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## **β-Arrestins: Structure, Function, Physiology, and Pharmacological Perspectives**

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### **Running title**

β-Arrestins: Structure, Function, and Physiology

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## **ABBREVIATIONS:**

5-FU, 5-fluorouracil

ACKR, atypical chemokine receptor

AD, Alzheimer's disease

AgRP, agouti-related peptide

AMPH, amphetamine

AP2, adaptor protein 2

AR, androgen receptor

AT1R, angiotensin II AT<sub>1</sub> receptor

β-AR, β-adrenergic receptor

βarr1, β-arrestin-1

βarr2, β-arrestin-2

CLPD-NK, chronic lymphoproliferative disorder of natural killer cells

CML, Chronic myelogenous leukemia

CNO, clozapine N-oxide

CNS, central nervous system

CPP, conditioned place preference

CRC, colorectal cancer

CRPC, castration-resistant prostate cancer

CXCR7, C-X-C chemokine receptor 7

DA, dopamine

DOR, δ-opioid receptor

DREADD, designer receptor exclusively activated by a designer drug

FFAs, free fatty acids

FXS, fragile X syndrome

GCGR, glucagon receptor

GPBAR, G protein-coupled bile acid receptor

GPCR, G protein-coupled receptor

GRKs, GPCR kinases

GSIS, glucose-stimulated insulin secretion

HFD, high-fat diet

HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$   
hTERT, human telomerase reverse transcriptase  
IL-PFC, infralimbic prefrontal cortex  
IFN- $\gamma$ , interferon- $\gamma$   
iWAT, inguinal white adipose tissue  
JNK, Jun N-terminal kinase  
KO, knockout  
KOR,  $\kappa$ -opioid receptor  
MAPK, mitogen-activated protein kinase  
MB, medulloblastoma  
MELK, maternal embryonic leucine zipper kinase  
mGluR, metabotropic glutamate receptor  
MOR,  $\mu$ -opioid receptor  
MSNs, medium spiny neurons  
Mstn, myostatin  
NK1R, neurokinin-1 receptor  
NSCLC, non-small cell lung cancer  
NTSR1, neurotensin receptor 1  
OE, overexpression  
OTR, oxytocin receptor  
RCC, renal cell carcinoma  
SHH-MB, MB driven by aberrant sonic-hedgehog/Gli signaling  
SKM, skeletal muscle  
SOC, serous ovarian cancer  
SU, sulphonylurea drug  
T-ALL, T-cell acute lymphoblastic leukemia  
T2D, type 2 diabetes  
TNBC, triple negative breast cancer  
UCP1, uncoupling protein 1  
WAT, white adipose tissue  
WT, wild type

**Abstract** - The two  $\beta$ -arrestins,  $\beta$ -arrestin-1 and -2 (systematic names: arrestin-2 and -3, respectively), are multifunctional intracellular proteins that regulate the activity of a very large number of cellular signaling pathways and physiological functions. The two proteins were discovered for their ability to disrupt signaling via G protein-coupled receptors (GPCRs) via binding to the activated receptors. However, it is now well recognized that both  $\beta$ -arrestins can also act as direct modulators of numerous cellular processes via either GPCR-dependent or -independent mechanisms. Recent structural, biophysical, and biochemical studies have provided novel insights into how  $\beta$ -arrestins bind to activated GPCRs and downstream effector proteins. Studies with  $\beta$ -arrestin mutant mice have identified numerous physiological and pathophysiological processes regulated by  $\beta$ -arrestin-1 and/or -2. Following a short summary of recent structural studies, this review will primarily focus on  $\beta$ -arrestin-regulated physiological functions, with particular focus on the central nervous system and the roles of  $\beta$ -arrestins in carcinogenesis and key metabolic processes including the maintenance of glucose and energy homeostasis. This review will also highlight potential therapeutic implications of these studies and discuss strategies that could prove useful for targeting specific  $\beta$ -arrestin-regulated signaling pathways for therapeutic purposes.

**Significance Statement** - The two  $\beta$ -arrestins, structurally closely related intracellular proteins that are evolutionarily highly conserved, have emerged as multifunctional proteins able to regulate a vast array of cellular and physiological functions. The outcome of studies with  $\beta$ -arrestin mutant mice and cultured cells, complemented by novel insights into  $\beta$ -arrestin structure

and function, should pave the way for the development of novel classes of therapeutically useful drugs capable of regulating specific  $\beta$ -arrestin functions.

## I. Introduction

Arrestins represent a family of relatively small cytoplasmic proteins (average size: ~45 kDa) that consist of four distinct subtypes. The name “arrestin” stems from the observation that these proteins function to terminate (“arrest”) signaling through G protein-coupled receptors (GPCRs) (DeWire et al., 2007; Gurevich and Gurevich, 2020). Two arrestin subtypes, also known as arrestin-1 and -4, or rod and cone arrestins, respectively, are referred to as visual arrestins since their expression is largely confined to photoreceptors of the eye. Arrestin-2 and -3 are ubiquitously expressed and can bind to and regulate the activity hundreds of different GPCRs (DeWire et al., 2007; Peterson and Luttrell, 2017). These two arrestins are also referred to as  $\beta$ -arrestin-1 ( $\beta$ arr1) and  $\beta$ -arrestin-2 ( $\beta$ arr2), respectively, since their function was first studied by analyzing their role in regulating the activity of  $\beta$ -adrenergic receptors ( $\beta$ -ARs) (DeWire et al., 2007; Peterson and Luttrell, 2017).

The two  $\beta$ -arrestins play key roles in the homologous desensitization of GPCRs, which involves two steps: phosphorylation of the activated receptor by one or more specialized GPCR kinases (GRKs), followed by the binding of arrestin(s) to the active phosphorylated receptor, thus interfering with productive receptor/G protein coupling (DeWire et al., 2007; Gurevich and Gurevich, 2020). After a brief summary of recent insights into structural and functional aspects of  $\beta$ -arrestins, this review will focus on the physiological and pathophysiological roles of  $\beta$ arr1 and  $\beta$ arr2, with particular emphasis on their ability to regulate various functions of the central nervous system (CNS), tumor formation, and several important metabolic functions. Due to space limitations, we will refer the reader to several excellent reviews that cover other important

physiological  $\beta$ -arrestin functions. Wherever possible, we will emphasize the potential translational relevance of  $\beta$ -arrestin-mediated cellular functions.

## II. Arrestin Structure and Function

### A. Conformational Flexibility of Arrestins

Different conformational states of most proteins have distinct biological functions. Therefore, it is important to elucidate conformational states of arrestins and determine their functional capabilities. Available evidence suggests that arrestins exist in four different states: free monomers, free oligomers, GPCR-bound, and microtubule-bound. Among the four arrestin subtypes, only arrestin-4 does not oligomerize (Gurevich and Gurevich, 2022).

Arrestins are elongated molecules consisting of two domains, usually termed N- and C-domains (Fig. 1). In their basal conformation, all four vertebrate arrestins have a very similar structure (Chen et al., 2018; Scheerer and Sommer, 2017). Receptor binding induces similar, but not identical, conformational rearrangements in arrestin-1 and  $\beta$ -arrestins, as revealed by biophysical methods (Hanson et al., 2006b; Zhuang et al., 2013; Zhuo et al., 2014) and high-resolution structures of arrestin/GPCR complexes. It should be noted in this context that all high-resolution structures of arrestin-GPCR complexes solved so far contained either arrestin-1 (Kang et al., 2015)(Zhou et al., 2017) or  $\beta$ arr1 (Bous et al., 2022; Cao et al., 2022; Lee et al., 2020; Staus et al., 2020; Yin et al., 2019). No high high-resolution structures of  $\beta$ arr2 bound to GPCRs have been published so far.

Structural studies have identified two major GPCR/arrestin interaction sites. The arrestin N-domain binds to phosphorylated regions of the receptor (Cahill et al., 2017; Shukla et al.,

2013; Thomsen et al., 2016; Zhuo et al., 2014), and the arrestin finger loop region inserts into the cytoplasmic cavity formed by the GPCR transmembrane core (Böttke et al., 2020; Bous et al., 2022; Cao et al., 2022; Kang et al., 2015; Staus et al., 2020; Yin et al., 2019). Moreover, recent studies have demonstrated that loops within the C-edge of arrestin can function as a membrane anchor, thus enhancing the stability of GPCR/arrestin complexes (Lally et al., 2017; Staus et al., 2020).

Structural studies suggest that  $\beta$ -arrestins can bind to activated GPCRs in two different modes. One mode of binding involves the interaction of arrestins with the phosphorylated C-terminus of GPCRs only ('hanging' complex) (Cahill et al., 2017; Nguyen et al., 2019; Shukla et al., 2014). The other one represents a high affinity  $\beta$ -arrestin/GPCR complex involving interactions with the finger loop region and the C-edge loops (Bous et al., 2022; Cao et al., 2022; Kang et al., 2015; Lee et al., 2020; Staus et al., 2020; Yin et al., 2019; Zhou et al., 2017). While this latter complex interferes with the binding of heterotrimeric G proteins, the GPCR– $\beta$ -arrestin complex in the 'hanging' configuration still allows for productive receptor-G protein interactions (Thomsen et al., 2016). In this structure, arrestin interacted exclusively with the phosphorylated C-terminal segment of the receptor, whereas the G protein engaged the cytoplasmic cavity formed by the transmembrane receptor core. It is of considerable interest to explore whether agonist-bound native GPCRs form similar trimeric complexes. The outcome of such studies is expected to have important implications for the concept of biased GPCR signaling, which implies that GPCRs can preferentially engage either G proteins or arrestins, depending on the structural properties of the activating ligand (Gurevich and Gurevich, 2020; Lefkowitz and Shenoy, 2005; Rajagopal et al., 2010b; Seyedabadi et al., 2022; Smith et al., 2018).



The conformation of microtubule-bound arrestins appears to be different from those of both basal and GPCR-bound conformations (Hanson et al., 2007; Hanson et al., 2006a). Biochemical and biophysical studies have shown that arrestins are endowed with considerable conformational flexibility, suggesting that this feature contributes to the ability of  $\beta$ -arrestins to interact with a vast array of cellular proteins (see (Gurevich and Gurevich, 2014a; Peterson and Luttrell, 2017) and references therein). In addition to several subtle structural rearrangements, GPCR binding to arrestins induces the release of the arrestin C-terminus and a twist of the two arrestin domains relative to each other (Hanson et al., 2006b; Maharana et al., 2022; Scheerer and Sommer, 2017; Shukla et al., 2013; Zhuo et al., 2014). The release of the C-terminus makes the arrestin binding sites for clathrin and adaptor protein 2 (AP2) more accessible, ensuring that free arrestins do not compete with the receptor-arrestin complexes that should be internalized. In contrast to arrestin-1, where the C-terminus released upon receptor binding appears to “flop around” without any preferred position (Hanson et al., 2006b), the C-terminus in receptor-bound  $\beta$ -arrestins appears to occupy a particular position (Zhuo et al., 2014), possibly more than one (Asher et al., 2022), a finding predicted to be of functional significance.

GPCR-bound arrestins are often called active, implying that free arrestins are not. Existing evidence suggests that this notion is unfounded. Arrestins in the so-called basal conformation can affect cellular activity. For example, some cellular proteins including E3 ubiquitin ligases Mdm2 (Song et al., 2006) and parkin (Ahmed et al., 2011) preferentially interact with the basal arrestin conformation. Moreover, certain cellular proteins, exemplified by JNK3, do not show a clear preference for either free or receptor-bound arrestins (Song et al., 2006). An arrestin mutant “frozen” in a basal-like state that cannot bind GPCRs facilitates JNK3 activation in cells, whereas another mutant with significantly enhanced ability to bind GPCRs is inactive in this

regard (Breitman et al., 2012). In the same cells where the activation status of endogenous  $\beta$ -adrenergic receptor ( $\beta$ 2-AR) greatly affects the level of ERK1/2 activation, it does not affect the activation of JNK3, which is solely determined by a particular form of  $\beta$ arr2 expressed (Breitman et al., 2012).

GPCRs show significant sequence differences in arrestin binding elements. Also, GPCRs vary considerably in the position of serine and threonine residues present within these regions that are potential targets for GRK-mediated phosphorylation required for efficient arrestin recruitment (Ranjan et al., 2017). These differences might explain recent observations that arrestins can display distinct conformations when bound to different GPCRs or after stimulation of the same GPCR by structurally different activating ligands (Ranjan et al., 2017). It is conceivable that distinct conformations of GPCR-bound arrestins lead to different cellular responses by interacting with different sets of intracellular signaling proteins. More detailed structural and biochemical studies are needed to elucidate the molecular mechanisms by which arrestins can efficiently interact with different GPCRs.

Most GPCRs have many more potential phosphorylation sites on their cytoplasmic regions than required for tight arrestin binding. This observation has led to the proposal that different phosphorylation patterns of the same receptor can determine the functional outcome of  $\beta$ -arrestin recruitment (barcode hypothesis (Kim et al., 2005; Nobles et al., 2011)). The same GPCR might exhibit cell type-specific phosphorylation patterns, depending on which GRKs are preferentially expressed in a certain cellular context (Butcher et al., 2012). The results of a recent study are consistent with the barcode hypothesis. Using sophisticated biophysical techniques, the authors showed that  $\beta$ arr1 and  $\beta$ arr2 can adopt distinct phosphorylation-dependent conformations after recruitment by the same GPCR (parathyroid hormone 1 receptor) (Haider et al., 2022).

Additional work is needed to explore whether the findings obtained with this receptor subtype apply to other GPCRs.

### *B. Biological Functions of $\beta$ -Arrestins*

The two  $\beta$ -arrestins have no enzymatic activity. Their only function is to bind other proteins, often several at the same time, as exemplified by arrestin scaffolding of three-tiered MAP kinase activation cascades (see (DeWire et al., 2007; Gurevich and Gurevich, 2006; Peterson and Luttrell, 2017) and references therein). Thus, if one of the arrestin binding partners has a particular localization,  $\beta$ -arrestins localize the other partners that simultaneously interact with them to the same sub-cellular compartment. Since GPCRs are integral membrane proteins, proteins that bind GPCR-associated  $\beta$ -arrestins are predicted to act in proximity to the plasma membrane. Similarly, via binding to polymerized tubulin,  $\beta$ -arrestins localize signaling proteins to microtubules, directing their activity to microtubule-associated targets (Hanson et al., 2007).

$\beta$ -Arrestins regulate a remarkable array of cellular functions (see (Gurevich and Gurevich, 2019b; Lefkowitz and Shenoy, 2005; Peterson and Luttrell, 2017) and references therein). Numerous studies revealed that receptor-bound  $\beta$ -arrestins can initiate another wave of GPCR-driven signaling and that free  $\beta$ -arrestins in the cytoplasm also regulate many signaling pathways (reviewed in (Gurevich and Gurevich, 2006; Lefkowitz and Shenoy, 2005; Peterson and Luttrell, 2017)). It has been demonstrated that both  $\beta$ arr1 and  $\beta$ arr2 can interact with hundreds of different GPCRs, certain growth factor receptors (Dalle et al., 2001; Girnita et al., 2014; Lin et al., 1998), as well as >100 non-receptor signaling and trafficking proteins (Xiao et al., 2007). The list of arrestin-interacting partners includes the trafficking proteins clathrin and clathrin adaptor AP2, as well as numerous signaling proteins, including kinases (e.g. members of the Src

family, ERK1/2, p38, or JNK1/2/3), phosphatases, and E3 ubiquitin ligases ((Gurevich and Gurevich, 2014b; Peterson et al., 2015) and references therein). Moreover, the two  $\beta$ -arrestins have been implicated in regulating the post-endocytic fate of GPCRs via ubiquitination and deubiquitination (Jean-Charles et al., 2016; Shenoy et al., 2001), cell spreading and motility (Cleghorn et al., 2015; Cleghorn et al., 2018), apoptotic cell death (Kook et al., 2019; Kook et al., 2014), and many other fundamental cellular processes.

### *C. $\beta$ -Arrestins and ERK1/2 Activation*

One of the most common responses observed after GPCR-mediated arrestin recruitment is the activation of ERK1/2 (DeWire et al., 2007; Peterson and Luttrell, 2017). As discussed in the previous paragraph,  $\beta$ arr1 and  $\beta$ arr2 can act as scaffolding proteins to facilitate ERK1/2 activation (DeWire et al., 2007; Peterson and Luttrell, 2017). The molecular mechanisms underlying  $\beta$ -arrestin scaffolding of the Raf1-MEK1-ERK1/2 cascade (Qu et al., 2021) and likely differences between  $\beta$ arr1 and  $\beta$ arr2 in mediating this process (Perry-Hauser et al., 2022a) are currently the subject of intense investigation. Interestingly, recent data suggest that different GPCR/ $\beta$ arr1 complexes, besides acting as scaffolding proteins, can directly activate, in an allosteric fashion, the protein kinases Src and C-Raf (Pakharukova et al., 2020; Zang et al., 2021).

Somewhat surprisingly, recent studies showed that deletion or inactivation of multiple functional G protein  $\alpha$ -subunits abolished ERK1/2 activation by essentially all GPCR–ligand pairs examined (Grundmann et al., 2018), suggesting that  $\beta$ -arrestin-mediated ERK1/2 activation requires the presence of functional G proteins. