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Regulation of α_2AR trafficking and signaling by interacting proteins

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ABSTRACT

The continuing discovery of new G protein-coupled receptor (GPCR) interacting proteins and clarification of the functional consequences of these interactions has revealed multiple roles for these events. Some of these interactions serve to scaffold GPCRs to particular cellular micro-compartments or to tether them to defined signaling molecules, while other GPCR–protein interactions control GPCR trafficking and the kinetics of GPCR-mediated signaling transduction. This review provides a general overview of the variety of GPCR–protein interactions reported to date, and then focuses on one prototypical GPCR, the α_2AR, and the *in vitro* and *in vivo* significance of its reciprocal interactions with arrestin and spinophilin.

It seems appropriate to recognize the life and career of Arthur Hancock with a summary of studies that both affirm and surprise our preconceived notions of how nature is designed, as his career-long efforts similarly affirmed the complexity of human biology and attempted to surprise pathological changes in that biology with novel, discovery-based therapeutic interventions. Dr. Hancock's love of life, of family, and of commitment to making the world a better place are a model of the life well lived, and truly missed by those who were privileged to know, and thus love, him.

1. Introduction

The α_2 adrenergic receptor (AR) belongs to the G protein-coupled receptor (GPCR) superfamily. *In vivo*, activation of the α_2AR by endogenous ligand, epinephrine and norepinephrine, leads to decrease in epileptogenesis [1] and anxiety [2]. In response to α_2-agonists, the α_2AR activation can lower blood pressure by central mechanisms [3,4], evoke sedation [5], reduce pain perception [5,6], and improve working memory [7–9]. There are three subtypes of α_2ARs, α_{2A}-, α_{2B}- and α_{2A}AR, which are encoded by three different genes but all couple to the $G_{i/o}$ subfamily of G proteins to inhibit adenylyl cyclase and voltage-gated Ca^{2+} channels and to activate receptor-operated K^+ channels and mitogen-activated protein kinase (MAPK) in native cells [10–12].

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Abbreviations: GPCR, G protein-coupled receptor; α_2AR, α_2-adrenergic receptor; RGS, regulatory of G protein signaling; MAPK, mitogen-activated protein kinase; GRK, G protein-coupled receptor kinase; eIF, eukaryotic translation initiation factor; APLP, amyloid precursor like protein; APP, amyloid precursor protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun amino-terminal kinase; MEF, mouse embryonic fibroblast; PP1, protein phosphatase 1; PDZ, PSD-95/Discs large/ZO-1 homology

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Discovery of regulatory of G protein signaling (RGS) proteins a decade ago has changed the historic view of GPCR signaling as simplistic coupling of receptors to G proteins to effectors. Additionally, protein–protein interactions are now appreciated as a critical means to fine tune GPCR functions. GPCR–interacting proteins can regulate both receptor trafficking and receptor-mediated signaling. The α_2AR has been identified to interact with a number of proteins, including GPCRs, kinases and scaffolding proteins such as 14-3-3ζ, arrestin and spinophilin (see Table 1). Interactions of the α_2AR with some of these proteins have been implicated in controlling α_2AR surface retention, internalization, signaling desensitization, response sensitivity as well as scaffolding α_2AR-mediated intracellular signaling. In this review, we focus on α_2AR interaction with scaffolding proteins and the functions these proteins play in regulating α_2AR trafficking and signaling.

2. Proteins interacting with the α_2AR

2.1. GPCRs

Accumulating evidence indicates that homo- and hetero-dimerization (or oligomerization) of GPCRs exist *in vivo* and may represent an important mechanism for complex GPCR functions [13,14]. The α_{2A}AR has been reported to form homodimers [15,16] as well as to interact with some other GPCRs to form heterodimers. Compared to the α_{2A}AR homo-dimerization, heterodimerization of the α_{2A}AR subtype with the α_{2C}AR subtype significantly attenuates agonist-induced phosphorylation of the α_{2A}AR subtype, leading to a reduction of arrestin binding as well as arrestin-mediated Akt activation by α_2-agonist [16]. Heterodimerization of α_{2A}AR and β_1AR in HEK cells significantly alters ligand binding properties of the β_1AR but that of not the α_{2A}AR, and leads to internalization of β_1AR in response to α_2-agonists [17]. On the other hand,

