPA receptor desensitization is restored by re-introducing arrestin 3 to the null background, the transactivation-dependent signal becomes transient, contributing significantly to cellular ERK1/2 activity only during the first 10 min of stimulation. At the same time, expression of arrestin 3 confers a long-lasting EGF receptor-independent ERK1/2 signal that presumably reflects activation of the arrestin pathway. Whereas most of the early LPA-stimulated transcriptional responses in arrestin 2/3 null MEFs are EGF receptor-dependent, expression of arrestin 3 permits LPA to elicit ERK1/2-dependent responses that do not require the EGF receptor, suggesting that dissociation of the LPA receptor–arrestin complex upon internalization permits ERK1/2 activated by the arrestin pathway to enter the nucleus [63].

The contribution of G protein-dependent and arrestin 3-dependent signals to the temporal components of AT1a receptor-stimulated ERK1/2 activation have been elegantly dissected using isoform-selective arrestin RNA interference, pharmacologic inhibitors, G protein-uncoupled receptor mutants, and arrestin pathway-selective ligands [66]. When arrestin 3 expression in HEK293 cells is downregulated, AT1a receptor-stimulated ERK1/2 activation becomes transient and sensitive to PKC inhibition, indicating that it is mediated by a Gq/11-phospholipase (PL)Cβ-PKC pathway. In a reciprocal manner, inhibiting PKC in the presence of arrestin 3 blocks the initial spike in ERK1/2 activity, but does not prevent the persistent late phase response. Exposing the G protein-uncoupled DRY-AAY AT1a receptor mutant to AngII, or the wild-type AT1a receptor to [Sar^1-Ile^4-Ile^8]-AngII generates only the sustained signal, which is insensitive to PKC inhibition and abolished by RNA interference targeting arrestin 3.

c-Jun N-terminal kinase 3

The JNKs are stress-activated kinases that regulate apoptosis by stimulating mitochondrial cytochrome C release during cellular stress [73] and control transcription by phosphorylating the transcription factor c-Jun. There are three JNK isoforms, of which JNK1/2 are widely expressed, while JNK3 is highly expressed only in brain, heart, and testes, where it is known to play important roles in glutamate excitotoxicity [74] and neuronal apoptosis due to cerebral ischemia [75]. JNK2 and JNK3 were originally found to interact with arrestin 3 in yeast two-hybrid screens, but only the JNK3 interaction has been observed in mammalian cells [76]. All four arrestins can bind JNK3, along with the JNK pathway-specific MAPKKK, Ask1, and the MAPKK, MKK4 [77], but only arrestin 3 can assemble a functional scaffold complex that potentiates JNK3 phosphorylation when all of the components are co-expressed [76, 77]. This has prompted speculation that arrestin 2 and 3 perform opposing functions, with arrestin 2 acting as a physiologic 'sink' for JNK3. Both the N- and C-domains of arrestin 3 contribute to Ask1, MKK4, and JNK3 binding [77]. The arrestin 3 C-domain contains an RSS motif not present in arrestin 2. Mutating this motif to the corresponding arrestin 2 sequence eliminates its ability to activate JNK3 [78], indicating that specific points of contact are required to hold the pathway components in the proper orientation for JNK3 activation. A negative regulator of JNK, the MAP kinase phosphatase, MKP7, also interacts with arrestin 3 [79]. MKP7 constitutively interacts with cytosolic arrestin 3 repressing the associated JNK. Upon AT1a receptor activation, MKP7 dissociates from the arrestin complex allowing for JNK3 activation. As with ERK1/2, active JNK3 bound to arrestin is restricted to the cytoplasmic compartment [76, 78, 80], and it is not known whether JNK3 activated in this manner can phosphorylate c-Jun.

Casein kinase 2

Casein kinase II (CK2) is a ubiquitously expressed, constitutively-active Ser/Thr protein kinase that performs diverse functions related to cell survival and tumorigenesis. The catalytic subunit of CK2 was identified in a proteomic screen of arrestin 3 binding proteins [28]. Since CK2 has been implicated in phosphorylation of Thr383 of arrestin 3, which destabilizes the interaction between arrestin 3 and β2 adrenergic receptors [81], it is possible that arrestin-recruitment of CK2 plays a role similar to that proposed for ERK1/2-mediated phosphorylation of arrestin 2 Ser 412 [68].

Protein phosphatase 2A-Akt-glycogen synthase kinase 3β

The Ser/Thr phosphatase PP2A is ubiquitously expressed and has a broad range of substrates. The PP2A holoenzyme

is heterotrimeric, composed of regulatory A and B subunits that target the catalytic C subunit to specific intracellular locations. An arrestin 3 complex containing the PP2A catalytic subunit, Akt, and glycogen synthase kinase 3β (GSK3β) has been copurified from the D_2 dopamine receptor-rich striatum of mice [82]. Within the D2 receptor–arrestin 3 signalsome, PP2A dephosphorylates Akt Thr^{308} rendering it inactive. Since Akt phosphorylation of GSK3α/β on Ser^9 inhibits its activity, signalsome formation releases this inhibition, leading to the activation of GSK3α/β. Active GSK3β, in turn, phosphorylates β catenin, accelerating its degradation (Fig. 4a). Predictably, therefore, striatal extracts from arrestin 3 null mice show higher levels of β catenin expression, presumably resulting from the loss of signalsome-mediated Akt inhibition and GSK3β activation [83]. Conversely, PAR-1 receptor stimulation reportedly produces rapid activation of Akt by an arrestin 2-dependent mechanism [84].

PP2A also plays an important role in receptor trafficking and resensitization, and some of these effects may be arrestin-dependent. Arrestin 2-bound PP2A reportedly dephosphorylates Ser412 on arrestin 2, a step that regulates the interaction between arrestin and the clathrin-coated pit and subsequent internalization [85]. Dephosphorylation of GRK-phosphorylated receptors, a prerequisite for receptor resensitization, also involves PP2A. A 150-kDa oligomeric form of PP2A catalyzes the dephosphorylation of β2 and α2 adrenergic receptors [86]. The acidic microenvironment of endosomes is important for PP2A association with internalized β2 adrenergic receptors, their dephosphorylation, and subsequent recycling to the plasma membrane [87]. At present, it is not clear whether arrestins

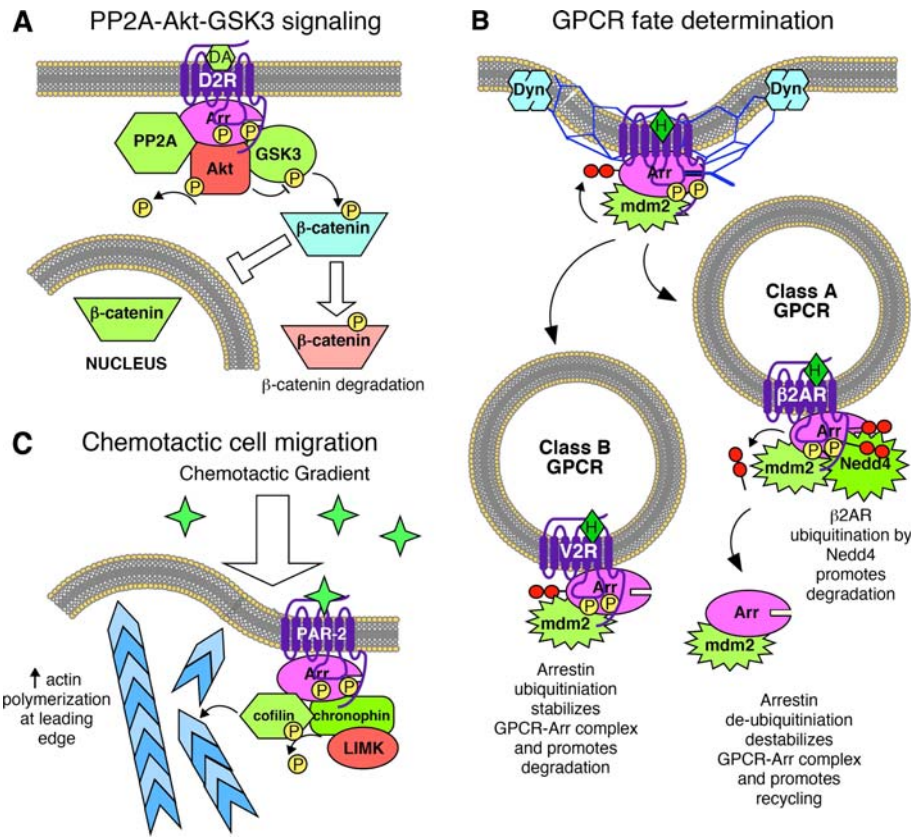

Fig. 4 Diverse functions of GPCR-arrestin signalsomes. **a** Negative regulation of β-catenin signaling by dopamine D2 receptors. PP2A activity in arrestin3-PP2A-Akt-GSK3β complexes promotes dephosphorylation and inactivation of co-scaffolded Akt. This releases Akt-mediated negative regulation of GSK3β activity. GSK3β phosphorylates β-catenin, accelerating its degradation [83]. Without arrestin scaffolding, free Akt phosphorylates and inactivates GSK3β resulting in nuclear accumulation of β-catenin. **b** Determination of receptor fate by arrestin and GPCR ubiquitination. Arrestin3 binds the E3 ligase, mdm2. Upon recruitment to the receptor, mdm2 ubiquitinates arrestin 3, stabilizing the receptor–arrestin complex [22]. After the receptor internalizes, de-ubiquitination of arrestin 3 destabilizes the complex producing a Class A pattern of arrestin binding, e.g., β2 adrenergic receptor. Stable arrestin ubiquitination produces a Class B pattern leading to accumulation of internalized receptor–arrestin signalsomes, e.g., V2 vasopressin receptor. In some cases, GPCR ubiquitination by other E3 ligases, e.g., Nedd4, promotes receptor degradation over recycling [25]. **c** Actin filament assembly during chemotaxis. In a chemotactic gradient (*arrow*), arrestin binding promotes the assembly of a complex containing, cofilin, chronophin, and LIMK on PAR-2 receptors. Activation of chronophin and inhibition of LIMK within the complex increases cofilin activity, forming free actin barbed ends for filament elongation [138]

recruit the PP2A involved in receptor dephosphorylation, since dissociation of arrestin from the receptor should be required to expose the GRK sites for dephosphorylation.

Nuclear factor-κB

Phosphorylation of Inhibitor of nuclear factor (NF)-κB (IκBα) by IκBα kinases accelerates its proteosomal degradation, leading to enhanced NF-κB activity. IκBα binds to a region in the first 60 amino acids of the arrestin 3 N-terminus [88], and IκB kinase α/β and NR-IκB-inducing kinase interact with arrestins 2 and 3 [89]. Both arrestins appear to act as negative regulators of NF-κB-dependent transcription. Arrestin 2 constitutively interacts with IκBα, and downregulating arrestin 2 expression increases NF-κB activation in response to tumor necrosis factor α [89], suggesting that arrestins tonically inhibit NF-κB signaling by protecting IκBα from degradation. β2 receptor stimulation increases arrestin 3 binding to IκBα, preventing its phosphorylation and degradation and inhibiting IL-8-stimulated NF-κB activity [88]. In HEK-293 cells, downregulating endogenous arrestins attenuates Toll-like receptor 4-mediated ERK1/2 activation while at the same time enhancing NFκB reporter activity, suggesting that arrestins exert opposing effects on the ERK1/2 and NFκB pathways [90].

E3 ubiquitin ligases

E3 ubiquitin ligases catalyze the transfer of ubiquitin to the ε-amino group of lysine residues in substrate proteins. Arrestins interact with at least three different E3 ubiquitin ligases: Mdm2, Nedd4, and AIP4. Arrestin-dependent recruitment of E3 ligases into the signalsome complex regulates ubiquitination of both receptors and arrestins, affecting signalsome stability and receptor trafficking.

The N-terminal half of arrestin 3 interacts with residues 383–410 of Mdm2, the E3 ligase responsible for arrestin 3 ubiquitination [22]. Mdm2-mediated arrestin ubiquitination is reversible, and the stability of the modification has profound affects on receptor internalization and trafficking (Fig. 4b). Agonist stimulation of β2 adrenergic receptors leads to transient Mdm2-dependent ubiquitination of arrestin 3. This initial step is necessary for receptor internalization, since genetic ablation of Mdm2 prevents β2 receptor endocytosis. A lysine-less arrestin3 mutant retains the ability to bind clathrin and AP-2, but is unable to hold onto the receptor, suggesting that ubiquitination is required to stabilize the receptor–arrestin interaction [49]. Conversely, de-ubiquitination appears to release arrestin from the receptor after internalization, since an arrestin 3-ubiquitin fusion protein fails to dissociate from the β2 receptor, traffics with it into early endosomes and enhances β2 receptor degradation [23]. Studies with the angiotensin AT1a receptor, which normally remains arrestin-bound after internalization, support the model [91]. In contrast to β2 adrenergic receptors, arrestin 3 is persistently ubiquitinated following AT1a receptor stimulation. Expression of a K11/12R mutant of arrestin 3, which is only transiently ubiquitinated upon binding the AT1a receptor, transforms the pattern of arrestin binding such that the AT1a receptor adopts the β2 adrenergic receptor pattern of transient arrestin binding at the plasma membrane, followed by dissociation upon internalization.

Arrestin ubiquitination therefore determines whether the receptor exhibits a Class A or B pattern of intracellular trafficking and recycling. The different kinetic patterns have been characterized in living cells expressing Class A (β2 adrenergic) or Class B (V2 vasopressin) receptors by measuring arrestin ubiquitination using bioluminescence resonance energy transfer (BRET) [92]. In both cases, the arrestin ubiquitination is detectable within 2 min, but within 5 min the β2 receptor-bound arrestin is de-ubiquitinated, while V2 receptor-bound arrestin remains stably ubiquitinated beyond 10 min. Stability of the arrestin-ERK1/2 signalsome is also controlled by arrestin ubiquitination. The arrestin 3 K11/12R mutant is impaired in its scaffolding function and does not retain active ERK1/2 in the cytosol [91], while a K11/12R arrestin 3-ubiquitin chimera, which cannot be de-ubiquitinated, restores the wild-type pattern and inhibits AT1a receptor-stimulated transcription of *Egr1*, an ERK1/2-mediated nuclear signal. Conversely, expressing arrestin 3-ubiquitin confers upon the β2 adrenergic receptor the ability to target active ERK1/2 to endosomes [49]. Arrestin 3 also regulates other functions of the oncoprotein Mdm2 [93]. Mdm2 is a major regulator of the p53 tumor suppressor, since ubiquitination of p53 promotes its degradation by the proteosome. The Mdm2-arrestin 3 interaction prevents Mdm2 self-ubiquitination and p53 ubiquitination, and increases p53-mediated apoptosis.

Although receptor ubiquitination is arrestin-dependent, it does not involve Mdm2 [22]. The β2 receptor is ubiquitinated by the E3 ligase Nedd4, which is recruited to the receptor by binding to arrestin 3. Nedd4 promotes β2 receptor downregulation by increasing proteosomal degradation [25]. The chemokine receptor CXCR4 is ubiquitinated by the E3 ligase atrophin-interacting protein 4 (AIP4), which binds to the amino terminal half of arrestin 2 [26]. Interestingly, the type 1 insulin growth factor receptor (IGF-1R), which is not a heptahelical receptor, also appears to be regulated by arrestin-dependent ubiquitination [94]. In this case, arrestin-scaffolded Mdm2 catalyzes IGF-1R polyubiquitination, which flags it for degradation.

Vesicle trafficking proteins

Several proteins that regulate the trafficking of membrane-bound vesicles also interact with arrestins and regulate internalized receptor–arrestin complexes. The ATPase N-ethylmalimide-sensitive factor (NSF) interacts with both arrestin 2 and 3 and is important for β2 adrenergic receptor internalization [95]. Ral is a monomeric small G protein in the Ras family that regulates cytoskeletal dynamics. Ral is activated by the GEF activity of Ral-GDP dissociation simulator (Ral-GDS). Ral-GDS constitutively interacts with cytoplasmic arrestin 2 and 3. Upon activation of the formyl-Met-Leu-Phe (fMLP) receptor and arrestin recruitment, Ral-GDS is released from the arrestin complex, whereupon it regulates cytoskeletal rearrangement and exocytic granule release in polymorphonuclear neutrophilic leukocytes [96]. ADP-ribosylation factor 6 (ARF6) and ARF nucleotide binding site opener (ARNO) bind arrestins and are also important for β2 adrenergic receptor endocytosis [97]. As with Ral and Ral-GDS, ARF6 is a monomeric small G protein that regulates vesicular trafficking and ARNO acts as an ARF6 GEF.

cAMP phosphodiesterases and diacylglycerol kinase

Two enzymes involved in second messenger breakdown have been reported to bind arrestins: type 4D cAMP phosphodiesterases (PDE4D) and diacylglycerol kinase (DGK). Arrestin-dependent recruitment of these enzymes to the locus of second messenger production may serve as an additional means of limiting the magnitude and duration of G protein-mediated signaling. Arrestins 2 and 3 interact with all five PDE4D isoforms, PDE4D1–5. The Gs-coupled β2 adrenergic receptor forms a signaling complex with arrestin 3 and PDE4D3 and PDE4D5 [98], leading to accelerated second messenger degradation. Recruitment of PDE4D into the signalsome appears to be highly receptor-specific, since the closely related β1 adrenergic receptor was recently shown to recruit a different alternative-spliced isoform, PDE4D8, and to do so without the aid of arrestin [99]. Arrestin-dependent recruitment of DGK appears to dampen M1 muscarinic receptor-mediated protein kinase C activity [100]. DGK converts diacylglycerol produced by PLC-β to phosphatidic acid. Besides terminating PKC activity, this mechanism may generate physiologically relevant concentrations of phosphatidic acid, a precursor of the multifunctional lipid second messenger, LPA.

Transcriptional regulators

Arrestin 2 and 3 differ in their partitioning between the cytosol and nucleus. Arrestin 3, but not arrestin 2, has a discrete nuclear export signal (NES) [101, 102]. As a result, free arrestin 3 is excluded from the cell nucleus, whereas arrestin 2 distributes uniformly between the cytosolic and nuclear compartments. When the NES in arrestin 3 is mutated or changed to the corresponding sequence in arrestin 2, it accumulates in the nucleus, suggesting that it may constitutively shuttle across the nuclear membrane. However, arrestin 3 lacks a classical nuclear localization sequence, raising the possibility that one or more binding partners confers nuclear targeting of the complex.

These differences in nuclear import/export permit arrestins to exert diverse transcriptional effects. Nuclear export of arrestin 3 may repress the nuclear functions of NFκB, JNK3, and Mdm2 [77, 88–90, 93]. Arrestin 3 binding to Mdm2 promotes its export from the nucleus [76, 77, 103]. Since Mdm2 ubiquitinates the transcription factor p53, leading to its downregulation by the proteosome pathway, arrestin-mediated nuclear export of Mdm2 could upregulate nuclear level p53 activity [104]. Effects on ERK1/2 nuclear signaling are variable. In overexpression systems, arrestin 3 consistently promotes cytosolic retention of active ERK1/2 and inhibits transcription [62]. On the other hand, the transient association of arrestin 3 with Class A GPCRs may support arrestin-dependent activation of nuclear ERK1/2 [63].

Some evidence suggests that arrestin 2 engages directly in nucleo-cytoplasmic shuttling as a means of conveying information between GPCRs on the plasma membrane and transcriptional regulatory elements in the nucleus. Activation of the δ-opioid receptor causes arrestin 2 to move into the nucleus, where it interacts with the p27 and c-Fos promoters and stimulates transcription by recruiting the histone acetyltransferase p300, enhancing local histone H4 acetylation [105, 106].

Specificity in arrestin–effector interactions

The wide range of signaling events that are reportedly regulated by arrestins seems incongruous with their lack of molecular diversity. In contrast even to heterotrimeric G proteins, with 16 mammalian Gα subunit, five Gβ subunit, and 12 Gγ subunit genes [107], there are only two non-visual arrestins; they are ubiquitously expressed outside the retina, and they bind to the vast majority of GPCRs. How then is signalsome composition determined? It seems clear that GPCR-bound arrestins adopt different conformations depending on which GPCR they bind and which GRK phosphorylated the receptor. Evidence of the former comes from characterization of arrestin ubiquitination. Although lysines 11 and 12 of arrestin 3 must be ubiquitinated to maintain stable binding to AT1a receptors, the K11/12R arrestin 3 mutant is stably ubiquitinated when recruited to the V2 receptor [91]. Similarly, all 31 lysines must be

mutated before arrestin 3 ubiquitination is lost upon β2-adrenergic receptor binding [49]. This variability suggests either that the conformation or the accessibility of surface epitopes on arrestin differ depending on the GPCR binding partner. Biophysical evidence obtained using an intramolecular arrestin 3 BRET probe indicates that different ligands acting on the same receptor can also induce different arrestin conformations [108]. GRKs also play a role in dictating arrestin function. Whereas V2 receptors phosphorylated by GRK2 or GRK3 undergo arrestin-dependent desensitization, arrestin-dependent ERK1/2 activation occurs only if the receptor is phosphorylated by GRK5 or GRK6 [109]. Similar results have been reported for the AT1a receptor [110]. The physical basis for this specialization remains unclear, but it may indicate that interaction with specific GRK sites on the receptor induce different desensitizing and signaling conformations in arrestin.

Selectivity and bias among GPCR ligands

Traditionally, GPCRs have been considered to exist in spontaneous equilibrium between two states: an inactive uncoupled state (R), and an active state (R*) capable of catalyzing G protein guanine nucleotide exchange [111]. According to the model, ligand binding affects this equilibrium by preferentially stabilizing R or R*. Thus, agonists exhibit high affinity for R*, whereas neutral antagonists, which have no net effect on the equilibrium, bind both R and R* equally, and inverse agonists, which possess the ability to inhibit the basal activity of constitutively active receptors, preferentially stabilize the inactive R state [112]. The corollary of this two-state model is that, for any set of ligands binding the same receptor in the same cell type, rank order of potency would look the same regardless of the assay used to detect R*, i.e., ligand binding can change signal quantity by producing more or less R* but not its quality (Fig. 5a). Nonetheless, the phenomenon of reversal of potency, where rank order of potency differs depending on the response being measured, has long been recognized and taken as evidence for the existence of more than one 'active' state of the receptor [7, 113, 114]. The discovery of arrestin signaling has added weight to the argument for a multi-state model, as recently characterized ligands for a number of receptors exhibit reversal of efficacy, where a ligand acting as an antagonist or inverse agonist for G protein coupling functions as an arrestin pathway-selective agonist, or vice versa (Fig. 5b). Such behavior can only be modeled based on the assumption that the receptor can adopt more than one 'signaling' conformation. Moreover, the demonstration that functionally-selective or 'biased' ligands can activate or inhibit only a subset of the full signaling repertoire suggests that the quality of hepthelical receptor signaling, not just its quantity, is subject to pharmacologic manipulation.

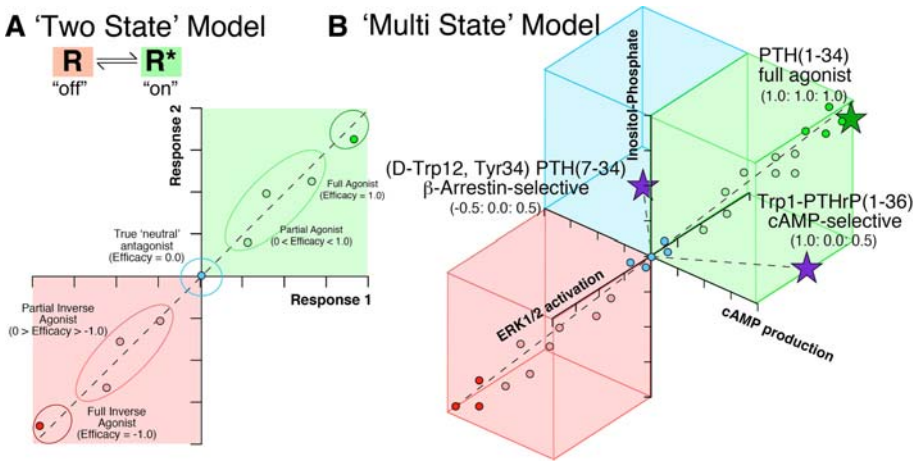

Fig. 5 Models of GPCR agonist efficacy. **a** In a two-state model, the receptor exists in equilibrium between inactive (R) and active (R*) conformations. Ligands exhibit varying degrees of preferential binding to R and R*, such that full agonists preferentially stabilize R* while inverse agonists stabilize R. True neutral antagonists bind indiscriminately to both conformations with no net effect on the equilibrium. According to the model, any given ligand should have similar properties, e.g., agonist, antagonist or inverse agonist, regardless of the assay used to detect receptor activation state. **b** Examples of biased agonism in a multi-state model. Cartesian plot depicting the relationship between three different readouts of PTH1 receptor activity; cAMP production, PI hydrolysis, and ERK1/2 activation. The line of unity predicted by a two-state model, from full agonist (efficacy 1:1:1), through neutral antagonist (efficacy 0:0:0), to full inverse agonist (efficacy −1:−1:−1) is shown, as are the efficacy profiles produced by three PTH1 receptor ligands (stars); the full agonist PTH(1-34), a cAMP pathway-selective, agonist Trp1-PTHrP(1-36), and an arrestin pathway-selective agonist, (D-Trp12, Tyr34)PTH(7-34), that acts as an inverse agonist for receptor-Gs coupling. Such 'reversal of efficacy' can only be modeled assuming the existence of more than one signaling conformation. Figure based on [64]

Angiotensin receptors

The octapeptide, angiotensin II, regulates vascular tone and systemic blood pressure by coupling angiotensin AT1a receptors to the Gq/11-PLCβ pathway. A synthetic AngII analogue, [Sar^{1}Ile^{4}Ile^{8}]AngII (SII), antagonizes Gq/11 coupling but promotes GRK phosphorylation, arrestin recruitment, and receptor endocytosis [115]. In transfected HEK cells, SII promotes sustained ERK1/2 activation via an arrestin-dependent mechanism that is independent of PKC activity [50]. In vascular smooth muscle cells, SII induces ERK1/2 activation and proliferation by promoting AT1a receptor-dependent EGF receptor transactivation without stimulating G protein activity [116]. In primary cardiomyocytes, SII activates cytosolic, but not nuclear, ERK1/2, leading to activation of p90 ribosomal S6 kinase (p90RSK), but not the nuclear transcription factor Elk1, which requires nuclear translocation of ERK1/2. In this system, SII stimulates cardiomyocyte proliferation, but not hypertrophy, which requires Gq/11 [70]. SII also produces positive inotropic and lusitropic effects on isolated murine cardiomyocytes [117]. Interestingly, these effects require GRK6 and arrestin 3 while GRK2 appears to oppose them, consistent with the apparent role of GRK phosphorylation in determining arrestin function described in a transfected system [110].

Parathyroid hormone receptors

The type 1 parathyroid hormone receptor (PTH1R) couples to Gs and Gq/11 and is activated by two endogenous ligands, parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP). PTH1R is expressed in bone, cartilage, and kidney and is critical for homeostatic control of blood calcium levels and for bone remodeling. [D-Trp^{12},Tyr^{34}]PTH(7-34) was originally classified as a PTH1R antagonist, but later reclassified as an inverse agonist based on its ability to reduce basal activity of the naturally-occurring constitutively-active H223R PTH1R mutant [118]. Despite stabilizing a G protein-uncoupled receptor conformation, [D-Trp^{12},Tyr^{34}]PTH(7-34) promotes arrestin recruitment, PTH1R internalization, and arrestin-dependent ERK1/2 activation [64]. This antagonism of one signaling pathway combined with activation of another reflects true reversal of efficacy. [D-Trp^{12},Tyr^{34}]PTH(7-34) is, in effect, an inverse agonist for Gs-coupling, a neutral antagonist for Gq/11 coupling, and an arrestin pathway-selective agonist for receptor sequestration and ERK1/2 activation. Another PTH analog, Bpa^{1}-PTHrP(1-36), illustrates the point that ligand bias runs both ways. In parallel assays, it activates Gs-dependent cAMP production without coupling the PTH1R to Gq/11 or stimulating arrestin-dependent desensitization and signaling [64].

Adrenergic receptors

The β2 adrenergic receptor couples to Gs and Gi and is activated by the catecholamines epinephrine and norepinephrine. Several antagonists of β2 adrenergic receptor-Gs coupling act as arrestin-selective agonists [119]. Carvedilol is a nonselective β1/2 and α1 adrenergic receptor antagonist used clinically for the treatment of heart failure. Originally characterized as an antagonist, it demonstrates inverse agonist activity toward β2 receptor-Gs coupling, stimulates GRK-mediated phosphorylation of the receptor C-terminus, and arrestin-mediated ERK1/2 activation [120]. Bucindolol has even greater efficacy for ERK1/2 activation than carvedilol, but it is not known whether this signal is arrestin-mediated [114]. Similarly, propanolol has inverse agonist effects on cAMP induction from both the β1 and β2 adrenergic receptors yet positively regulates ERK1/2. The β2 receptor-selective agent ICI 118551, another inverse agonist for cAMP production, stimulates G protein switching from Gαs to Gαi coupling [121], and arrestin-dependent ERK1/2 activation [122].

Opioid receptors

The μ, δ and κ opioid receptor subtypes are regulated by endogenous endorphins and are the targets of opiate drugs. The μ opioid receptor (MOR) exhibits marked ligand selectivity. Morphine promotes Gi-dependent inhibition of adenylyl cyclase activity and G protein-dependent activation of a nuclear ERK1/2 pool without producing arrestin-mediated desensitization [123–125]. In contrast, etorphine promotes arrestin recruitment and arrestin-dependent activation of cytosolic ERK1/2 [123]. A G protein-uncoupled ^{276}RRITR^{280} mutant MOR with five-residue deletion in the C-terminus of the third intracellular loop fails to activate ERK1/2 in response to morphine, but promotes arrestin-dependent ERK1/2 activation in response to etorphine [126, 127]. Interestingly, morphine-stimulated G protein activation occurs predominantly in lipid rafts, whereas etorphine-stimulated arrestin binding causes the MOR to exit from rafts, suggesting that G protein- and arrestin-dependent signals originate in different cellular compartments [126].

Chemokine receptors

Functional selectivity also exists amongst naturally occurring GPCR ligands. The chemokine receptor, CCR7, is expressed in B, T, Natural Killer, and dendritic cells. Its two endogenous ligands, CCL19 and CCL21 are constitutively released from lymph nodes and promote homing of circulating CCR7-bearing cells. Both ligands have similar receptor binding affinities in CCR7-transfected HEK 293

cells and both induce chemotaxis with equal potency [128]. However, CCL19 induces Gi activation, receptor phosphorylation, desensitization, and arrestin recruitment, whereas CCL21 activates Gi without leading to receptor desensitization. CCL19 also promotes arrestin-dependent ERK1/2 activation whereas CCL21 does not [129].

How physiologically relevant is arrestin-signaling?

It is clear that arrestins play a crucial role in determining cellular responsiveness by regulating GPCR desensitization, sequestration, and recycling. Likewise, their ability to function as ligand-regulated scaffolds appears to greatly expand the GPCR signaling repertoire. But what physiologic functions can be unambiguously ascribed to arrestin signaling? The fundamental duality of arrestin function makes this a difficult question to answer, since observed phenotypes in vivo might derive from either prolonged G protein activation or loss of arrestin signaling. Whereas arrestin 2/3 double knockout mice exhibit early embryonic lethality, individual arrestin 2 and 3 knockouts have surprisingly mild phenotypes, often attributable to impaired heptahelical receptor desensitization [130, 131]. Nonetheless, there are several examples of altered heptahelical receptor function in vivo that are at least consistent with a physiologic role for arrestin signaling.

Cell migration and chemotaxis

Chemotaxis is a receptor-mediated process whereby migrating cells follow a concentration gradient to its source. Chemoattractant receptor activation induces actin cytoskeletal rearrangement forming leading and trailing edges. A dominant pseudopodium forms at the leading edge and protrudes forward driven by F-actin polymerization and actin-myosin contraction forces [132, 133]. Splenocytes derived from arrestin 3 null mice exhibit strikingly impaired chemotaxis to stromal cell-derived factor-1, CXCL12 [134]. While impaired gradient sensing due to the loss of arrestin-mediated desensitization may contribute [135], considerable data suggest that arrestin-dependent regulation of ERK1/2 and cortical actin cytoskeletal assembly at the leading edge is required for chemotaxis by PAR-2 [69], and the AT1a [136], fMLP [96], and CXCR4 receptors [134].

PAR-2-induced chemotaxis in MDA breast cancer cells requires both arrestin 2 and 3 [137]. During chemotaxis, a PAR-2 receptor–arrestin–ERK1/2 complex localizes to the leading edge that activates actin cytoskeleton reorganization [69]. In addition, arrestin-mediated scaffolding of a complex containing the actin filament-severing protein, cofilin, LIM kinase, and the cofilin-specific phosphatase, chronophin, is required for PAR-2-mediated dephosphorylation and activation of cofilin (Fig. 4c) [138]. Cofilin activation is independent of Gq/Ca^{2+} signaling. It binds to both arrestin 2 and 3 and redistributes from the cytosol to membrane protrusions upon receptor activation, where it creates the free barbed ends on actin filaments that permit filament extension. Interestingly, although arrestin 3 localizes predominantly to the back of F-actin-rich protrusions, arrestin 2 is found predominantly at the tips, suggesting distinct scaffolding roles for the two isoforms. In AT1a receptor-expressing HEK 293 cells, Ang II, as well as the biased agonist SII, stimulate chemotaxis by an arrestin 3-dependent mechanism that is independent of G protein activity [139].

Actin bundling proteins, like filamin A, are important for organizing actin filament structures in filopodia and microvilli. Assembly of an AT1a receptor-arrestin-ERK1/2-Filamin A complex is required for the formation of membrane ruffles in Hep2 cells [140]. Filamin A also interacts with other GPCRs, including PAR-2 [69], the D_2 and D_3 dopamine receptors [141, 142], and the calcium sensing receptor [143, 144], but it is not known whether arrestins regulate filamin function. However, the fMLP chemokine receptor utilizes an arrestin 2-Ral-GDS-filamin pathway to regulate actin cytoskeleton reorganization [96].

Dopamine-dependent behavior

Dopamine is a neurotransmitter that regulates behavioral responses such as locomotor activity and neural reward mechanisms. Loss of dopaminergic cells in the substantia nigra leads to a loss of locomotor control in Parkinson's disease. Conversely, D2 dopamine receptor antagonists are effective neuroleptic drugs used for the treatment of schizophrenia and attention deficit hyperactivity disorder.

Several lines of evidence suggest that arrestins positively regulate dopamine dependent behaviors. Locomotor hyperactivity induced by the dopaminergic drug apomorphine, a D2 receptor agonist, is reduced in arrestin 3 knockout mice [83]. The hyperactivity displayed by dopamine transporter knockout mice, which results from increased synaptic dopamine concentration, is paradoxically reduced when DAT knockout mice are cross-bred with arrestin 3 knockouts. A G protein-mediated response should be enhanced by the loss of arrestin-dependent desensitization. Similarly, GRK6 knockout mice have augmented locomotor responses to dopaminergic drugs [145]. GRK6 is the most abundant GRK in the striatum, the target of substantia nigra dopaminergic cells and a region with high D2 receptor density, and has been implicated in arrestin signaling via the AT1a receptor [70, 117].

The molecular basis of these effects may lie in the scaffolding functions of arrestins. D2 receptors have been

reported to positively [146] and negatively [147] regulate the prosurvival kinase, Akt. D2 receptor-mediated phosphatidylinositol-3 kinase (PI3K)-independent Akt activation is not fully understood, but D2 receptor-mediated inhibition of Akt involves the formation of a D2 receptor–arrestin 3–PP2A–Akt complex [147]. Amphetamine treatment, which increases synaptic dopamine release, increases the PP2A–Akt association in wild-type, but not arrestin 3 knockout, mice [83], suggesting that arrestins mediate the interaction. PP2A inhibition by fostriecin antagonizes dopamine-induced Akt dephosphorylation, and GSK3β inhibitors produce a dose-dependent inhibition of hyperactive locomotor activity in DAT knockout mice, suggesting that dopamine-mediated activation of GSK3β results from PP2A-dependent Akt inhibition that is scaffolded by arrestin 3 [82].

Modulation of arrestin scaffolding may play a role in the mechanism of action of lithium, a mood stabilizer used in the treatments of schizophrenia, a disease hypothesized to result from overstimulation of the dopaminergic system. Lithium modulates dopamine-dependent behavior in mice such as horizontal activity. GSK3β haploinsufficient mice exhibit augmented lithium-induced antidepressant and anxiolytic effects compared to wild-type animals, suggesting that lithium acts by inhibiting GSK3β. In addition to directly inhibiting GSK3β, therapeutic concentrations of lithium disrupt the interaction between arrestin, Akt, and PP2A, relieving PP2A-mediated negative regulation of Akt allowing it to phosphorylate and inactivate GSK3β [83]. The arrestin 3–PP2A–Akt complex requires magnesium, and lithium is thought to destabilize the complex by competing for magnesium binding.

Cardiac hypertrophy and failure

In murine models, chronic stimulation of cardiac angiotensin or adrenergic receptors leads to a pathologic form of cardiac hypertrophy. Evidence suggests that both G protein- and arrestin-mediated signals are involved in these responses.

The role of G protein-independent AT1a receptor signaling in the heart has been probed using an AT1a receptor second intracellular loop mutant (i2m) that lacks G protein coupling, but activates a Src–Ras–ERK1/2 pathway leading to cytosolic ERK1/2 and p90RSK activation [71]. Cardiomyocyte-specific overexpression of the i2m mutant produces greater cardiomyocyte hypertrophy, bradycardia, and fetal cardiac gene expression than comparable overexpression of the wild-type receptor [148]. Conversely, wild-type AT1a receptors generate greater cardiomyocyte apoptosis and interstitial fibrosis than the mutant, suggesting that G protein-dependent and -independent AT1a receptor signals mediate different aspects of the hypertrophic response. Perhaps significantly, expression of either arrestin 2 or 3 rescues arrestin 2/3 null fibroblasts from AT1a, fMLP, and V2 vasopressin receptor-induced apoptosis [149]. The apoptotic signal is G protein-dependent and involves activation of PI3K, MAP kinases and c-Src. Arrestins appear to negate this pathway, either by promoting desensitization or initiating G protein-independent pro-survival signaling pathways. Another AT1a receptor mutant sheds further light on the processes by which angiotensin II promotes cardiomyocyte hypertrophy. Metalloprotease-dependent transactivation of EGF receptors is an important factor in the hypertrophic response to pressure overload [150]. EGF receptor transactivation by angiotensin requires the presence of an intact ^{319}YIPP motif in the AT1a receptor C-terminal tail [151]. Transgenic mice with cardiomyocyte-specific expression of a Y319F-mutant AT1a receptor develop less severe cardiac hypertrophy, less fetal cardiac gene expression, less interstitial fibrosis, and less cardiomyocyte apoptosis than wild-type receptor-expressing controls [152]. Moreover, expression of a dominant negative EGF receptor suppresses cardiac hypertrophy in bigenic mice overexpressing wild-type AT1a receptors. Thus, both transactivation of EGF receptors and G protein-independent signals appear to contribute to cardiac hypertrophy in AT1a receptor overexpression models.

A very different picture has been painted for the role of arrestins in β1 adrenergic receptor-mediated cardiomyopathy. Whereas AT1a receptors activate the Gq/11-PLCβ-PKC pathway and form stable Class B receptor–arrestin complexes, β1 receptors couple to Gs–adenylyl cyclase–PKA and exhibit a transient Class A pattern of arrestin binding. In β1 receptor-expressing HEK 293 cells, EGF receptor transactivation and ERK1/2 activation are inhibited by siRNA-mediated downregulation of arrestin 2 or 3, or GRK 5 or 6, inhibiting Src kinase or matrix metalloprotease activity, or by exposure to a heparin-binding (HB)-EGF neutralizing antibody [153], suggesting that β1 receptor-mediated EGF receptor transactivation is arrestin-dependent. Consistent with this, a mutant β1 receptor lacking 14 GRK phosphorylation sites in its C-terminal tail ($^{-GRK}\beta$1), which cannot undergo arrestin-dependent desensitization, fails to transactivate the EGF receptors despite exaggerated G protein activation. In response to chronic isoproterenol stimulation, transgenic mice expressing the $^{-GRK}\beta$1 receptor develop more severe dilated cardiomyopathy, with significantly increased LV end-diastolic dimension, decreased fractional shortening, and increased myocardial apoptosis, than wild-type β1 receptor transgenic mice. In this model, inhibiting EGF receptors worsens the dilated cardiomyopathy, suggesting a protective, rather than a deleterious, role for EGF receptors in the heart. While the authors cannot exclude a role for

exaggerated cAMP production by the non-desensitizing $^{-GRK}\beta 1$ receptor, they speculate that arrestin-dependent EGF receptor transactivation mediated by $\beta 1$ receptors exerts a cardioprotective effect that may be of therapeutic benefit in heart failure.

Retinal degeneration

Several forms of hereditary retinal degeneration result from mutations in the visual signal transduction system. Oguchi disease, for example, a form of stationary night blindness, results from null mutations in the arrestin 1 or rhodopsin kinase (GRK1) genes that impair rhodopsin desensitization [154]. Retinitis pigmentosa has several causes, among them rhodopsin mutations that result in either constitutive receptor signaling or constitutive receptor–arrestin interaction. Arrestin-mediated signaling may underlie an autosomal dominant form of retinitis pigmentosa associated with a K296E mutation in rhodopsin. This mutation, which leads to photoreceptor cell degeneration, causes rhodopsin to be constitutively phosphorylated and bound to arrestin [155]. JNK3 and Mdm2 interact with both visual (arrestin 1) and cone (arrestin 4) arrestin [80], and arrestin interactions with Jnk3 and Mdm2 have been implicated in the induction of retinal cell degeneration and neuronal apoptosis [103].

Studies in *Drosophila* suggest that G protein-dependent pathways inhibited by arrestin lead to photoreceptor necrosis, while G protein-independent signals transmitted by arrestin mediate photoreceptor apoptosis [156]. Unlike vertebrates, which use a transducin-activated cGMP phosphodiesterase for phototransduction, *Drosophila* uses a Gq-coupled pathway. A loss-of-function mutation in the RdgA gene in *Drosophila* results in photoreceptor cell degeneration and blindness [157]. RdgA encodes a DGK that is required for rhodopsin desensitization in the invertebrate eye. DGKs catalyze the conversion of diacylglycerol (DAG) to phosphatidic acid reducing DAG levels and dampening the PKC pathway. Similar to the RdgA mutant, rhodopsin activation will induce necrosis if arrestin is lost [158]. Retinal degeneration in arrestin null flies cannot be rescued by eye-specific expression of the baculoviral p35 caspase inhibitor protein [159], but it can be rescued by disruption of Gq function [160], indicating that the protection from necrosis arises from arrestin-mediated desensitization of a Gq-dependent pathway. In contrast, the RdgC loss-of-function mutation in *Drosophila* leads to light-dependent photoreceptor cell apoptosis [161]. RdgC is a calcium-dependent kinase that promotes dissociation of the rhodopsin–arrestin complex. This form of retinal degeneration is enhanced by a loss-of-function Gq mutation or by deleting the arrestin phosphorylation domain [160], both of which stabilize the rhodopsin–arrestin interaction. Triple inactivation of Gαq, arrestin, and RdgC rescues the phenotype [160], as does expression of the p35 caspase inhibitor [161], implicating an arrestin-dependent apoptotic signal transmitted by a stable rhodopsin–arrestin complex. Similarly, deletion of the eye-specific PLC gene in *Drosophila* results in the constitutive formation of rhodopsin–arrestin complexes and retinal degeneration by apoptosis. This degeneration is rescued in the PLC/arrestin double mutant fly [159]. Blocking receptor endocytosis by an inactivating mutation of the *Drosophila* dynamin homolog also rescues retinal degeneration in the PLC mutant fly [159].

Conclusions

Arrestins are clearly vital to the proper regulation of heptahelical receptor signaling. They control the desensitization, endocytosis, and recycling/degradation of the vast majority of GPCRs. At the same time, they function as ligand-regulated scaffolds that recruit functionally diverse proteins to the receptor, conferring novel signaling properties. In many cases, signals transmitted by arrestin binding are demonstrably independent of heterotrimeric G protein activation. Limited but growing evidence suggests that these ‘arrestin-dependent’ signals are involved in a number of physiologic processes in vivo, among them cell migration, neurotransmission, hypertrophy, and apoptosis. Moreover, the discovery that in some cases arrestin-mediated signals can be initiated or antagonized independent of G protein activation has rekindled interest in the concept of biased agonism, pathway-selective drugs that activate only a subset of the heptahelical receptor signaling repertoire. Much additional work will be required to define the optimal efficacy profile needed to capitalize on this phenomenon for the development of novel therapeutics with greater efficacy or more favorable side effect profiles.

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