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Serotonin receptor signaling and regulation via β-arrestins

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Abstract

Serotonin receptors are the product of 15 distinct genes, 14 of which are G protein-coupled receptors. These receptors are expressed in a wide range of cell types, including distinct neuronal populations, and promote diverse functional responses in multiple organ systems. These receptors are important for mediating the *in vivo* effects of their cognate neurotransmitter, serotonin, as well as the endogenous tryptamines. In addition, the actions of many drugs are mediated, either directly or indirectly, through serotonin receptors, including antidepressants, antipsychotics, anxiolytics, sleep aids, migraine therapies, gastrointestinal therapeutics and hallucinogenic drugs. It is becoming increasingly evident that serotonin receptors can engage in differential signaling that is determined by the chemical nature of the ligand and that ligands that demonstrate a predilection for inducing a particular signaling cascade are considered to have "functional selectivity". The elucidation of the cellular signaling pathways that mediate the physiological responses to serotonin and other agonists is an active area of investigation and will be an onward-looking focal point for determining how to effectively and selectively promote beneficial serotonergic mimicry while avoiding unwanted clinical side effects. This review highlights the modulation of serotonin 2A, 2C, and four receptors by β-arrestins, which may represent a fulcrum for biasing receptor responsiveness *in vivo*.

Keywords

G protein-coupled receptor; functional selectivity; 5-hydroxytryptamine (5-HT); receptor trafficking; homologous desensitization

Introduction

The serotonin receptors, also known as the 5-hyrdoxytryptamine (5-HT) receptors, are expressed in the periphery as well as in the central nervous system and are activated by the neurotransmitter serotonin. The receptor family is divided into seven main types, comprising 15 genetically unique receptors. All of the serotonin receptors are G protein-coupled receptors (GPCR), with the exception of the serotonin 3 receptor (5-HT3R), which is a

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ligand-gated ion channel. Serotonin receptors expressed in the central nervous system are involved in mediating diverse biological processes, including the modulation of mood, cognition, learning, memory, aggression, feeding behavior, sleep, and pain processing and transmission mechanisms. Receptors expressed in the periphery mediate smooth muscle contraction, platelet aggregation, gastrointestinal function and also impact upon pain processing. Further, activation of serotonin receptors modulates the release of a number of neurotransmitters and hormones. Considering their potential for influencing wide-ranging functions throughout the body, the serotonin receptors are prominent targets for pharmacotherapies, including antidepressants, antipsychotics, appetite suppressants, migraine therapies and drugs for the treatment of gastrointestinal disorders. They are also the direct target of abused drugs, such as the serotonergic hallucinogens lysergic acid diethylamide (LSD) and dimethyltryptamines (DMT) and also indirect targets of psychostimulants such as cocaine and amphetamines.

Canonical regulation of GPCR signaling by β-arrestins

The canonical model of GPCR regulation posits that the agonist bound receptor is regulated through interactions with β-arrestins (reviewed in Ferguson, 2001; Pierce and Lefkowitz, 2001; Luttrell and Lefkowitz, 2002). Upon activation, GPCRs are phosphorylated by GPCR kinases (GRKs), thus initiating the termination of G protein-mediated signaling and promoting the recruitment of β-arrestins to the receptors. The interaction between β-arrestins and GPCRs has been measured by confocal microscopy, co-immunoprecipitation, bioluminescence/fluorescence resonance energy transfer (BRET/FRET), gene reporter and enzyme fragment complementation assays (Barak *et al.*, 1997; Hamdan *et al.*, 2005; Groer *et al.*, 2007; von Degenfeld *et al.*, 2007; Drake *et al.*, 2008; McDonald and Bohn, 2010). There are two forms of β-arrestin, β-arrestin1 and β-arrestin2, which are for the most part, ubiquitously expressed. The two isoforms have very similar amino acid structures and in some cases can substitute in function for each other; however, there is some indication that certain GPCRs may be preferentially regulated by one isoform over the other (Oakley *et al.*, 2000; Kohout *et al.*, 2001).

Following β-arrestin binding to an agonist–receptor complex, structural evidence indicates that β-arrestins sterically block the receptor–G protein interaction and thus play a critical role in the process of homologous desensitization (Gurevich and Gurevich, 2006). Therefore, if the β-arrestin interaction is disrupted, the potential for receptor desensitization may be diminished and relative agonist efficacy may be enhanced. The Lefkowitz group has shown, using mouse embryonic fibroblasts (MEF) derived from genetically modified mice lacking both β-arrestins (βarr1/2-KO MEF), that agonist-induced signaling of β_2 adrenergic receptor to adenylyl cyclase and angiotensin II receptor to phospholipase C is enhanced (Kohout *et al.*, 2001). This group has also demonstrated this phenomenon utilizing siRNA gene silencing, wherein β-arrestin ablation enhances agonist induced G protein-signaling through these receptors in HEK293 cells (Ahn *et al.*, 2003).

Role of β-arrestins in facilitating GPCR signaling

In addition to their roles in the desensitization of GPCRs, β-arrestins have also been shown to mediate receptor signaling by acting as scaffolding elements. In this manner, β-arrestins can couple GPCRs to signaling proteins in a manner that is independent of G protein mediated activation (Lefkowitz and Shenoy, 2005; Kovacs *et al.*, 2009). Cell culture studies have shown that β-arrestins can facilitate signaling for many pathways, including the MAP kinase family of serine/threonine kinases, specifically ERK1/2, p38 kinase and c-Jun N-terminal kinases, the Src family of tyrosine kinases, phosphatidylinositol 3 kinase (PI3-K), and protein kinase B (Akt) (Daaka *et al.*, 1998; Luttrell *et al.*, 1999; McDonald *et al.*, 2000; Luttrell and Lefkowitz, 2002). β-arrestin-mediated signaling has been demonstrated for a wide range of GPCRs, including the neurokinin-1, angiotensin, parathyroid hormone, vasopressin, β-adrenergic, chemokine and dopamine receptors (DeWire *et al.*, 2007). Further, a study by Stalheim *et al.* (2005) demonstrated that β-arrestins are involved in both the desensitization of G protein coupling and the activation of ERK1/2 for the protease-activated receptor-2 (PAR2) *in vitro*. However, mutation of the C-terminal tail of the receptor, a proposed β-arrestin interaction site, only affected the β-arrestin-mediated activation of ERK1/2, suggesting that the pro-signaling and signaling-arresting functions of β-arrestin are controlled by distinct interactions with the GPCRs.

