

CLASSICAL AND NEW ROLES OF β -ARRESTINS IN THE REGULATION OF G-PROTEIN-COUPLED RECEPTORS

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In the classical model of G-protein-coupled receptor (GPCR) regulation, arrestins terminate receptor signalling. After receptor activation, arrestins desensitize phosphorylated GPCRs, blocking further activation and initiating receptor internalization. This function of arrestins is exemplified by studies on the role of arrestins in the development of tolerance to, but not dependence on, morphine. Arrestins also link GPCRs to several signalling pathways, including activation of the non-receptor tyrosine kinase SRC and mitogen-activated protein kinase. In these cascades, arrestins function as adaptors and scaffolds, bringing sequentially acting kinases into proximity with each other and the receptor. The signalling roles of arrestins have been expanded even further with the discovery that the formation of stable receptor–arrestin complexes initiates photoreceptor apoptosis in *Drosophila*, leading to retinal degeneration. Here we review our current understanding of arrestin function, discussing both its classical and newly discovered roles.

Heptahelical or G-protein-coupled receptors (GPCRs) form a large superfamily of cell-surface receptors (>1,000 members in humans) that respond to an enormous array of stimuli, including light, hormones and neurotransmitters. This superfamily is responsible for processes that range from the perception of light, smell, taste and pain, to synaptic neurotransmission and the hormonal control of many physiological processes. Many drugs that are commonly used today, including analgesics, antihypertensives and antipsychotics, target GPCRs. Although these receptors respond to diverse agonists, the structural and functional features of the receptors are remarkably conserved. As was first elucidated for rhodopsin and the β_2 -adrenoceptor, all GPCRs have seven membrane-spanning domains, an extracellular amino terminus and an intracellular carboxyl terminus. Agonist occupancy of these receptors promotes the activation of heterotrimeric G proteins by catalysing the exchange of GDP for GTP on the G-protein α -subunit. The α - and $\beta\gamma$ -subunits of the G protein dissociate from each other, and separately activate several classical effectors, including adenylyl cyclases, phospholipases and ion channels.

In addition to switching on these classical effectors, receptor activation also initiates other highly conserved processes that lead to the desensitization or turning off of classical receptor signalling. Two types of kinase phosphorylate the receptor and participate in desensitization. One type — the second-messenger-dependent kinases, such as cAMP-dependent protein kinase and protein kinase C — is involved in heterologous receptor desensitization. This form of desensitization does not require agonist occupancy of the receptor; instead, it simply depends on kinase activation by many different stimuli. Second-messenger-dependent phosphorylation is thought to alter receptor conformation such that the affinity of the receptor for the G protein is greatly reduced¹. The second type of kinase — GPCR kinases (GRKs) — is involved in homologous receptor desensitization. This desensitization is referred to as homologous because only agonist-occupied receptors are substrates for GRKs, ensuring that only those receptors that have been activated will be desensitized.

So far, seven GRKs (GRK1–7) have been cloned. GRK1, also known as rhodopsin kinase, is primarily

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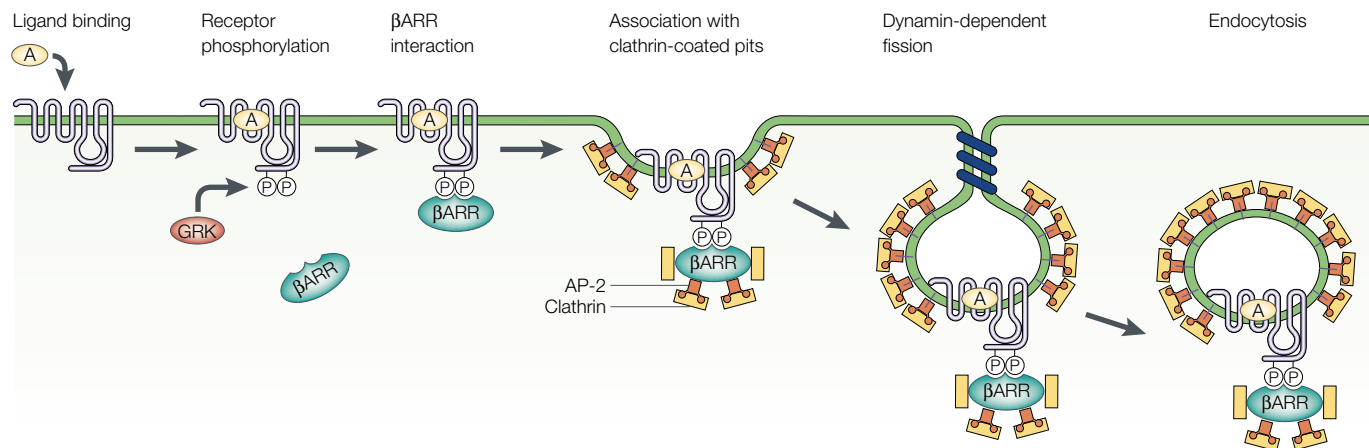


Figure 1 | **β -Arrestin-dependent internalization of GPCRs.** After agonist (A) binding to G-protein-coupled receptors (GPCRs), GPCR kinases (GRK) phosphorylate residues in the third intracellular loop and carboxyl tail of GPCRs, leading to the recruitment of β -arrestins (β ARR). The β -arrestins recruit clathrin and the AP-2 complex, which target GPCRs for clathrin-mediated endocytosis.

responsible for phosphorylating rhodopsin. It is expressed almost exclusively in the eye², and is important for adaptation to light. GRK2 and GRK3, also known as β ARK1 and β ARK2, are among the most widely distributed GRKs, and phosphorylate a wide range of receptors. In studies of genetically modified mice, it has been found that knocking out GRK2 leads to embryonic lethality, and that heterozygotes show altered cardiac contractility³. GRK3 knockout mice show impaired odorant receptor desensitization⁴, and GRK5 knockout mice are supersensitive to the effects of muscarinic receptor activation, including salivation and tremor⁵. Relatively little is known about the other GRKs. The expression of GRK4 is largely limited to the testis, whereas GRK6 is highly expressed in a wide range of tissues⁶. The tissue distribution of GRK7 is limited to the eye, and might specifically regulate cone opsins⁷.

In the late 1980s, during attempts to purify β ARK from bovine brain, a surprising finding was made: the more purified the kinase, the less effective it was in desensitizing the β_2 -adrenoceptor *in vitro*⁸. When purified β_2 -adrenoceptors in phospholipid vesicles that contained the adenylyl cyclase stimulatory G protein, G_s , were co-incubated with partially purified β ARK, the agonist-stimulated GTPase activity of G_s was robustly inhibited. But when more purified preparations of β ARK, which were able to phosphorylate the receptor with the same stoichiometry as the partially purified fractions, were included in the reactions, the inhibition of agonist-stimulated GTPase activity was largely lost. So, even though the receptor was phosphorylated, it was not desensitized. As receptor phosphorylation was clearly not the only determinant of receptor desensitization, it was concluded that another protein present in the partially purified fractions must be crucial in regulating GPCR desensitization. Surprisingly, *in vitro* desensitization of the β_2 -adrenoceptor could be partially restored by the addition of visual arrestin⁹, a protein that had previously been implicated in the desensitization of rhodopsin. However, large amounts of arrestin were required to partially recover of β_2 -adrenoceptor desensitization,

indicating that visual arrestin was not the protein responsible for desensitizing β_2 -adrenoceptors *in vivo*.

Homologues of arrestin that might be more selective for β_2 -adrenoceptors were then sought. In the following years, several arrestin homologues, along with a few splice variants, were cloned from bovine and rat brain. **β -arrestin 1** (REF. 10) and **β -arrestin 2** (REF. 11), also known as arrestin 2 and arrestin 3, share high homology with each other and with visual arrestin. Both proteins can robustly desensitize β_2 -adrenoceptors *in vitro*, by physically preventing the interaction between the phosphorylated receptor and its cognate heterotrimeric G protein. A third homologue — **cone arrestin** — has been cloned. The distribution of this arrestin is limited to cones and to the pineal gland¹². By contrast, both β -arrestin 1 and β -arrestin 2, assessed by immunohistochemistry, are widely expressed throughout the body, although there are some differences in their distribution¹¹. For example, in the rat brain, β -arrestin 2 is more widely expressed overall than β -arrestin 1. However, the expression of β -arrestin 1 is higher in the olfactory bulb and tract, whereas β -arrestin 2 is expressed at particularly high levels in the striatum, hypothalamus and hippocampus. Immunoreactivity to both β -arrestins is especially strong at postsynaptic densities, probably reflecting the high density of GPCRs in these regions.

Classical roles of β -arrestins

After the initial identification of β -arrestins and their roles in desensitizing GPCRs, these proteins were also found to participate in initiating the internalization of several GPCRs, including β_2 -adrenoceptors. It had been known for many years that agonist stimulation of GPCRs, including β_2 -adrenoceptors, led to the formation of a pool of receptors that was inaccessible to hydrophilic drugs, a process variously known as sequestration, internalization or endocytosis. It was later found that this inaccessibility to hydrophilic ligands correlated with the subcellular redistribution of agonist-stimulated β_2 -adrenoceptors from the cell surface into **ENDOSOMES** that also contained the protein **TRANSFERRIN**¹³.

ENDOSOME

An organelle that carries materials ingested by endocytosis and passes them to lysosomes for degradation or recycles them to the cell surface.

TRANSFERRIN

A metal-binding glycoprotein involved in ferric ion uptake into the cell. The pathway followed by transferrin bound to its receptor defines a classical recycling pathway.

Subsequent studies have established that β -arrestins are essential in the internalization of many GPCRs, acting as adaptors that link the receptors to CLATHRIN-coated pits¹⁴. Both β -arrestin 1 and β -arrestin 2 directly interact with at least two components of the endocytotic machinery: clathrin itself¹⁵ and the β 2-adaptin subunit of the AP-2 COMPLEX¹⁶ (FIG. 1). So, after the GRK-mediated phosphorylation of GPCRs, β -arrestins bind to the receptor, and recruit clathrin and AP-2, leading to the co-localization of the GPCR and β -arrestin in punctated pits at the cell surface. In addition, β -arrestin 1 and β -arrestin 2 interact with membrane phospholipids, which might help to target β -arrestins to clathrin-coated pits¹⁷. Soon afterwards, the receptors, either alone or together with β -arrestin (see below), internalize to acidic endosomes, where they are either dephosphorylated and recycled to the cell surface, or degraded in a process known as downregulation, which leads to a reduction in the total number of receptors. Although many receptors internalize through clathrin-coated pits, there are additional pathways of GPCR internalization, including CAVEOLAE¹⁸, which are independent of both clathrin and β -arrestin.

As β -arrestin 1 and β -arrestin 2 share such a high degree of homology, and both proteins can functionally desensitize the β_2 -adrenoceptor *in vitro*, it was initially assumed that these two proteins served the same functions. However, more recent studies have shown overlapping, but not identical, functions of β -arrestin 1 and β -arrestin 2 in regulating both GPCR signalling and internalization. An important tool for delineating the roles of these two proteins has been the development of mouse embryo fibroblasts (MEFs) obtained from embryos that lack β -arrestin 1, β -arrestin 2 or both proteins¹⁹. In studies of second-messenger generation, cyclic AMP accumulation stimulated by β_2 -adrenoceptor activation was enhanced in MEFs lacking one or other β -arrestin isoform, but was even more robustly enhanced in cells lacking both β -arrestins. Likewise, in MEFs expressing the angiotensin AT_{1A} receptor, inositol phosphate accumulation elicited by angiotensin application was only modestly enhanced in the single knockouts, but was robustly enhanced in the double-knockout MEFs. These data indicate that, at least for desensitization of these two receptors, the β -arrestins might be interchangeable. However, there are differences in the ability of β -arrestin 1 and β -arrestin 2 to support internalization of the β_2 -adrenoceptor. As compared with wild-type MEFs, β_2 -adrenoceptor sequestration is intact or enhanced in β -arrestin-1 knockout cell lines, inhibited in β -arrestin-2 knockout cells, and abolished in the double knockouts. In reconstitution experiments in which one or other isoform was selectively transfected into the double-knockout cells, about 100-fold less β -arrestin 2 than β -arrestin 1 was required to restore sequestration, showing that, at least for the β_2 -adrenoceptor, the β -arrestins are not functionally redundant. It therefore seems that both β -arrestin 1 and β -arrestin 2 can support GPCR desensitization and internalization. However, depending on the particular GPCR, β -arrestin 2 might be more efficacious than β -arrestin 1 in supporting internalization. There are undoubtedly many reasons for differences in the ability of β -arrestins 1

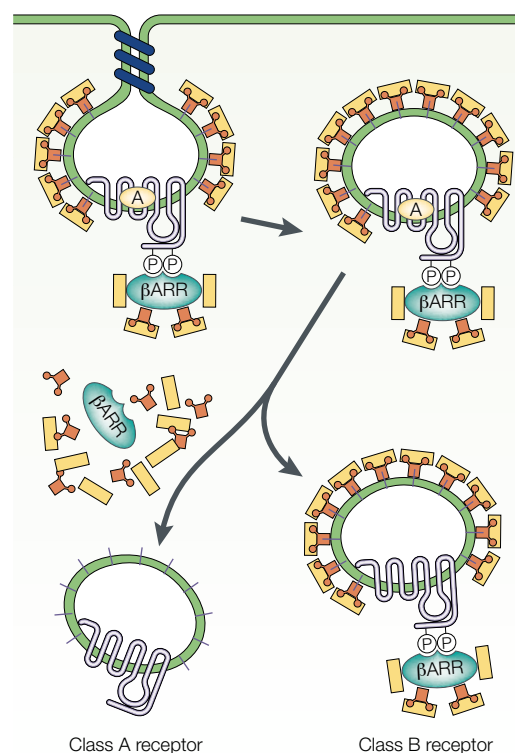


Figure 2 | Internalization properties define two classes of GPCRs. G-protein-coupled receptors (GPCRs) of class A, such as the β_2 -adrenoceptor, preferentially internalize through a β -arrestin-2-dependent mechanism, whereas class B receptors, such as the angiotensin receptor AT_{1A}, have no preference for β -arrestin 2 over β -arrestin 1. In the case of class A receptors, the β -arrestin–GPCR interaction is transient, and β -arrestin does not localize with the GPCR in endosomes. For class B receptors, the β -arrestin–GPCR interaction is more stable, and the receptor and β -arrestin co-localize in endosomes. A, agoist; β ARR, β -arrestin.

and 2 to support β_2 -adrenoceptor internalization, including differences in their binding to components of the endocytotic machinery, such as clathrin and AP-2.

Differences in the translocation of β -arrestin 1 and β -arrestin 2 to GPCRs have also been examined in confocal microscopy studies using β -arrestins tagged with green fluorescent protein (GFP). These studies have shown that GPCRs can be divided into two classes — A and B — on the basis of their internalization properties²⁰ (FIG. 2). In the case of class A receptors, such as the β_2 -adrenoceptor, β -arrestin 2 translocates to the receptor more readily than β -arrestin 1. The receptor and β -arrestin co-localize in clathrin-coated pits at or near the cell surface, and rapidly dissociate before the GPCR travels to endosomes. In the case of class B receptors, such as the angiotensin receptor AT_{1A} or the vasopressin V2 receptor, the translocation of β -arrestin 1 to the receptor is indistinguishable from that of β -arrestin 2. Moreover, the receptor and β -arrestin co-localize throughout the internalization processes, and can be found together in endosomes. The carboxy-terminal tail of the receptor is important in determining into which class a given GPCR fits²¹. So, a chimeric GPCR with the seven transmembrane domains of the β_2 -adrenoceptor

CLATHRIN

A major structural component of coated vesicles that are implicated in protein transport. Clathrin heavy and light chains form a triskelion, the main building element of clathrin coats.

AP-2 COMPLEX

A heterotetrameric complex composed of subunits called adaptins. It is one of the main components of the coats formed during membrane endocytosis.

CAVEOLAE

Flask-shaped, cholesterol-rich invaginations of the plasma membrane that might mediate the uptake of extracellular materials, and are probably involved in cell signalling.

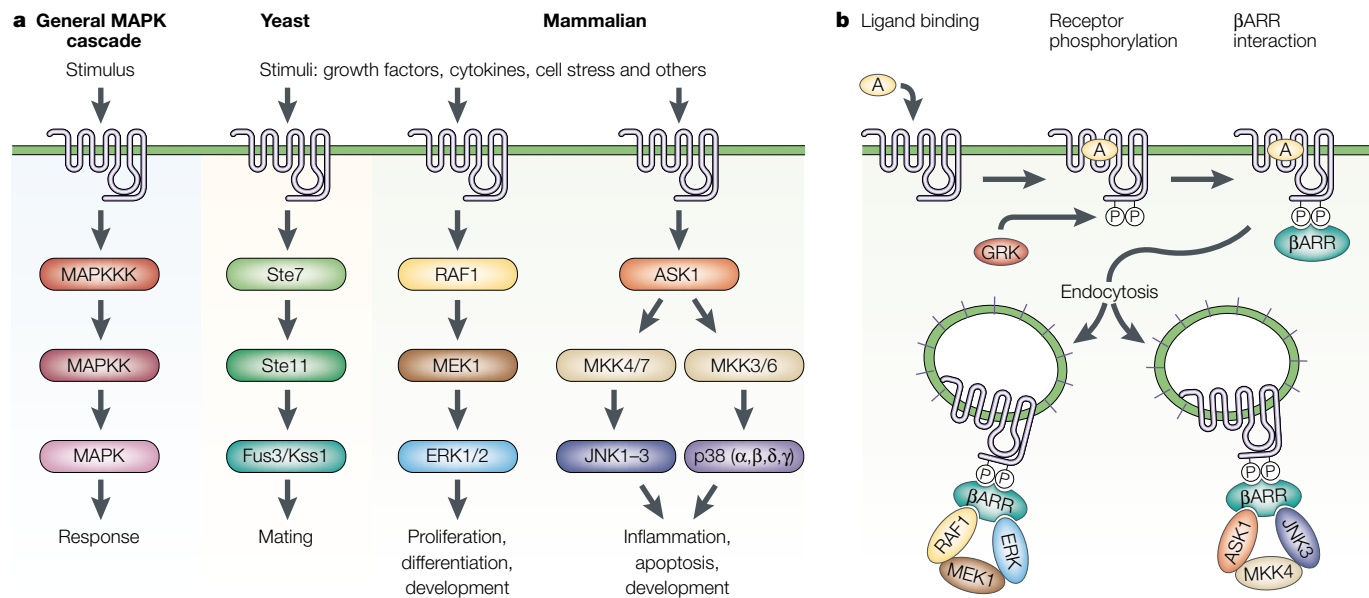


Figure 3 | **β-Arrestin and the GPCR activation of MAPK cascades.** **a** | G-protein-coupled receptor (GPCR) activation of mitogen-activated protein kinase (MAPK) cascades proceeds by the sequential activation of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. In yeast, the activation of pheromone receptors leads to the Gβγ-dependent activation of the MAPK cascade. In the case of the mammalian extracellular-signal-regulated kinase (ERK) cascade, receptor activation leads to both Gα- and Gβγ-dependent activation of ERK. Little is known about the early intermediates in the activation of the c-Jun N-terminal kinase (JNK) and p38 MAPK cascades. **b** | β-Arrestin (βARR) acts as a scaffold for GPCR activation of both the ERK and the JNK3 cascades. Agonist (A) binding leads to the GPCR kinase (GRK)-mediated phosphorylation of the receptor, the recruitment of β-arrestin, and the activation of both ERK and JNK3. β-Arrestin scaffolding is similar in these cascades. The expression of β-arrestin enhances overall ERK and JNK3 activation, and it seems that the interaction with the MAPKKK — apoptosis-signal-regulating kinase 1 (ASK1) or v-raf-1 murine leukaemia viral oncogene homologue 1 (RAF1) — and the MAPK (ERK or JNK) is direct, whereas interaction with the MAPKK — MAPK/ERK kinase 1 (MEK1) or MAPKK 4 (MKK4) — is indirect. Both β-arrestin 1 and β-arrestin 2 can scaffold the ERK cascade, whereas only β-arrestin 2 can scaffold the JNK3 cascade.

and the carboxy-terminal tail of the V2 vasopressin receptor shares internalization properties with the vasopressin receptor. The opposite is true when the tail of the β₂-adrenoceptor is switched to the body of the V2 receptor. For these two proteins, switching only the carboxy-terminal tails determines both the affinities for β-arrestin 2 and β-arrestin 1, and whether the receptor internalizes with β-arrestin or not. In initial studies with just a few GPCRs, clusters of serines and threonines were identified in the carboxy-terminal tail of class B, but not class A, receptors. Mutating these residues to alanines confers the receptors with class A properties. Interestingly, all GPCRs that couple to the adenylyl cyclase inhibitory G protein, G_i, that have been examined so far, such as the **μ-opioid receptor**, are class A receptors, whereas GPCRs that couple to G_s or G_q, such as the β₂-adrenoceptor or the V2 vasopressin receptor, fit into both classes.

Functionally, the class a GPCR fits into dictates its trafficking pattern²². Class A receptors recycle rapidly; they are de-phosphorylated, resensitized and returned to the cell surface in about 30 minutes. Class B receptors, by contrast, recycle much more slowly, and most of the receptors remain in endosomes, even after an hour, and are still associated with β-arrestin. The physiological significance of the differences between the class A and class B receptors is not yet known, but it might relate to differences in signalling pathways activated by the GPCRs (see below).

Several questions remain about the roles of β-arrestins in receptor desensitization, internalization and downregulation. For example, what determines whether a receptor

will internalize through clathrin-coated pits or through alternative pathways such as caveolae? Are desensitization and internalization parts of the same pathway, or can these two functions of β-arrestin be dissociated? Evidence to suggest that these functions are distinct is accumulating. For example, the muscarinic acetylcholine **M2 receptor** is phosphorylated by GRKs, β-arrestins subsequently bind and the receptor desensitizes. However, the M2 muscarinic receptor internalizes primarily through β-arrestin-independent mechanisms²³. What is the relationship between receptor internalization and downregulation? In some cases, internalization has been implicated as a prerequisite for receptor downregulation, but this is not a general rule. These questions have recently been addressed in β-arrestin knockout MEFs. In cells that lack either β-arrestin 1 or β-arrestin 2, β₂-adrenoceptor downregulation is intact. However, downregulation is abolished in the double knockouts, implicating β-arrestins in this process¹⁹. Surprisingly, this downregulation might not depend on receptor internalization. So, β₂-adrenoceptor internalization is largely lost in β-arrestin-2 knockout MEFs, whereas downregulation remains intact. Alternatively, as β₂-adrenoceptor internalization and downregulation are both completely abolished in the double-knockout MEFs, perhaps the small amount of internalization that remains in the β-arrestin-2 knockout is sufficient to support downregulation.

Classical roles of β-arrestins in vivo

With the recent development of animals that lack either β-arrestin 1 or β-arrestin 2 (the double knockout is

CARDIAC EJECTION FRACTION

A measure of the pumping efficiency of the heart. The ejection fraction is the amount of blood ejected from the left ventricle with each heartbeat, expressed as a percentage of the total amount of blood in the heart at the beginning of contraction. The normal ejection fraction lies between 55% and 75%.

MITOGEN-ACTIVATED PROTEIN KINASE CASCADE

A signalling cascade that relays signals from the plasma membrane to the nucleus. Mitogen-activated protein kinases (MAPKs), which represent the last step in the pathway, are activated by a wide range of proliferation- or differentiation-inducing signals. Extracellular-signal-regulated kinases (ERKs) are among the best-characterized MAPKs.

SRC

The first proto-oncogene to be identified. It codes for a non-receptor protein tyrosine kinase.

DYNAMIN

A GTPase that is involved in endocytosis. It is thought to be involved in severing the connection between the nascent vesicle and the donor membrane.

CHEMOKINES

Small, secreted proteins that stimulate the motile behaviour of leukocytes.

POLYMORPHONUCLEAR NEUTROPHIL

A phagocytic cell that has an important role in the inflammatory response, undergoing chemotaxis towards sites of infection or wounding. It is characterized by the presence of cytoplasmic granules that are not stained by acidic or basic dyes, hence the name 'neutrophil'.

C-JUN N-TERMINAL KINASES

A family of kinases, distantly related to extracellular-signal-regulated kinases (ERKs), that are activated by dual phosphorylation on tyrosine and threonine residues. JNK3 is mainly found in neurons and might participate in the regulation of apoptosis.

embryonic lethal), it has become possible to study the physiological roles of these proteins *in vivo*. β -arrestin-1 knockout animals seem to be largely normal, except that they have a slightly greater CARDIAC EJECTION FRACTION after β -adrenoceptor stimulation than their wild-type littermates²⁴. Although β -arrestin-2 knockout animals also seem to be grossly normal, striking physiological alterations have been discovered in their responses to the μ -opioid receptor agonist morphine^{25,26}. Both the duration of action and the analgesic potency of single doses of morphine are markedly enhanced in the β -arrestin-2 knockout mice compared with wild-type littermates. Moreover, the β -arrestin-2 knockout mice, unlike wild-type animals, fail to develop tolerance to the repeated administration of morphine. In the mice lacking β -arrestin 2, daily doses of morphine produce the same analgesic effect after seven days as after one day. By contrast, the same dose of morphine produces almost no analgesia in the wild-type littermates by the seventh day. In wild-type mice, but not the β -arrestin-2 knockouts, the morphine dose-response curve is shifted to the right by almost an order of magnitude after seven days of treatment. So, to elicit the same analgesic effect as on the first day of treatment, it takes almost ten times the dose of morphine on the seventh day. This repeated morphine administration did not alter the number of μ -opioid receptors in either wild-type or knockout mice, although it did uncouple the μ -opioid receptor from G proteins in the brainstem and the periaqueductal grey, brain regions that express significant amounts of β -arrestin 2. This finding indicates that β -arrestin-dependent uncoupling from the G protein is involved in the development of tolerance to morphine. Interestingly, in several studies in transfected cell lines, the μ -opioid receptor does not internalize after morphine treatment, although it does internalize in response to stronger opioids such as etorphine²⁷. If morphine also fails to promote agonist-induced internalization of the μ -opioid receptor *in vivo*, then tolerance to morphine might simply involve β -arrestin-dependent uncoupling from the G protein, and not agonist-induced internalization or downregulation of the receptor.

So, β -arrestin 2 prevents the development of tolerance to morphine. Is it also involved in the development of physical dependence to morphine? Surprisingly, the answer is no²⁶. Wild-type and β -arrestin-2 knockout mice were implanted with a morphine pellet, which provided a continuous supply of morphine for three days, and then treated with the μ -opioid receptor antagonist naloxone. Naloxone triggered the same withdrawal symptoms, including weight loss, diarrhoea and paw tremors, in the wild-type and β -arrestin-2 knockout mice. Previously, tolerance to morphine had been thought of as a stepping stone to physical dependence. These data, however, clearly dissociate the two phenomena.

Although it is not known at the present time, it will be interesting to discover whether the β -arrestin-1 knockout mice develop tolerance to morphine. We predict that they will, just like the wild-type mice. As the μ -opioid receptor is a class A receptor, it has higher affinity for β -arrestin 2 than for β -arrestin 1. If the latter is knocked out, sufficient

β -arrestin 2 should be expressed to compensate for the loss, especially in the relevant brain regions. In any case, these studies have revealed the utility of the β -arrestin-2 knockout mice for understanding some of the sequelae of addiction, including the development of tolerance and withdrawal. It is hoped that these studies will help to identify targets that could enhance the long-term efficacy of morphine and other opioids for pain relief, and limit the addictive potential of the opioids.

New roles of β -arrestins

In the past few years, our appreciation of the roles of β -arrestins has expanded markedly with the discovery that they can serve as adaptor proteins that facilitate the activation and subcellular localization of signalling cascades, particularly MITOGEN-ACTIVATED PROTEIN KINASE CASCADES (MAPK cascades) (FIG. 3a). The first hint that β -arrestins might regulate GPCR signalling beyond their roles in desensitization and internalization came with the discovery that β -arrestin 1 interacts with the non-receptor tyrosine kinase SRC²⁸. Members of the SRC family regulate cell proliferation and differentiation, and are also involved in the GPCR activation of MAPK/extracellular-signal-regulated kinase (ERK) cascades²⁹. Moreover, SRC also regulates the internalization of GPCRs³⁰, and of receptor tyrosine kinases such as the epidermal growth factor receptor³¹, by phosphorylating DYNAMIN and clathrin.

As some pathways of GPCR-stimulated ERK activation (such as the β_2 -adrenoceptor) involve both β -arrestin-dependent internalization³² and SRC activation, it was proposed that SRC might co-localize with β -arrestins in clathrin-coated pits after the agonist stimulation of β_2 -adrenoceptors. Indeed, SRC was found to co-immunoprecipitate with β_2 -adrenoceptors in an agonist- and β -arrestin-dependent manner, indicating a possible direct interaction between the two proteins²⁸. Mutations of SRC that disrupt its ability to interact with β -arrestin 1 disrupt both the tyrosine phosphorylation of dynamin and the dynamin-dependent internalization of the β_2 -adrenoceptor³³. Furthermore, mutations in β -arrestin that prevent its interaction with SRC inhibit β_2 -adrenoceptor stimulation of the ERK cascade²⁸. Moreover, the interaction of β -arrestin 1 with other SRC family members regulates CHEMOKINE-receptor-mediated granule release from POLYMORPHONUCLEAR NEUTROPHILS³⁴.

Recently, these roles of β -arrestins in stimulating signalling have been further expanded with the discovery that β -arrestins act as agonist-regulated scaffolds for two MAPK cascades — the ERK cascade^{35–37} and the C-JUN N-TERMINAL KINASE type 3 (JNK3) cascade³⁸ (FIG. 3b). Scaffolding proteins have several important roles in regulating signalling pathways, especially MAPK cascades. MAPK scaffolds were first appreciated in yeast, in which activation of the pheromone receptor (a GPCR) leads to the $G_{\beta\gamma}$ -dependent activation of a MAPK (Fus3 or Kss1) via the scaffold protein Ste5. Subsequently, several mammalian MAPK scaffolds have been discovered, including the JUN-KINASE-INTERACTING PROTEIN (JIP) family of JNK scaffolds. A general feature of MAPK scaffolds is that they bring together the sequential kinases of a MAPK cascade

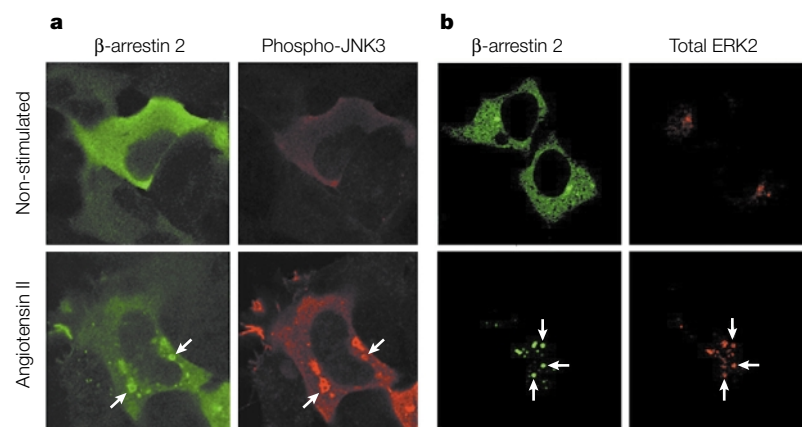


Figure 4 | Similarity in the angiotensin-stimulated redistribution of ERK and JNK3 in cells that express β -arrestin 2. **a** | Human embryonic kidney (HEK)-293 cells were transfected with the AT_{1A} angiotensin receptor, β -arrestin 2 tagged with green fluorescent protein (GFP), and phospho-JNK3 (c-Jun N-terminal kinase 3). In the absence of an AT_{1A} angiotensin receptor agonist, β -arrestin 2 and phospho-JNK3 were diffusely distributed throughout the cytosol (upper panel). Receptor stimulation with angiotensin II led to an increase in the amount of phospho-JNK3, and to the redistribution of both β -arrestin 2 and phospho-JNK3 to endosomes, where they co-localized (lower panel, arrows). Adapted with permission from REF. 38 © 2000 American Association for the Advancement of Science. **b** | In HEK-293 cells transfected with the AT_{1A} receptor, β -arrestin 2 tagged with GFP, and ERK2 tagged with red fluorescent protein, angiotensin II causes both β -arrestin 2 and ERK2 (extracellular-signal-regulated kinase 2) to redistribute to endosomes (arrows), which also contain the AT_{1A} receptor (not shown). Adapted with permission from REF. 37 © 2001 National Academy of Sciences, USA. These data indicate that β -arrestin 2 targets both activated ERK and JNK3 to endosomes, where they might phosphorylate non-nuclear substrates.

(FIG. 3a): a MAPK kinase kinase (MAPKKK, such as **RAF1**), a MAPK kinase (MAPKK, such as **MEK1**) and a MAPK (such as ERK). In doing so, scaffolds facilitate protein–protein interactions, ensure signalling specificity, and maintain the correct subcellular distribution of proteins. For both the JNK3 and ERK cascades, β -arrestin performs all of these functions. A notable difference between the two β -arrestin scaffolds is that only β -arrestin 2 can scaffold the JNK3 pathway, whereas both β -arrestin 1 and β -arrestin 2 can scaffold ERK.

The β -arrestin-scaffolded JNK3 and ERK pathways share many common features (FIG. 3). For example, co-expression of β -arrestins leads to an enhancement in the AT_{1A}-receptor-mediated stimulation of both JNK3 and ERK^{37,38}. As illustrated by JNK3, both scaffolds contain a MAPKKK (**ASK1**) and a MAPKK (**MKK4**), in addition to the MAPK (JNK3). Both scaffolds are highly selective. Although β -arrestin 2 markedly facilitates ASK1-mediated JNK3 activation, it does not facilitate the activation of **JNK1**, another JNK family member. Interestingly, for both scaffolds, β -arrestin seems to interact directly with both the MAPKKK and the MAPK, but might interact indirectly with the MAPKK^{36,37} (FIG. 3b). So, one way in which β -arrestin regulates the MAPK cascade is by bringing the MAPKKK and the MAPK together, facilitating the agonist-dependent interaction with the MAPKK. It is noteworthy that, in the case of the β -arrestin–ERK scaffold, some of the component kinases might vary. For both the protease-activated receptor 2 (**PAR2**) and the AT_{1A} angiotensin receptor, agonist stimulation leads to a complex that contains the receptor, β -arrestin 1, the MAPKKK (**RAF1**) and ERK^{35,37}. However, in the case of the neurokinin NK1 receptor,

Raf is not detectable; instead, the scaffold contains SRC along with ERK³⁶. For both the JNK3 and ERK scaffolds, agonist activation of GPCRs (clearly shown for the AT_{1A} angiotensin receptor) leads to both the co-localization of β -arrestin and activated JNK3 or ERK in endosomal vesicles, and the exclusion of ERK and JNK3 from the nucleus^{37,38} (FIG. 4).

In addition to its role in resensitizing receptors, GPCR internalization also targets the active ERK and JNK3 to specific substrates. The data available so far indicate that one function of β -arrestin is to target both ERK and JNK away from the nucleus and into the cytosol, facilitating the phosphorylation of non-nuclear substrates. Further evidence in support of this idea has come from the study of the PAR2 scaffold. In this case, the β -arrestin-dependent pathway of ERK activation is associated both with a failure of ERK to translocate to the nucleus, and a failure to stimulate cellular proliferation, a common consequence of ERK activation³⁵.

What, if any, is the role for G-protein activation in these β -arrestin-scaffolded ERK pathways? How are the β -arrestin scaffolds regulated? What are the cytosolic substrates of ERK and JNK3? These and other exciting questions remain to be answered.

New roles of β -arrestins *in vivo*

At present, we have a very limited understanding of the physiological roles of β -arrestin-scaffolded MAPK cascades. One particularly interesting feature of the β -arrestin 2–JNK3 scaffold is that, although the distribution of JNK3 is rather limited, this protein is highly expressed in brain regions that also express substantial levels of β -arrestin 2. Furthermore, endogenous JNK3 can be immunoprecipitated from bovine brain lysates using β -arrestin-selective antibodies³⁸. Specifically, both JNK3 and β -arrestin 2 are highly expressed in the hippocampus, indicating that the β -arrestin 2–JNK3 scaffold might be particularly important in regulating hippocampal function. However, the effect of knocking out β -arrestin 2 has not been explored in the context of learning, memory, spatial navigation and other hippocampal functions.

In addition to the scaffolding roles of β -arrestins, the *Drosophila* arrestin protein (**Arr2**) has recently been found to have a surprising role in regulating retinal cell apoptosis in the fly. Like many mammalian GPCRs, *Drosophila* rhodopsin is desensitized. After activation, rhodopsin is phosphorylated; Arr2 is then recruited to the receptor, and turns off signalling by interfering with G-protein coupling. Two separate studies have reported that the stable recruitment of Arr2 to activated rhodopsin leads to retinal cell apoptosis^{39,40}. This apoptosis requires rhodopsin phosphorylation, phosphorylation of Arr2 by calcium/calmodulin-dependent protein kinase II, and clathrin-mediated endocytosis. Moreover, mutant forms of *Drosophila* dynamin that block rhodopsin internalization can also block apoptosis. So, like the β -arrestin-scaffolded ERK and JNK3 complexes, this Arr2-dependent apoptotic pathway is another example of GPCR internalization that is associated with signalling. It will be of particular importance to determine whether the

JUN-KINASE-INTERACTING PROTEINS

Proteins that bind to c-Jun N-terminal kinase (JNK) *in vitro* and *in vivo*. They can also bind to upstream components of the JNK pathway, indicating that they might function as a scaffold proteins that regulate the specificity of JNK signalling.

RETINITIS PIGMENTOSA

An inherited condition of the retina, in which rods degenerate. The loss of rods diminishes the patient's ability to see in dim light and can also diminish peripheral vision.

formation of stable rhodopsin–arrestin complexes also leads to mammalian retinal cell degeneration, as a similar mechanism of retinal cell degeneration has been implicated in the human disease known as RETINITIS PIGMENTOSA. Up to 25% of the mutations associated with autosomal-dominant **retinitis pigmentosa** affect the rhodopsin gene. As constitutively active mutants of rhodopsin lead to retinitis pigmentosa in humans, it was thought that constitutive G-protein signalling might be the cause of the disease. However, it was recently found that the constitutively active rhodopsin mutants were highly phosphorylated and were associated with a large amount of visual arrestin, indicating that it is not G-protein signalling *per se*, but rather the constitutive association of rhodopsin with arrestin that triggers the retinal degeneration⁴¹. It is therefore possible that in humans, as in *Drosophila*,

arrestin-triggered apoptosis might be a cause of retinal cell degeneration leading to retinitis pigmentosa, despite the fact that mammalian rhodopsin is not internalized.

Conclusions

Once thought of simply as proteins that desensitize GPCRs by uncoupling them from their cognate G proteins, we are now aware of a rapidly expanding list of signalling functions for β -arrestins. Just five years ago, the now paradigmatic concept of β -arrestin-dependent internalization of GPCRs was discovered. We predict that five years from now, the manifold roles of β -arrestins in heptahelical receptor signalling will be much more fully understood, and the new roles of β -arrestins will include various adaptor and scaffold functions that are, at present, unimagined.

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Online links

DATABASES

The following terms in this article are linked online to:
LocustLink: <http://www.ncbi.nlm.nih.gov/LocustLink/>
 β_2 -adrenoceptor | Arr2 | β -arrestin 1 | β -arrestin 2 | ASK1 | AT $_{1A}$ receptor | cone arrestin | Fus3 | GRK1–7 | JIP | JNK1 | JNK3 | Kss1 | M2 receptor | MEK1 | MKK4 | μ -opioid receptor | PAR2 | RAF1 | Ste5 | V2 receptor
OMIM: <http://www.ncbi.nlm.nih.gov/Omim/>
retinitis pigmentosa