Table 1 – α_2AR-interacting proteins

Interacting protein(s)	α_2AR subtype(s)	Functional role(s) implicated	Reference(s)
GPCR dimerization			
Homo-dimerization			
	α_{2A}	Precoupled to G proteins	[15]
Hetero-dimerization			
α_{2C}AR	α_{2A}	Attenuating agonist-induced α_{2A}AR phosphorylation, reducing arrestin binding to the α_{2A}AR	[16]
β_1AR	α_{2A}	Altering ligand binding properties of β_1AR, leading to internalization of β_1AR in response to α_2-agonist	[17]
β_2AR	α_{2C}	Enhancing surface expression and internalization of α_{2C}AR, enhancing ERK activation by α_{2C}AR	[18]
μOR	α_{2A}	Enhancing morphine-induced GTPγS binding and ERK activation; however, no transactivation of G proteins, no interdependent internalization	[19,20]
δOR	α_{2A}	Enhancing δOR-mediated neurite outgrowth	[21]
Kinases			
GRK2	α_{2A}, α_{2B}	Mediating agonist-induced phosphorylation and homologous desensitization	[26,27,29]
PKC	α_{2A}	Mediating heterologous desensitization; affecting constitutive activity of α_{2A}AR	[30–33]
Proteins interacting with the C-tail of α_2AR			
eIF-2B	α_{2A}, α_{2B}, α_{2C}	Function unknown	[35]
APLP1	α_{2A}, α_{2B}, α_{2C}	Increasing α_{2A}AR-mediated inhibition of adenylyl cyclase activity	[36]
Proteins interacting with the 3i loop of α_2AR			
14-3-3ζ	α_{2A}, α_{2B}, α_{2C}	Function unknown, competed by phosphorylated Raf peptide	[43,47]
Spinophilin	α_{2A}, α_{2B}	Stabilizing receptor at surface, attenuating phosphorylation, decelerating ERK signaling rate, decreasing *in vivo* response sensitivity	[44,47,51,86]
	α_{2C}	Function unknown	[44]
Arrestin 3	α_{2A}, α_{2B}	Stabilizing receptor phosphorylation, mediating endocytosis and desensitization, accelerating ERK signaling rate, enhancing *in vivo* response sensitivity	[45–47,51]
	α_{2C}	Function unknown	[46]
Arrestin 2	α_{2B}	Affinity is lower than arrestin 3	[46]

heterodimerization of α_{2C}AR with β_2AR notably reduces the intracellular pool of α_{2C}AR in HEK cells, and such increased α_{2C}AR surface expression results in enhanced α_{2C}AR internalization and ERK activation in response to α_2-agonist stimulation [18].

Adrenergic-opioid synergism in induction of spinal analgesia has long been noted [6]. Interactions of the α_{2A}AR with the mu [19,20] and delta [21] opioid receptors (OR) have been observed in both heterologous cells and neurons. In cells coexpressing MOR with the α_{2A}AR, morphine (MOR agonist) treatment led to a significant higher level of GTPγS binding and ERK activation than in cells expressing MOR alone [19]. Coexpression of α_{2A}AR with DOR also enhanced DOR-mediated neurite outgrowth [21]. These data suggest that physical interaction between opioid receptors and the α_{2A}AR leads to functional interaction between these receptors at the cellular level [19,21]. Intriguingly, simultaneous stimulation with a combination of opiods and α_2-agonists caused a decrease of MOR–α_{2A}AR interaction as well as a remarkable reduction of MOR-mediated signaling in cells coexpressing these two receptors [19]. Also, hetero-oligomers of α_{2A}AR and MOR did not lead to transactivation of G proteins or interdependent redistribution on one another following agonist treatment [20]. Taken together, while physical interaction between α_{2A}AR and MOR represents a potential molecular mechanism contributing to the observed adrenergic-opioid synergism, additional complex mechanisms likely underlie functional cross-talk of adrenergic-opioid system *in vivo*.

2.2. *Kinases*

Like most GPCRs, the α_2AR is substrate of G protein-coupled receptor kinases (GRKs). The GRKs represent a family of seven members with serine threonine kinase activity. GRKs 1 and 7 phosphorylate rhodopsin and iodopsin, respectively, in the visual system. GRK4 is mainly expressed in testes. GRKs 2, 3, 5 and 6 are ubiquitously expressed and regulate most GPCRs [22,23]. Through interaction of its C-terminus with G protein βγ subunits, GRK translocates to plasma membrane [24] and specifically phosphorylates agonist-occupied or conformationally activated GPCRs [22,23]. GRK-catalyzed phosphorylation, which leads to subsequent arrestin binding, represents a major mechanism for homologous desensitization of GPCRs [22,23].

GRK2 phosphorylation occurs in both the α_{2A}- and α_{2B}AR subtypes. A tetraserine sequence (S-296-299) in the 3i loop of the α_{2A}AR [25] and non-contiguously distributed serines in the 3i loop of the α_{2B}AR subtype [26] have been identified as GRK phosphorylation sites. By contrast, the α_{2C}AR subtype does not appear to be a substrate for GRK-catalyzed phosphorylation, even though its 3i loop contains multiple serine and threonine residues organized in a putative GRK favored motif [27]. The fact that the α_{2A}AR bearing an α_{2C}AR 3i loop but not the α_{2C}AR bearing an α_{2A}AR 3i loop gets phosphorylated upon agonist stimulation suggests that agonist-induced conformational change of the receptor backbone determines the level of α_2AR phosphorylation by GRK [27].

Direct interaction of the α_{2A}AR with GRK2 involves the 2 and 3 intracellular (2i and 3i) loops but not the 1i loop or the C-tail of the α_{2A}AR [28]. Within the α_{2A}AR 3iloop, several basic residues at the membrane proximal N- and C-terminal regions as well as in a region adjacent to the phosphorylation sites mediate association with GRK2 [28].

The α_{2A}AR also can be phosphorylated by PKC [29,30] at residues located at the N- and C-terminal regions of the 3i loop [30–32]. Phosphorylation of α_{2A}AR by PKC can cause heterologous desensitization of the α_{2A}AR [29], and may also affect constitutive coupling of G proteins [30]. Therefore, PKC phosphorylation may represent a mechanism for crosstalk between α_{2A}AR-mediated and other signaling pathways.

2.3. *Proteins interacting with the C-terminus of the α_2AR*

The α_2AR has a relatively short C-terminal cytoplasmic tail that has been implicated in receptor downregulation [33]. The C-terminal domains of all three α_2AR subtypes also have been shown to interact with α-subunit of eukaryotic translation initiation factor 2B (eIF-2B) [34] as well as with the amyloid precursor like protein 1 (APLP1) [35], a homologue of the β-amyloid precursor protein (APP) involved in Alzheimer's disease [36]. Coexpression of APLP1 with the α_{2A}AR in HEK cells significantly increases α_{2A}AR-mediated inhibition of adenylyl cyclase activity [35]. The functional relevance of α_2AR interactions with these proteins in native tissues has not yet been established.

2.4. *Scaffolding proteins interacting with the 3i loop of the α_2AR*

In polarized renal epithelial cells, all three subtypes of α_2AR are primarily localized at the basolateral surface and manifest a surface half life ($t_{1/2}$) of 10–12 h [37,38]. In an effort to map sequence regions responsible for α_2AR targeting and retention at the basolateral surface, Keefer et al. found that deletion of the third intracellular loop (3i loop) resulted in a much fast turnover of the α_2AR on that surface [39,40]. Furthermore, the entire 3i loop seems to be involved in α_{2A}AR basolateral retention, since no single region within the loop can fully account for the accelerated turnover of the $\Delta 3i\alpha_{2A}$AR [41].

Given its importance in regulating α_2AR retention, the 3i loop has been used as a ligand to identify intracellular interacting proteins that may contribute to α_2AR retention at the cell surface. Two scaffolding proteins, 14-3-3ζ [42] and spinophilin [43], were identified to interact with the 3i loops of all three α_2AR subtypes by gel overlay analysis and GST pull-down assay. Interestingly, interaction of the α_2AR with these two proteins involves multiple, non-contiguous regions of sequence of the 3i loop, concurring with the fact that multiple regions of the 3i loop contribute to α_2AR surface retention. In addition to 14-3-3ζ and spinophilin, arrestin also binds to the 3i loop of α_2AR [44–46]. As discussed below, interaction of the 3i loop with these scaffolding proteins regulates both α_2AR trafficking and signaling.

3. Regulatory cycle of 14-3-3ζ, arrestin and spinophilin with the α_2AR

To understand the functional relevance of interactions of arrestin, 14-3-3ζ, and spinophilin with the 3i loop of α_2ARs, we

explored whether these proteins share protein–protein interaction domains within the 3i loop of the α_{2A}AR. Our data demonstrated that: (1) multiple, non-contiguous regions of sequence are needed for interactions of the α_{2A}AR loop with 14-3-3ζ, arrestin, and spinophilin, (2) the relative affinity of the unphosphorylated 3i loop for interacting proteins is: spinophilin ((Sp151–444) ≈ arrestin 3 ≫ 14-3-3ζ, 3) arrestin and 14-3-3ζ interact with regions in the 3i loop that are not involved in coupling to G proteins, but that spinophilin requires both the amphipathic helices at the base of transmembrane (TM) domains 5 and 6 as well as non-contiguous regions of the 3i loop for its interactions with the α_{2A}AR [46].

One important question was whether or not these interactions occurred independently of one another in different target cells or differing compartments of a given cell, or whether they were part of a regulatory cycle in the context of an individual cell. Fig. 1 suggests a functional relationship among these interactions. Interactions of 14-3-3ζ are proposed to occur with the α_2AR in its inactive state. This interpretation derives from the finding that α_2AR–14-3-3ζ interactions can be competed for by phosphorylated Raf peptides, but not by corresponding non-phosphorylated peptides [42]. Thus, the α_2AR appears to interact with 14-3-3ζ at a site shared by phosphorylated Raf, as initially demonstrated by Muslin and coworkers [47,48]. Since α_2AR activation leads to downstream Ras-Raf activation [49], it is reasonable to propose that α_2AR activation would ultimately disrupt interaction with 14-3-3ζ due to production of the endogenous competitor, phosphorylated Raf. In contrast to loss of α_2AR–14-3-3ζ interactions upon agonist binding to the receptor, agonist activation enhances the binding of both arrestin and spinophilin [43,46] with the α_2AR. Indeed, spinophilin and arrestin compete for interaction with the α_2AR [46]. It appears that the selection between α_2AR binding to arrestin or to spinophilin depends on the phosphorylation state of the receptor. Whereas arrestin binds more effectively to the GRK-phosphorylated receptor, spinophilin favors interactions with the non-phosphorylated receptor [46]. Thus, there is an equilibrium between agonist–receptor–arrestin and agonist–receptor–spinophilin interactions, and this equilibrium can be altered by changes in available concentrations of arrestin versus spinophilin as well as the phosphorylation state of the α_2AR in the target cell. Spinophilin appears to serve as a functional antagonist of arrestin functions, both those that lead to receptor deactivation and redistribution and those that contribute to receptor activation of signaling [50]. Below, we discuss the functional relevance of α_2AR interaction with arrestin and spinophilin, respectively.

4. Arrestin plays multiple roles in regulating α_2AR trafficking and signaling

Arrestin is a critical regulator for almost all known GPCRs. Arrestin was first discovered for its role in desensitization (“arresting”) of phototransduction [51]. In response to light, rhodopsin undergoes conformational changes induced by light-evoked electronic changes in its covalently bound ligand, retinol. These changes result in activation of the visual G protein, transducin. In addition to activation of molecular pathways that mediate the visual response, signal activation also results in activation of rhodopsin kinase, phosphorylation of rhodopsin, and stabilization of rhodopsin’s interaction with arrestin (the “48K antigen”) [52–54]. Arrestin binding to rhodopsin leads to stabilization of rhodopsin’s phosphorylated state and to functional uncoupling from transducin, thus terminating activation.

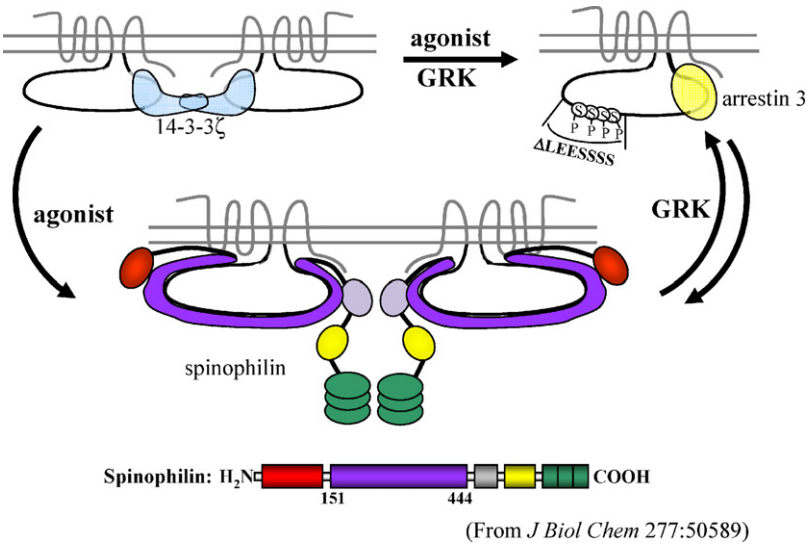

Fig. 1 – Postulated regulatory cycle for interactions of spinophilin, 14-3-3ζ and arrestin 3 with the 3i loop of the α_{2A}AR. The interactions of the α_2AR with 14-3-3ζ likely occur when the receptor is in an agonist naïve state; these interactions might contribute to receptor targeting and retention at the cell surface, but the functional role of α_2AR–14-3-3ζ interactions has not been established. Agonist occupancy can stabilize the α_2AR interaction with arrestin as well as with spinophilin, depending on the phosphorylation state of the receptor. Arrestin preferentially binds to GRK-phosphorylated α_2AR, whereas spinophilin prefers to bind to the non-phosphorylated receptor. Spinophilin and arrestin compete for interaction with the α_2AR 3i loop.

Visual arrestin is a member of a protein family of arrestins [55]. Arrestins 1 (rods) and 4 (cones) are expressed in the visual system. Arrestins 2 (also known as β-arrestin 1) and 3 (β-arrestin 2) are ubiquitously expressed. A role of arrestin has been revealed not only in terminating signaling pathways [56], but also in serving as an adaptor protein bringing these receptors into association with clathrin-coated pits, thus facilitating agonist-evoked GPCR internalization [57,58]. Furthermore, arrestin has been implicated as a scaffold for GPCR interaction with other signaling pathways, such as extracellular signal-regulated kinase (ERK) [59] and c-Jun amino-terminal kinase (JNK) [60] pathways. Thus, arrestin appears to serve as a molecular switch for signal activation as well as deactivation and receptor redistribution.

All three α_2AR subtypes interact with arrestin 3 [44–46]. The α_{2B}AR also interacts with arrestin 2 with a relatively lower affinity than that with arrestin 3 [45]. Arrestin regulation of α_2AR functions discussed below are based on studies on α_{2A}- and α_{2B}AR subtypes.

4.1. *Arrestin stabilizes α_2AR phosphorylation*

Arrestin enhances agonist-elicited GRK-catalyzed phosphorylation of many GPCRs [61–63]. This also holds true for the α_2ARs. Overexpression of arrestin 3 in COSM6 cells, which have a relatively low level of endogenous arrestin, greatly increases the level of agonist-induced α_2AR phosphorylation [50]. Overexpression of arrestin 3 does not affect GRK2 interaction with the receptor [50], indicating that increased phosphorylation of α_2AR observed with overexpression of arrestin 3 is likely due to stabilization of phosphorylation (i.e. preventing receptor dephosphorylation, as shown previously for the paradigmatic GPCR, rhodopsin [62,64,65]), rather than to enhancement of GRK2 association. Further corroboration of the importance of arrestin in achieving detectable phosphorylation of agonist-occupied α_2AR is the evidence that in mouse embryonic fibroblasts (MEFs) derived from mice null for both arrestin 2 and arrestin 3 (Arr2,3$^{-/-}$), agonist-induced phosphorylation of the α_2AR is more difficult to detect than in MEFs isolated from WT mice expressing arrestin [50].

4.2. *Arrestin mediates α_2AR endocytosis*

Arrestin mediates agonist-evoked internalization of a number of GPCRs through clathrin-coated pits by directly interacting with clathrin [57] and AP2 [58]. Although the α_{2A}- and α_{2B}AR exhibit different internalization profiles in response to agonist [66,67], endocytosis of both subtypes requires the presence of arrestin, as revealed by immunofluorescence and cell surface ELISA approaches ([50]; Wang, Brady and Limbird, unpublished data). In WT cells, ~20% of the α_{2A} and ~40% of the α_{2B}AR undergo endocytosis after agonist treatment. However, in MEFs without arrestin expression (Arr2,3$^{-/-}$), no significant loss of surface α_2AR following agonist stimulation is detected ([50]; Wang, Brady and Limbird, unpublished data).

Based on their abilities to associate with arrestin post endocytosis, GPCRs are divided into two classes. Endocytosis of so-called class A GPCRs is not accompanied by arrestin co-localization with receptors in an endocytosed compartment and receptor endocytosis is followed by recycling and resensitization [63,68]. On the other hand, following internalization of class B GPCRs, arrestin trafficks with the receptor to intracellular compartments, where subsequent GPCR trafficking to lysosomes leads to receptor degradation and receptor down-regulation [55,63,68]. The α_2AR appears to be a class A receptor. Thus, arrestin-mediated internalization of the α_2AR serves as a prelude for receptor recycling. As discussed below, arrestin-mediated endocytosis and subsequent recycling of the α_2AR may lead to an acceleration of α_2AR-evoked signaling processes.

4.3. *Arrestin regulates spatiotemporal properties of α_2AR-activated ERK signaling*

GPCRs can activate ERK1/2 signaling through G protein-dependent and arrestin-dependent pathways [69]. G protein-dependent activation of ERK occurs transiently and active ERK signal is translocated into nuclei, whereas arrestin-dependent, G protein-independent activation of ERK has a sustained signal and is restricted to the cytosol [69,70]. Our studies have demonstrated that α_{2A}AR endogenously expressed in MEFs evokes ERK signaling in a G protein-dependent manner [50,71]. In this process, arrestin seems to be dispensable, since α_2-agonists are able to evoke ERK activation in arrestin-deficient MEFs with a potency similar to that observed in WT cells [71].

Although not required for α_2AR-evoked ERK activation *per se*, arrestin tightly regulates the time course of this process. Without arrestin expression, the α_{2A}AR-evoked ERK signal is much extended [50,71], consistent with the role of arrestin in desensitization. Deletion of the GRK phosphorylation sites from the α_{2A}AR, which decreases the receptor binding affinity for arrestin, also leads to prolonged ERK activation [50]. More intriguingly, the rate of α_{2A}AR-evoked ERK activation appears to be slower in arrestin deficient cells than that in WT cells, especially when cells are restimualted after a 5-min stimulation and a 30-min wash (a protocol that allows monitoring resensitization) [50,71]. These findings suggest that arrestin plays a role in acceleration of α_2AR-mediated ERK signaling, presumably through mediating endocytosis which leads to recycling and replenishment of the surface α_{2A}AR pool for agonist activation. Similarly, arrestin-mediated endocytosis has been previously suggested as a prerequisite for β_2AR resensitization [72].

Arrestin also is critical in defining the spatial profiles of α_2AR-evoked ERK activation. In WT MEFs, α_{2A}AR-evoked ERK phosphorylation is first detected in cytosol (2 min) and then translocated into nuclei at later time points (10 min). However, in the absence of arrestin, α_{2A}AR-evoked ERK phosphorylation is enriched in the nuclei of target cells from the earliest time point (1 min) evaluated [71].

4.4. *Arrestin serves as a molecular switch determining Src involvement in α_2AR-activation of ERK*

Studies done by Dr. Lefkowitz and colleagues first identified arrestin as a signaling scaffold linking β_2AR, Src and MEK to ERK activation [73,74]. Using pharmacological inhibitor blocking and dominant negative inhibition approaches, we found that Src tyrosine kinase also is involved in endogenous α_{2A}AR-mediated ERK activation, and Src involvement in this process requires the

presence of arrestin [71]. Stimulation of α_{2A}AR enhances the complex formation of Src and arrestin, and promotes Src activation. When arrestin is absent, the α_{2A}AR is still able to elicit ERK signaling, but through a Src-independent pathway [71]. Src-dependent and -independent pathways both converge on the Ras-Raf-MEK pathway [75] to activate ERK phosphorylation [71]. Therefore, in the G protein-dependent ERK activation by α_{2A}AR, arrestin serves as molecular switch, linking α_{2A}AR to the Src-dependent but not to the Src-independent pathway. The receptor tyrosine kinase, EGFR, is not required for α_{2A}AR-evoked ERK signaling [71], despite their reported involvement in ERK activation by many other GPCRs [74].

5. Regulation of the α_2AR by the multi-domain protein, spinophilin

Spinophilin (also known as Neurabin II) is a ubiquitous protein named for its enrichment in dendritic spines [76]. Spinophilin has multiple domains including an actin-binding domain, protein phosphatase 1 (PP1)-binding and regulatory domain, a PSD-95/Discs large/ZO-1 homology (PDZ) domain, and three coiled-coil domains. In the nervous system, spinophilin targets PP1 to ionotropic glutamate receptors, thus modulating synaptic plasticity [77,78]. Through its interaction with F-actin, spinophilin also has been implicated in spine morphogenesis [77,79] and neuronal migration [80,81]. Studies by Smith et al. [82] and Richman et al. [43] demonstrated that the sequence region encompassed by spinophilin amino acids 151–444 (Sp151–444) interacts with GPCRs, specifically the G_i/G_o-coupled D2 dopamine receptor [82] and all three α_2AR subtypes [43], *via* their 3i loops. More recently, interaction of spinophilin with the 3i loop of α_1AR also has been reported [83]. In contrast, neurabin I, which is highly homologous to neurabin II/spinophilin except in the ''GPCR binding domain'', does not interact in a detectable fashion with these GPCRs [43,84].

5.1. *Spinophilin tethers the α_2AR at the cell surface*

In renal epithelial cells (including MDCKII cells), endogenous spinophilin is enriched at the basolateral surface, where the α_2AR subtypes localize [79]. Taking advantage of the unique targeting property of the α_{2B}AR subtype in polarized MDCK cells (random delivery to both apical and basolateral surfaces with rapid turnover at the apical surface), Brady et al. found that redirection of the receptor binding domain of spinophilin to the apical surface significantly extends the half life of α_{2B}AR at this surface compartment [85], consistent with the interpretation that endogenous spinophilin is responsible for, at least in part, surface retention of the α_2AR at the basolateral surface. Moreover, in MEFs without spinophilin expression (derived from $Sp^{-/-}$ mouse embryos), turnover of the α_{2B}AR from the cell surface following agonist treatment is much accelerated and occurs to a larger extent than that in WT cells expressing spinophilin [85]. Since spinophilin and arrestin both interact with the α_2AR 3i loop and are mutually exclusive for this interaction, the accelerated internalization of the α_2AR is presumably due to unimpeded arrestin binding to the receptor in the absence of spinophilin, which leads to receptor endocytosis.

5.2. *Spinophilin competes for agonist-induced GRK2 binding to the α_2AR–$G_{\beta\gamma}$ complex*

Spinophilin interaction with the α_{2A}AR requires the N- and C-terminal ends of the 3i loop of the receptor, which form amphipathic helices predicted to lie just beneath surface membrane [46]. These helices also have been implicated in receptor–G protein coupling. However, spinophilin binding seems not to perturb α_2AR–G protein coupling (Wang and Limbird, unpublished finding). Thus, spinophilin likely recognizes the α_2AR–G protein complex. In support of this hypothesis, we found that agonist-enhanced interaction of spinophilin with the α_2AR is diminished when cells are pretreated with pertussis toxin or expressing GRK2-C terminus [50], both conditions which impair functional interaction between GPCR and the $G_{i/o}$ subfamily of G proteins and block $G_{\beta\gamma}$-mediated signal transduction. Thus, spinophilin interaction with the α_2AR seems to require α_2AR–$G_{\beta\gamma}$ interactions and/or G protein-mediated signaling events. Whether or not spinophilin directly interacts with the $\beta\gamma$ subunits of G proteins has not yet been established.

The N- and C-terminal proximal regions of the α_2AR 3i loop that interact with spinophilin [46] are also involved in GRK2 binding [28], raising the possibility of competition between spinophilin and GRK2 for the agonist-occupied receptor. Indeed, agonist-induced GRK2 association with the α_2AR is prevented when spinophilin is overexpressed in the same cell background [50]. However, under this circumstance the amount of GRK2 translocated to the cell membrane following agonist treatment is not perturbed [50], indicating that spinophilin blocks binding to receptor–$\beta\gamma$ interactions but does not perturb GRK2 translocation.

5.3. *Spinophilin attenuates α_2AR phosphorylation*

Spinophilin competes for α_2AR interaction with GRK2 and arrestin, which are responsible for catalyzing and stabilizing α_2AR phosphorylation, respectively. Therefore, association of spinophilin with the α_2AR leads to alteration of α_2AR phosphorylation following agonist stimulation. In MEFs without spinophilin expression (where arrestin action is unopposed), agonist stimulation causes a significant increase in agonist-elicited phosphorylation of the α_2AR as compared to that in WT cells [50]. On the other hand, overexpression of spinophilin in cells significant attenuates agonist-induced α_2AR phosphorylation [86]. Intriguingly, this spinophilin effect requires the presence of arrestin to be detected, since in cells with low levels of endogenous arrestin expression, such as COS M6 cells, the effect of spinophilin on α_2AR phosphorylation is negligible unless arrestin is heterologously expressed in these cells (which stables the α_2AR phosphorylation) [50]. These findings suggest that spinophilin principally functions as an antagonist of arrestin actions, or as ''arrestin's nemesis''.

5.4. *Spinophilin regulates α_2AR signaling duration and response sensitivity*

Arrestin association with the α_2AR results in desensitization as well as acceleration of α_2AR-mediated ERK signaling. Through competing for GRK and arrestin association with

the α_2AR, spinophilin counteracts these arrestin-mediated functions. In spinophilin-deficient MEFs, ERK activation by endogenously expressed α_{2A}AR desensitizes significantly faster than that in WT MEFs [50]. Also, the rate of α_{2A}AR-mediated ERK is more rapid in $Sp^{-/-}$ cells than in WT cells, especially when cells are restimualted after a 5-min stimulation and a 30-min wash (representing resensitization) [50]. Using combined gene knockout and RNA silencing strategies, we confirmed that both desensitization and acceleration of α_{2A}AR-mediated ERK signaling in $Sp^{-/-}$ cells depends on the presence of arrestin [50]. As discussed earlier, arrestin-dependent acceleration of ERK stimulation is conceivably due to recycling of α_{2A}AR to the surface following arrestin-dependent endocytosis, increasing the receptor population available for reactivation. Consistent with this hypothesis, blockade of clathrin-coated pit-mediated α_{2A}AR internalization by incubation of MEFs in K^+-depleted medium (low $[K^+]_o$) eliminates the accelerated rate of α_{2A}AR-mediated ERK activation seen in $Sp^{-/-}$ cells when compared to WT MEFs. Taken together, through antagonizing arrestin functions, spinophilin regulates both α_2AR signaling duration and response sensitivity.

5.5. *Other potential functions of spinophilin*

Spinophilin contains multiple domains. However, the receptor binding domain of spinophilin alone seems to be sufficient to compete for arrestin binding to the α_2AR [86]. How the other domains of spinophilin may contribute to antagonism of arrestin functions and/or link the receptor to signaling and regulatory pathways has not been rigorously explored. The PP1 binding domain of spinophilin may recruit PP1 to regulate the phosphorylation level of the α_2AR, thus affecting the interaction equilibrium between spinophilin and arrestin with the receptor [46]. The PDZ domain of spinophilin has been shown to interact with C-terminal end of the $p70^{S6}$ kinase ($p70^{S6K}$) [87,88]. Stimulation of α_{2A}AR can result in activation of $p70^{S6K}$ [86]. Therefore, spinophilin may serve as a scaffold linking the α_{2A}AR to the $p70^{S6K}$ signaling pathway.

By analogy to arrestin enrichment at the cell surface following stimulation of GPCRs, α_2AR activation also leads to enrichment of spinophilin at the surface [89]. This phenomenon requires the actin-binding domain of spinophilin and appears to involve G protein-mediated signaling pathways, since it can be eliminated by pertussis toxin treatment of target cells [89]. Intriguingly, only agonist activation of the α_{2A} but not the α_{2B}AR subtype can cause this effect in a detectable fashion, despite the fact that both subtypes interact with spinophilin [89]. This subtype-selective enrichment of spinophilin may represent a mechanism contributing to subtype signaling diversity between the α_{2A} and the α_{2B}AR, which may serve as a base for different physiological functions elicited by these two subtypes [90].

6. *In vivo* relevance of the reciprocal regulation of α_2AR by spinophilin and arrestin

We have identified spinophilin antagonism of multiple arrestin functions in regulating the α_2AR [50]. Fig. 2 provides a schematic diagram of the reciprocal regulation of α_2AR by spinophilin and arrestin. Spinophilin interaction with α_2ARs is

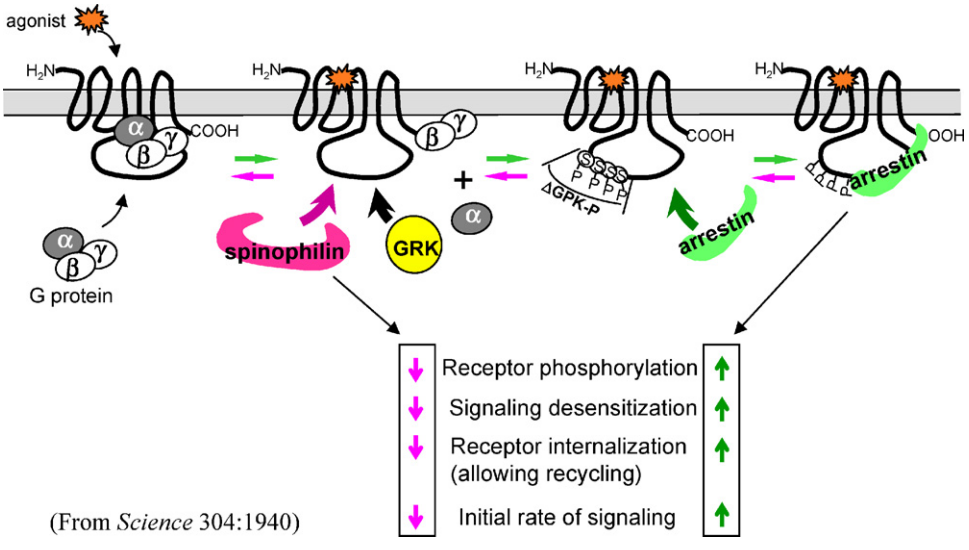

Fig. 2 – Model of α_2AR interactions with arrestin, and spinophilin, and their functional consequences. GRK2 interacts with the α_2AR–$G_{\beta\gamma}$ complex and mediates α_2AR phosphorylation. Spinophilin, which also recognizes the α_2AR–$G_{\beta\gamma}$ complex and shares the interacting sites on the 3i loop with GRK2, competes for GRK2 association with the α_2AR. GRK2-catalyzed phosphorylation of the α_2AR is stabilized by arrestin 3 binding to the receptor, and is diminished by increasing the relative concentration of spinophilin compared to arrestin in target cells. Arrestin binding to the α_2AR induces desensitization by disrupting coupling to G proteins and mediates endocytosis by facilitating binding to the clathrin-coated pit machinery. One outcome of α_2AR endocytosis is receptor recycling, which may facilitate signaling activation and resensitization through replenishment of surface α_2AR available for activation. These arrestin-mediated functions are antagonized by spinophilin interaction with the α_2AR.

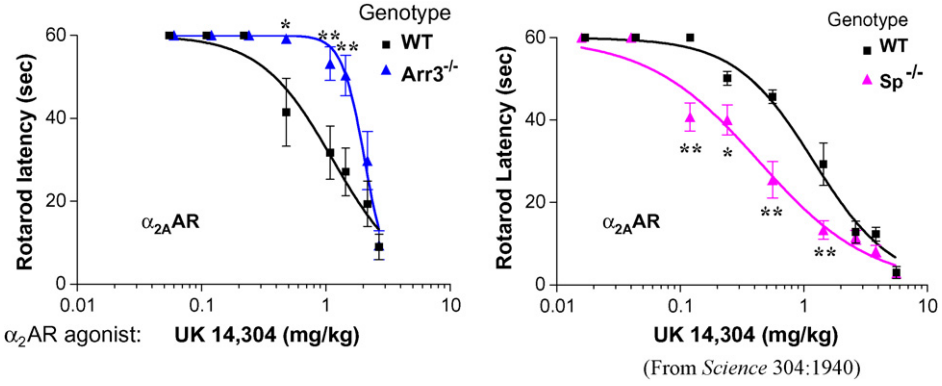

Fig. 3 – Reciprocal effects of spinophilin and arrestin 3 on α_2AR-mediated sedation *in vivo*. Sedation in response to α_2AR-agonists was assessed *via* rotarod latency following administration of increasing doses of the α_2-agonist UK 14,304. The EC_{50} values for sedation in Arr3$^{-/-}$ and corresponding WT litter mates (n = 5 for each genotype) are 2.1 and 1.1 mg/kg, respectively, and the EC_{50} values for sedation in Sp$^{-/-}$ and corresponding WT mice (n = 6 for dose 0.56 mg/kg and n = 11 for the rest doses for each genotype) are 0.4 and 1.2 mg/kg, respectively. $^{*}p < 0.01$; $^{**}p < 0.05$.

enhanced by agonist occupancy but diminished by GRK2-catalyzed receptor phosphorylation. This is qualitatively different from arrestin interactions with α_2AR, which are enhanced by phosphorylation of the receptor [55,91]. The ability of spinophilin to compete for GRK2 binding to the agonist-α_2AR–$G_{\beta\gamma}$ complex, thus blocking receptor phosphorylation and enhanced arrestin binding, may be the sole mechanism by which spinophilin antagonizes arrestin functions. However, we cannot rule out direct competition for arrestin binding by spinophilin, albeit diminished by α_2AR phosphorylation [46]. Regardless of whether either or both of these mechanisms dominate in intact cells, it is clear that spinophilin serves as a functional antagonist of the multiple functions of arrestin in receptor signaling and trafficking.

The data shown in Fig. 3 suggest that our *in vitro* findings indicating reciprocal regulation of α_2AR functions by spinophilin and arrestin have *in vivo* relevance. UK 14,304, an α_2-agonist, evokes sedation in WT mice *via* activation of the α_{2A}AR subtype [5]. In Sp$^{-/-}$ mice, α_2-agonists become more efficacious, manifest by a leftward shift in the UK 14,304 dose response curve for sedation (Fig. 3, left panel). In contrast, Arr3$^{-/-}$ mice are more resistant to UK 14,304-evoked sedation, manifest by a rightward shift in the dose response curve (Fig. 3, right panel). Despite the unknown mechanism of α_{2A}AR elicited sedation, these *in vivo* data strongly suggest that spinophilin antagonism for arrestin functions in regulating α_{2A}AR exist in *native* target cells.

7. Conclusion

Interactions of the α_2AR with various surface and cytoplasmic proteins would represent an important mechanism regulating α_2AR-elicited physiological and pharmacological functions in response to different ligands, in different cell types and under different disease conditions. While heterologous systems are powerful in studying α_2AR interaction with other proteins, the ultimate goal is to identify the functional relevance of such interaction in native cells. Identification of more interactors of the α_2AR and understanding of functional roles of the observed association would eventually provide therapeutic insights on manipulating α_2AR function in a variety of clinical settings, such as hypertension, pain diminution, attentional focus (as in attention deficit and hyperactivity disorder) and cognition enhancement.

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