β-arrestin-mediated internalization of GPCRs

Another important step in the β-arrestin-mediated regulation of GPCRs is the internalization of the agonist-bound receptor into intracellular vesicles. Depending on which path the receptor takes following internalization (whether it facilitates coupling to signaling components or acts to isolate the receptor from signaling components), β-arrestin-mediated internalization can positively or negatively impact GPCR signaling. Interactions with β-arrestins facilitate receptor endocytosis for many GPCRs, including the β-adrenergic, angiotensin, muscarinic, dopamine, mu-opioid and serotonin 2 A receptors (5-HT2AR) (reviewed in Ferguson, 2001; Schmid *et al.*, 2008). Both β-arrestin1 and β-arrestin2 have been shown to directly interact with clathrin and the β2-adaptin subunit of the AP-2 complex (Goodman *et al.*, 1996, Laporte *et al.*, 1999; Kim and Benovic, 2002). The recruitment of these proteins then promotes the assembly of the receptors into clathrin-coated endocytic vesicles. Not surprisingly, interfering with β-arrestin/GPCR interactions has been shown to impact receptor trafficking. For example, mutant β-arrestin proteins inhibit β_2-adrenergic receptor internalization (Ferguson *et al.*, 1996). Studies in cell lines demonstrate that the extent of agonist-induced receptor internalization can be influenced by the levels of GRK and β-arrestin protein expressed in the cells, such that overexpression of the proteins can facilitate internalization (Ferguson *et al.*, 1996; Menard *et al.*, 1997; Zhang *et al.*, 1998). The importance of β-arrestin-mediated trafficking of GPCRs is clearly demonstrated in cell lines that lack β-arrestins, wherein certain receptors fail to internalize in response to agonist (Kohout *et al.*, 2001; Stalheim *et al.*, 2005). Receptor endocytosis can serve as a means to compartmentalize receptor signaling, facilitate de-phosphorylation and resensitization or to shuttle the receptor towards a degradation pathway. Therefore, β-arrestins are key components in determining the function and life cycle of a G protein coupled receptor (Shenoy and Lefkowitz, 2003).

The use of β-arrestin knockout mice to study GPCR regulation in vivo

Since β-arrestins can regulate GPCR function by multiple mechanisms, it has been critically important to evaluate the physiological relevance of such regulation. The use of β-arrestin knockout mice (βarr1-KO or βarr2-KO) has been integral in determining how the responsiveness of certain GPCRs can be impacted upon by the removal of a particular β-arrestin *in vivo* (reviewed in Schmid and Bohn, 2009). In the knockout mice, some agonist-induced behaviors are enhanced, suggesting that for these receptor-mediated behaviors, β-arrestin may be acting as a negative regulator. For example, mice lacking β-arrestin2 display enhanced antinociception and greatly diminished antinociceptive tolerance compared to wild-type (WT) mice following treatment with the mu-opioid receptor agonist, morphine (Bohn *et al.*, 1999; 2000; 2002; 2004). In addition, DAMGO-induced [^{35}S]-GTPγS binding demonstrates that G protein coupling is enhanced in membranes from the spinal cords, periaqueductal gray and brainstem of βarr2-KO mice (Bohn *et al.*, 1999; 2000; 2002). These studies suggest that in the absence of β-arrestins, the agonist may display enhanced relative efficacy in activating the receptor and to produce the behaviors. Similarly, β-adrenergic receptor agonists produce exaggerated hemodynamic responses in βarr1-KO mice compared to their WT littermates suggesting that the β_2-adrenergic receptor is more responsive to agonist without the dampening actions of β-arrestin1 (Conner *et al.*, 1997).

Knockout mouse studies have also demonstrated that β-arrestins can facilitate GPCR-mediated actions. In these cases, a diminished behavioral response to a GPCR agonist may indicate a disruption of β-arrestin-mediated signaling. For example, the Limbird laboratory demonstrated that α_2-adrenergic receptor agonists induce sedation to a lesser extent in βarr2-KO mice than in WT mice, suggesting a signal-promoting role for β-arrestin2 (Wang *et al.*, 2004). The D2-dopamine receptor was the first GPCR shown to couple to β-arrestin2 to promote signaling *in vivo* and the stimulation of this receptor by dopamine or dopaminergic drugs leads to the formation of an Akt, β-arrestin2 and protein phosphatase 2A (PP2A) signaling complex in the mouse striatum. Furthermore, the disruption of this complex formation in the βarr2-KO mice is correlated with a reduction in the stimulant effects of amphetamine in these animals (Beaulieu *et al.*, 2005). The Caron laboratory went on to show that some of the antipsychotic effects of lithium can be attributed to a disruption of the β-arrestin2/Akt/PP2A signaling complex in WT mice (Beaulieu *et al.*, 2008). These studies suggest that decreased behavioral responses in βarr2-KO mice may be attributed to a disruption in β-arrestin2-mediated signaling. More recently, β-arrestin2 has been shown to be important in facilitating 5-HT2AR signaling in cells and *in vivo* and this will be discussed in greater detail below (Schmid *et al.*, 2008).

Functional selectivity of GPCR signaling

Agonist binding to a GPCR can stimulate the activation of multiple signaling pathways, including both G protein-mediated and β-arrestin-mediated/G protein-independent pathways. Moreover, the extent of the activation of each of the downstream pathways depends upon the ligand, with some ligands promoting activation of one pathway over another and vice versa. This concept of an agonist selectively stabilizing a receptor conformation, causing the receptor to preferentially interact with a subset of the multiple signaling pathways to which it is coupled, has been termed functional selectivity or biased agonism. (Figure 1) (Kenakin, 1995; Kobilka and Deupi, 2007; Urban *et al.*, 2007). Ligands can also bias receptors for interacting with β-arrestins. For example, morphine, methadone and fentanyl are all agonists at the mu-opioid receptor; however, while methadone and fentanyl robustly recruit both β-arrestins to the receptor, morphine appears to preferentially recruit β-arrestin2 (Bohn *et al.*, 2004). In addition to being ligand-directed, receptor-mediated signaling can also be directed by the cellular microenvironment in which the receptor is expressed. This also has been demonstrated for the muopioid receptor, wherein treatment with morphine does not lead to the recruitment of β-arrestin2 to the receptor in HEK293 cells, but does cause β-arrestin2 translocation in MEF cells and in HEK293 cells overexpressing GRK2 (Bohn *et al.*, 2004).

As GPCRs, the serotonin receptors are subject to regulation by β-arrestins. However, the role of β-arrestins in regulating serotonin receptors has only partially been determined. This review focuses on the modulation of serotonin 2A receptor (5-HT2AR), serotonin 2C receptor (5-HT2CR) and serotonin 4 receptor (5-HT4R) responsiveness by β-arrestins.

Serotonin 2A receptor signaling and regulation via β-arrestins

The 5-HT2AR is expressed in both the periphery and in the central nervous system and is involved in mediating many physiological responses, including platelet aggregation, vascular and nonvascular smooth muscle contraction and the modulation of mood and perception (Roth et al., 1998). Dysregulation of the 5-HT2AR has been implicated in a variety of mental health disorders, including depression, suicide and some of the symptoms of schizophrenia (Maes and Meltzer, 1995; Roth and Meltzer, 1995). Therefore, the 5-HT2AR is a prominent target for drug therapy; for example 5-HT2AR blockade has been shown to mediate, at least in part, the effects of atypical antipsychotic drugs such as clozapine, risperidone and olanzapine (Meltzer, 2002). In addition, serotonergic drugs that cause hallucinations in humans produce their pharmacological effects by activating the 5-HT2AR (Aghajanian and Marek, 1999; Nichols, 2004). Interestingly, not all agonists at the 5-HT2AR induce hallucinations in humans (Pieri *et al.*, 1978; Nichols, 2004), suggesting that agonists to the 5-HT2AR can promote diverse functional consequences. Moreover, studies by the Sealfon and Gingrich laboratories have shown that hallucinogens can elicit differential downstream gene response profiles than non-hallucinogens acting at the 5-HT2AR (Gonzalez-Maeso *et al.*, 2007).

The 5-HT2AR has been shown to interact with β-arrestins both *in vitro* and *in vivo*, implicating these proteins in the regulation of the receptor. The Roth group has demonstrated that both β-arrestin1 and β-arrestin2 are able to interact with fusion proteins encoding the third intracellular loop of the rat 5-HT2AR (Gelber *et al.*, 1999). The 5-HT2AR is highly expressed on pyramidal neurons in the prefrontal cortex (Willins *et al.*, 1997; Cornea-Hebert *et al.*, 1999; 2002) and both β-arrestin1 and β-arrestin2 are expressed in frontal cortex as well (Attramadal *et al.*, 1992; Gurevich *et al.*, 2002). To assess 5-HT2AR co-expression with β-arrestins *in vivo*, Gelber *et al.* (1999) utilized dual-label fluorescence confocal microscopy on tissue sections from the rat prefrontal cortex. Both β-arrestin1 and β-arrestin2 are shown to be co-expressed with the 5-HT2AR in some, but not all, cortical pyramidal neurons. Further analysis of β-arrestin1 expression revealed some colocalization with the 5-HT2AR in intracellular vesicles (Gelber *et al.*, 1999). These studies indicate that the 5-HT2AR can interact with β-arrestins *in vivo* and suggest that these interactions may have relevance to the regulation of the receptor in the frontal cortex.

Given that β-arrestins have been found to be colocalized with the 5-HT2AR in cortical neurons, studies have explored whether β-arrestins are recruited to the receptor in response to agonist treatment. Serotonin induces marginal translocation of β-arrestin1 to the plasma membrane of HEK293 cells transfected with the 5-HT2AR and β-arrestin1 tagged with GFP (βarr1-GFP) as determined by confocal microscopy. β-arrestin2 is also recruited to the 5-HT2AR following agonist treatment, as the 5-HT2AR agonist quipazine induces robust translocation of βarr2-GFP to the plasma membrane (Bhatnagar *et al.*, 2001). Further, a myc-tagged β-arrestin2 (myc-βarr2) co-immunoprecipitates with the 5-HT2AR following treatment with serotonin (Bhattacharya *et al.*, 2010). In Figure 2 we show that serotonin also induces β-arrestin2 translocation to 5-HT2AR expressed in HEK293 cells. Similarly, we have recently demonstrated by co-immunoprecipitation, that treatment with 5-hydroxytryptophan (5-HTP), the precursor to serotonin, induces associations between the 5-HT2AR and β-arrestin2 in the mouse frontal cortex (Schmid and Bohn, 2010).

β-arrestins as negative regulators of the 5-HT2AR

The 5-HT2AR has been shown to couple with $G\alpha_q$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ proteins both *in vitro* and *in vivo* (reviewed in Nichols, 2004). Stimulation of the $G\alpha_q$ pathway results in the activation of phospholipase C (PLC), the generation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), and the release of calcium from intracellular stores (Conn and Sanders-Bush, 1984; 1986; Roth *et al.*, 1984). Short-term exposure to agonist leads to a marked decrease in phosphoinositide (PI) hydrolysis *in vitro* (Ivins and Molinoff, 1991; Roth *et al.*, 1995) and therefore PI hydrolysis is often used to study the desensitization state of the 5-HT2AR. Prior to the availability of siRNA or genetic knockout cell models, dominant negative β-arrestins ($\beta arr1_{319-418}$; $\beta arr2_{284-409}$), which encode only for the C-terminal tail of the protein and contain the clathrin-binding domain and not the GPCR binding domain, have been utilized to inhibit the function of endogenously expressed β-arrestins *in vitro* (Krupnick *et al.*, 1997; Orsini and Benovic, 1998; Hanley and Hensler, 2002). This construct is expected to compete in receptor-pit assembly, and thereby disrupt normal trafficking and desensitization of the GPCR; for example, co-expression of $\beta arr1_{319-418}$, potentiates the isoproterenol-induced desensitization of the β_2-adrenergic receptor (Zhang *et al.*, 1997; Gray *et al.*, 2001). Studies of 5-HT2AR expressed in HEK293 cells tested the involvement of β-arrestin-regulation in the desensitization of quipazine-stimulated PI hydrolysis and found that $\beta arr1_{319-418}$ has no effect on quipazine-induced desensitization of PI hydrolysis, nor did it affect the resensitization of the pathway (Gray *et al.*, 2001). Though β-arrestin1 does not seem to be required for the desensitization of the 5-HT2AR in HEK293 cells, its function to negatively regulate the 5-HT2AR may be cell-type specific. For instance, in C6 glioma cells which endogenously express the 5-HT2AR, the $\beta arr1_{319-418}$ dominant negative mutant potentiated quipazine-induced 5-HT2AR receptor desensitization and inhibited resensitization of receptor-mediated PI hydrolysis (Gray *et al.*, 2001).

Facilitation of 5-HT2AR signaling by β-arrestins

β-arrestins can promote the activation of GPCR signaling by scaffolding components of the cascades to the receptors (DeWire *et al.*, 2007). Studies in our laboratory have demonstrated 5-HT2AR signaling through β-arrestins at the biochemical level (Schmid *et al.*, 2008).

Serotonin leads to the activation of ERK1/2 that is greatly potentiated in WT MEFs stably expressing the 5-HT2AR as compared to serotonin effects on 5-HT2AR expressed in βarr1/2-KO MEFs (Schmid *et al.*, 2008). In animal studies, serotonin activates ERK1/2 in frontal cortex of WT but not βarr2-KO mice, suggesting that serotonin utilizes a β-arrestin2-dependent mechanism to activate this cascade *in vivo* (Schmid *et al.*, 2008). In neuronal cultures, the 5-HT2AR agonist, α-methylserotonin, was shown to induce ERK1/2 phosphorylation in the dendrites of cortical neurons in a manner that requires β-arrestins as siRNA mediated knockdown of either β-arrestin inhibited the activation of ERK1/2 by α-methylserotonin (Yuen *et al.*, 2008). Interestingly, the presence of a cell-permeable dynamin inhibitory peptide also blocked the increase in ERK1/2 phosphorylation by α-methylserotonin, suggesting that 5-HT2AR endocytosis may be essential for β-arrestin-dependent, serotonin-induced ERK1/2 phosphorylation. Recently, we have found in cortex as well as in cortical cultures, that serotonin activates Akt via Src- and PI3-K-dependent mechanism that requires β-arrestin2 (Schmid and Bohn, 2010).

β-arrestin-mediated regulation of 5-HT2AR internalization

Similar to other GPCRs, agonist stimulation leads to the internalization of the 5-HT2AR, both *in vitro* and *in vivo* (Berry *et al.*, 1996; Gray and Roth, 2001). The involvement of β-arrestins in regulating agonist-induced internalization events has also been considered. The Roth group demonstrated that treatment of HEK cells with serotonin induces internalization of the 5-HT2AR, a process that is blocked by the dominant negative dynamin K44A (Bhatnagar *et al.*, 2001). Interestingly, the β-arrestin dominant negative mutants (βarr1$_{319\text{-}418}$; βarr2$_{284\text{-}409}$) have no affect on quipazine-induced internalization of a GFP-tagged 5-HT2AR (5-HT2AR-GFP), as analyzed by confocal microscopy. Further, quantification by a cell-surface biotinylation assay showed that βarr1$_{319\text{-}418}$ has no effect on the serotonin-induced 5-HT2AR internalization either (Bhatnagar *et al.*, 2001). However, it is not clear if these studies reflect the lack of involvement of β-arrestin or if they demonstrate the failure of the dominant negative mutant β-arrestins to effectively compete with this aspect of β-arrestin regulation.

In contrast to the studies reported above in HEK293 cells, studies from our laboratory have demonstrated that the 5-HT2AR can be internalized in a β-arrestin-dependent manner, both in primary neuronal cultures and in MEFs, which perhaps underscores the importance of the cellular environment in determining the function of the receptor in response to ligand (Schmid *et al.*, 2008). As previously reported, we found that the 5-HT2AR is predominantly localized in intracellular vesicles in neurons cultured from the frontal cortex of WT mouse neonates (Gelber *et al.*, 1999; Xia et al., 2003). However, in neurons cultured from βarr2-KO mice the 5-HT2AR primarily resides on the cell surface (Schmid *et al.*, 2008). This lack of 5-HT2AR internalization is rescued by transfection of βarr2-GFP in the βarr2-KO neurons. The differential localization of the 5-HT2AR depending upon the expression of β-arrestin2 was also observed in MEFs. In the absence of serum, the 5-HT2AR is localized to the cellular membrane of both WT and βarr1/2-KO MEFs while treatment with serotonin induces 5-HT2AR internalization in WT but not in βarr1/2-KO MEFs (Figure 3; Xia *et al.*, 2003; Schmid *et al.*, 2008).

While β-arrestins can interact with the 5-HT2AR to affect internalization, they are not absolutely required for agonist-induced internalization in all cell lines. To assess whether the 5-HT2AR can be internalized via a β-arrestin-dependent pathway in HEK293 cells, Gray *et al.* (2003) co-expressed a constitutively active mutant of β-arrestin1 with the 5-HT2AR and assessed trafficking of the receptor. The $\beta arr1_{R169E}$ mutant binds to GPCRs regardless of the phosphorylation state of the receptor, resulting in enhanced desensitization and internalization of the receptors in the absence of agonist (Gurevich *et al.*, 1995; Gray-Keller *et al.*, 1997; Kovoor *et al.*, 1999). Co-expression of $\beta arr1_{R169E}$ with the 5-HT2AR resulted in translocation of the mutant to the plasma membrane in the absence of agonist as assessed by confocal microscopy. Further, co-immunoprecipitation studies reveal agonist-independent associations between the flag-tagged 5-HT2AR with the mutant β-arrestin1. In addition, $\beta arr1_{R169E}$ expression induced significant constitutive internalization of the 5-HT2AR and decreased basal 5-HT2AR signaling, as assessed by PI hydrolysis (Gray *et al.*, 2003). These studies further indicate that the 5-HT2AR can be internalized by β-arrestins; however, the extent of their involvement appears to be cell-type specific.

Functional selectivity of 5-HT2AR agonists and β-arrestin regulation

While β-arrestins can play an integral role in the desensitization and internalization of the 5-HT2AR, their function may be influenced by the complement of intracellular proteins that are available to help initiate or modulate their interactions with the receptor. The chemical nature of the agonist also determines the role that β-arrestins play in the regulation of the 5-HT2AR (Figure 1). In WT MEFs expressing 5-HT2AR, agonists, such as serotonin, 2,5-dimethoxy-4-iodoamphetamine (DOI) and quipazine, induce internalization of the receptor (Figure 3; Schmid *et al.*, 2008). However, serotonin does not induce 5-HT2AR internalization in the βarr1/2-KO MEFs, while the effects of DOI and quipazine in the KO MEFs are identical to that seen in the WT MEFs (Figure 3; Schmid *et al.*, 2008). These findings underscore the impact that different ligands can make upon determining the dependence upon β-arrestins for inducing receptor internalization.

The differential contribution of β-arrestins is also evident when assessing agonist-directed signaling pathways. While both DOI and serotonin activate ERK1/2 in 5-HT2AR expressing WT MEFs, the extent of ERK activation is fourfold greater for serotonin. However, serotonin and DOI activate ERK1/2 in βarr1/2-KO MEFs to the same extent, which is similar to the levels induced by DOI in the WT cells, suggesting a β-arrestin-bias in the actions of serotonin at the receptor for activating ERK1/2. The contribution of the $G\alpha_q$ protein-coupled pathway to ERK stimulation was determined by treating MEFs with the PLC inhibitor, U73122; this was found to partially block serotonin-mediated ERK1/2 activation and fully block DOI effects in WT MEFs. In the βarr1/2-KO MEFs, U73122 pretreatment completely inhibits ERK1/2 phosphorylation induced by both agonists. Taken together, these data suggest that serotonin can promote ERK1/2 phosphorylation via a β-arrestin-mediated and a $G\alpha_q$/PLC-mediated pathway. Further, this is an example of ligand-directed signaling at the 5-HT2AR, as DOI induced activation of ERK1/2 occurs solely through the PLC-mediated pathway in these cells (Schmid *et al.*, 2008).

This differential 5-HT2AR signaling was also assessed *in vivo* where DOI and 5-HTP treatment induces ERK1/2 phosphorylation in the frontal cortex of WT mice. Consistent with the studies in the MEF cells, only DOI induces ERK1/2 phosphorylation in the cortex of βarr2-KO mice, suggesting that there is a disruption of serotonin-induced 5-HT2AR signaling to ERK when β-arrestin2 is absent (Schmid *et al.*, 2008). These differences between the ligands were also reflected in behavioral responses, where serotonin (injected as the precursor, 5-hydroxy-L-tryptophan) produces a head twitch response in WT but not in βarr2-KO mice, while DOI produces the behavior in both genotypes, suggesting that the deletion of β-arrestin2 only impacts serotonin-but not DOI-mediated signaling (Schmid *et al.*, 2008).

While the differences in agonist-directed internalization and signaling parallel the responses induced *in vivo*, a direct causal link between the internalization event and the signaling and behavioral events has yet to be made. However, other studies examining regulatory proteins in addition to β-arrestins, including PSD-95, may help to shed more light on the interplay between these systems (Abbas *et al.*, 2009). In regard to the interplay between the signaling and the behavior, we have recently found that serotonin and 5-HTP induce a β-arrestin/Src/Akt_2 signaling complex at the 5-HT2AR and that the formation of this complex is necessary for the head twitch response induced by these agonists. In contrast, N-methyltryptamines do not induce the formation of this signaling complex, and head twitch responses caused by these compounds are β-arrestin-independent. Furthermore, disruption of the signaling cascade, by inhibiting PI3-K or Src, prevents serotonin-induced Akt activation in neurons and attenuates serotonin-mediated head twitch responses in normal mice (Schmid and Bohn, in press).

Taken together, these studies demonstrate that the 5-HT2AR interacts with β-arrestins and that these interactions can dictate receptor signaling, trafficking and behavioral responses. Moreover, the studies demonstrate that ligands at the 5-HT2AR can be engineered to possess functional selectivity that can bias receptor signaling and trafficking. This may thereby be a means to impact upon the behavioral responses triggered by this receptor. While the variations seen in cellular model systems emphasize the need to study drug responses at the receptor *in vivo*, this approach is complicated by the fact that all serotonin receptors are activated by serotonin and that these GPCRs are likely regulated by β-arrestins. The serotonin 2C (5-HT2CR) and the serotonin 4 (5-HT4R) receptors have also been studied for their regulation by β-arrestins and these findings are discussed below.

Serotonin 2C receptor signaling and regulation via β-arrestins

The 5-HT2CR is expressed in numerous brain regions, including the choroid plexus, cortex, basal ganglia, hippocampus and hypothalamus and has been shown to play an essential role in the regulation of mood (Giorgetti and Tecott, 2004; Millan, 2005). The 5-HT2CR is the therapeutic target for many disorders, including obesity, schizophrenia, anxiety, depression, drug addiction and Parkinson’s disease (reviewed in Berg *et al.*, 2008; Iwamoto *et al.*, 2009). Like the 5-HT2AR, the 5-HT2CR has been shown to couple to multiple G proteins and activates PLC, PLA_2, phospholipase D (PLD) and ERK1/2 (reviewed in Leysen, 2004; Werry *et al.*, 2006).

Facilitation of 5-HT2CR signaling by β-arrestins

Activation of 5-HT2CR leads to ERK1/2 phosphorylation in several cell lines (Werry *et al.*, 2005; Labasque *et al.*, 2008). Moreover, serotonin activation of 5-HT2CR can lead to β-arrestin-dependent and -independent activation of ERK1/2. Using siRNA to knockdown $G\alpha_q$, $G\alpha_{13}$ and $G\alpha_{i/o}$ proteins, the Marin group showed that serotonin-mediated 5-HT2CR activation of ERK1/2 occurs via a G protein-independent pathway; pretreatment with the PLC inhibitor U73122 or with pertussis toxin also has no effect on ERK1/2 activation in this HEK293 cell line. Interestingly, siRNA to β-arrestin1 and β-arrestin2, as well as a dominant negative to β-arrestin1 ($\beta arr1_{319\text{-}418}$), inhibit serotonin-induced the ERK1/2 phosphorylation (Labasque *et al.*, 2008). Further, a YFP-tagged β-arrestin2 (βarr2-YFP) co-immunoprecipitates with the 5-HT2CR following a five minute treatment with serotonin. A dominant negative or siRNA to calmodulin reduced ERK1/2 phosphorylation in HEK293 cells and, interestingly, reduced the interaction between βarr2-YFP and the receptor. These data suggest that the 5-HT2CR activates ERK1/2 in a β-arrestin-dependent manner and that calmodulin and β-arrestins may act in concert to fully activate this signaling pathway in this cell line (Labasque *et al.*, 2008).

β-arrestin-mediated regulation of 5-HT2CR internalization

The second intracellular loop of the 5-HT2CR undergoes mRNA editing events, which produces distinct isoforms of the receptor that have varying degrees of constitutive activity (Burns *et al.*, 1997; Niswender *et al.*, 1998; Liu *et al.*, 1999). Trafficking profiles of the edited receptors differ such that while the non-edited 5-HT2CR (5-HT2C-INI receptor) is constitutively internalized, the edited versions, such as the 5-HT2C-VGV receptor, are mainly localized to the plasma membrane. While internalization of the edited receptors can be induced by agonist stimulation, a study by the Caron laboratory demonstrated that the unedited version of the 5-HT2CR is constitutively internalized due to its interactions with β-arrestins and that this receptor is highly associated with βarr2-GFP even in the absence of agonist (Marion *et al.*, 2004). Interestingly, mutation of proline 158 in the second intracellular loop of the receptor to an alanine relocalizes the receptor to the cellular membrane and dramatically decreases its constitutive interactions with β-arrestin2 (Marion et al., 2006). These studies demonstrate that the 5-HT2CR is capable of binding to β-arrestins *in vitro* and implicate that β-arrestins are responsible for the differences in constitutive activity observed for the various 5-HT2CR edited products.

Serotonin 4 receptor signaling and regulation via β-arrestins

The involvement of GRKs and β-arrestins in the desensitization and internalization of serotonin receptors has also been demonstrated for the 5-HT4R. The 5-HT4Rs are highly expressed in the limbic structures of the central nervous system, where they have been implicated in learning and memory, feeding control, the stress response and neurodegenerative diseases (Bockaert *et al.*, 2004). They are also highly expressed in the myenteric plexus and circular muscle layers of the colon, where they have been implicated in the pathogenesis of irritable bowel syndrome (Kim, 2009). The 5-HT4R has also been identified and studied for its role in regulating atrial fibrillation (Ouadid *et al.*, 1992; Yusuf *et al.*, 2003). Furthermore, there are spliced variants of the 5-HT4R, which are differentially expressed (Blondel *et al.*, 1998; Claeysen *et al.*, 1998).

Negative regulation of 5-HT4R by β-arrestins

The 5-HT4Rs predominantly couple to $G\alpha_s$ proteins and stimulate adenylyl cyclase (Dumuis *et al.*, 1988); cellular studies have demonstrated a prominent role for GRKs in the desensitization of the 5-HT4R *in vitro*. Ponimaskin *et al.* (2005) show that serotonin stimulation of 5-HT4AR in transfected *S. frugiperda* (Sf.9) cells leads to a dose-dependent phosphorylation of serine residues on the C-terminal domain of the receptor in a manner that is independent of second messenger-dependent kinases (such as PKA and PKC). Moreover, overexpression of GRK2 significantly decreases serotonin-induced cAMP generation in these cells, suggesting a negative regulatory role for GRK2 at the 5-HT4R. A similar effect was also seen for variants of the 5-HT4R, including the 5-HT4A, 5-HT4B, 5-HT4E and 5-HT4F receptors, which displayed less G protein-coupling when GRK2 was overexpressed in COS-7 cells (Barthet *et al.*, 2005).

In addition to their role in negatively regulating 5-HT4R-$G\alpha_s$ coupling, GRKs have also been shown to negatively impact on G protein-independent signaling via this receptor (Barthet *et al.*, 2005). Stimulation of primary colliculi neurons or HEK293 cells with serotonin or a selective 5-HT4R agonist (BIMU8) promotes ERK activation via a Src-mediated mechanism that is independent of PKA, PLC, $G\alpha_{i/o}$ and β-arrestins (Barthet *et al.*, 2007). Interestingly, overexpression of GRK5 inhibits the phosphorylation of ERK 1/2, but not cAMP production, in both HEK293 cells and colliculi neurons following treatment with either serotonin or BIMU8, an effect which is dependent upon both a serine/threonine cluster in the C-terminal tail of the receptor and β-arrestin1, but not β-arrestin2, expression. Furthermore, the phosphorylation of β-arrestin1 at Ser412 appears to be required for this effect as transfection of wild-type β-arrestin1, but not the S412A mutant, rescues this inhibition of ERK in βarr1/2-KO MEFs (Barthet *et al.*, 2009).

5-HT4R internalization via β-arrestins

Serotonin stimulation also has been shown to promote β-arrestin recruitment to the 5-HT4R and to induce internalization of the receptor. Immunoprecipitation of the 5-HT4R from HEK293 cells and immunoblotting for β-arrestins demonstrated that serotonin induces receptor interactions with β-arrestin1 and β-arrestin2 (Barthet *et al.*, 2009). Further, confocal microscopy reveals that treatment with serotonin leads to recruitment of β-arrestin2 to the 5-HT4AR in COS-7 cells and to the 5-HT4AR, 5-HT4BR and 5-HT4ER in HEK293 cells (Barthet *et al.*, 2005; Ponimaksin *et al.*, 2005). This recruitment of β-arrestin2 to the receptor is dependent upon the serine/threonine clusters in the 5-HT4R C-terminal domains and is inhibited by a dominant negative to GRK2, further implicating GRK-phosphorylation of the receptor as an integral component in the regulation of the 5-HT4R (Barthet *et al.*, 2005). Quantification of receptor internalization by radioligand binding of the 5-HT4R antagonist GR113808 on intact cells suggests that β-arrestin2 co-expression may increase receptor internalization induced by serotonin treatment (Ponimaskin *et al.*, 2005). In addition, expression of a dominant negative β-arrestin1 ($\beta arr1_{319\text{-}418}$) inhibits serotonin-induced internalization of the 5-HT4AR in HEK293 cells and in colliculus neurons; however, inhibiting β-arrestin2/5-HT4R interactions by the co-expression of a dominant negative GRK2 does not completely block endocytosis in HEK293 cells, suggesting the 5-HT4R may also be internalized by a β-arrestin2-independent mechanism (Barthet *et al.*, 2005).

In humans there are nine splice variants of 5-HT4Rs that are expressed in the central nervous system, each with a unique C-terminal tail. The isoforms have nearly identical pharmacological properties, but the differences in C-terminal splicing dictate the level of receptor constitutive activity as well as interactions with signaling, sorting and regulatory proteins (Claeysen *et al.*, 1999; 2001; Joubert *et al.*, 2004; Barthet *et al.*, 2005). While the data above indicate that 5-HT4Rs can be desensitized and internalized via classical GRK and β-arrestin-dependent mechanisms, a study by Mnie-Filali *et al.* (2010) suggests that at least two isoforms, 5-HT4AR and 5-HT4BR, depend on different mechanisms for internalization. For example, in HEK293 cells, the flag-tagged 5-HT4AR is constitutively internalized, even after serum-starving the cells. In contrast, the HA-tagged 5-HT4BR is localized predominantly to the cellular membrane in the absence of agonist. These differences in the subcellular distribution of the two isoforms were also observed in primary cortical neurons transfected with the tagged receptors. Interestingly, serotonin-induced internalization of the 5-HT4AR in HEK293 cells was inhibited by expression of dominant negative GRK2 and β-arrestin1 (β$arr1_{319\text{-}418}$), while 5-HT4BR internalization was only partially attenuated by β$arr1_{319\text{-}418}$. This study suggests that although β-arrestins are involved in the trafficking of 5-HT4Rs, there are isoform-specific differences that may impact on the overall regulation of these receptors (Mnie-Filali *et al.*, 2010).

Conclusion

The studies discussed in this review indicate that the regulation of serotonin receptor signaling by β-arrestins is extremely complex. β-arrestins can be desensitizers, facilitators of non-G protein signaling cascades and/or involved in the endocytosis of the receptor depending upon (1) the receptor isoform, (2) the agonist which is bound to the receptor and (3) the cellular environment in which the receptor is expressed. Further, the interactions between β-arrestins and the serotonin receptors may have major functional implications for the *in vivo* responsiveness of the receptors. Therefore understanding the consequences of β-arrestin interactions with the receptors could aid in the development of pharmacotherapies to selectively target the activation or the inhibition of specific serotonin receptor signaling cascades. This may also be important for fine tuning receptor function: by facilitating signaling in certain cell types, and by avoiding certain cascades in other systems, one might be able to preserve beneficial responses and avoid unwanted side effects. Certainly, from a pharmacological perspective, as we begin to assess receptor function and functional selectivity *in vivo*, serotonergic signaling has become even more complex.

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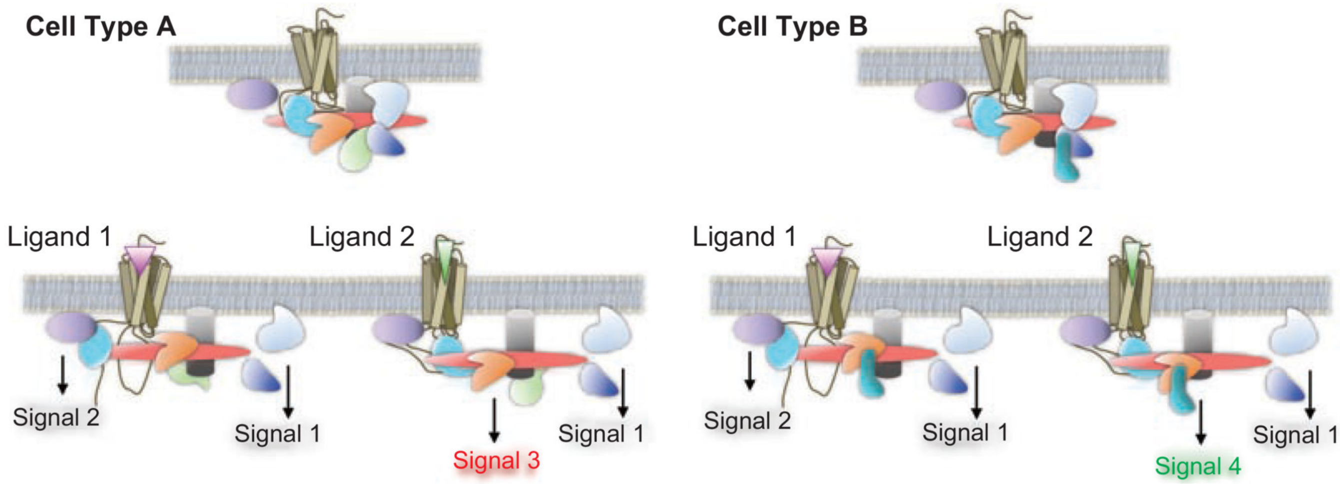

Figure 1.

Functional selectivity in GPCR signaling is determined by agonist as well as the cellular complement of proteins expressed in residence with the receptor. The diagram depicts a single GPCR type expressed in two distinct cell types (left: cell type A; right: cell type B). The different cell types express proteins in proximity to the receptor; some of which are the same between cell types and some of which are different (proteins are depicted as colored cartoon shapes clustered with the receptor). Upon agonist activation, the receptor engages some proteins in a conserved manner to produce the same signal regardless of the ligand or the cell type (Signal 1). However, some signaling cascades are specifically engaged by a particular ligand regardless of cell type (Signal 2) while other cascades are unique to the ligand and the cell type (Signal 3 and Signal 4). (See colour version of this figure online at www.informahealthcare.com/bmg)

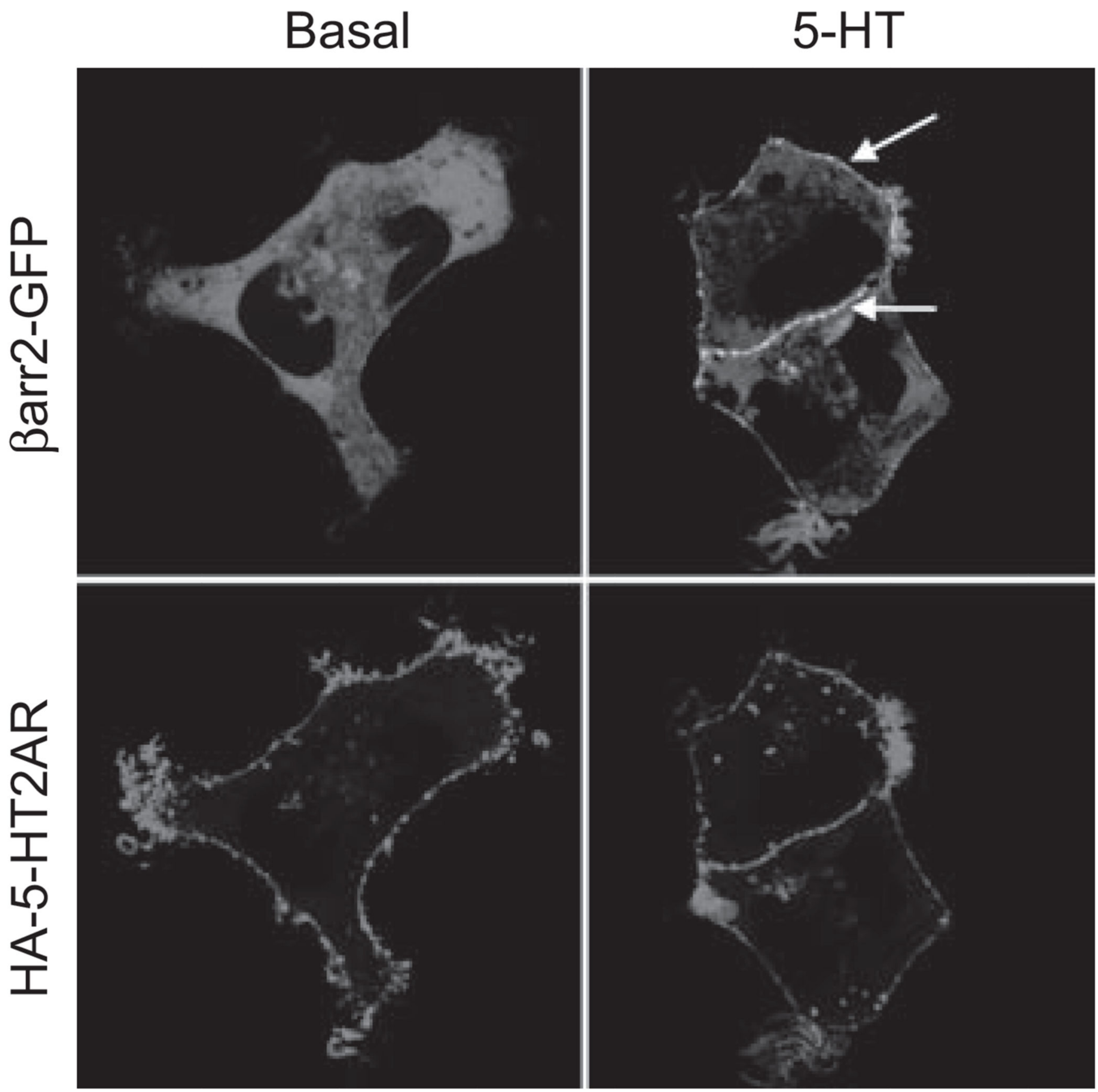

Figure 2.

Serotonin induces βarr2-GFP translocation to 5-HT2AR. HEK293 cells were transfected with HA-tagged mouse 5-HT2AR (HA-5-HT2AR) and GFP-tagged β-arrestin2 (βarr2-GFP). The cells were serum-starved for 2 hours and labeled with an HA-AlexaFluor 594 antibody for 15 minutes prior to live cell imaging with a confocal microscope. In the absence of agonist, βarr2-GFP is distributed throughout the cytosol. Treatment with serotonin (5-HT) (1 μM in 2 μM ascorbate) for 5 minutes leads to βarr2-GFP translocation to the plasma membrane outlining the cells as indicated by the arrows (top). Alexa-Fluor HA staining (bottom) is shown to demonstrate the location of the 5-HT2AR in the membrane.

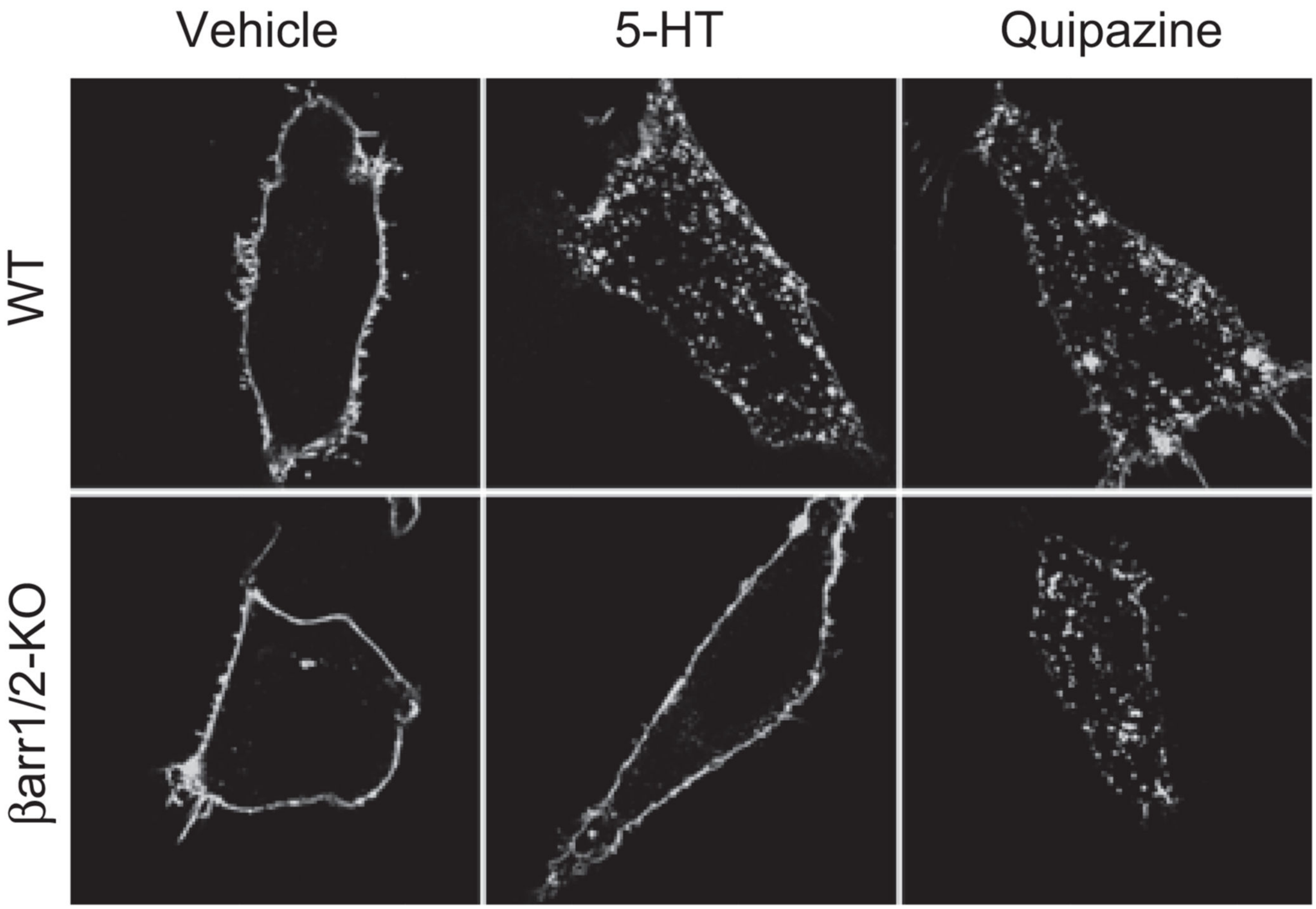

Figure 3.

Agonist-induced internalization of 5-HT2AR-YFP expressed in WT and βarr1/2-KO MEFs. WT and βarr1/2-KO MEFs were transfected with YFP-tagged 5-HT2AR (5-HT2AR-YFP) and serum-starved for 2 hours. Under these conditions, the 5-HT2AR-YFP is localized to the plasma membrane following treatment with vehicle (2 μM ascorbate) in both cell types. Treatment of WT cells with serotonin (5-HT, 1μM) or quipazine (1μM) induces receptor internalization within 30 minutes. While serotonin fails to internalize the receptor in the βarr1/2-KO MEFs (Schmid *et al.*, 2008), quipazine leads to internalization of the 5-HT2AR in the absence of β-arrestins.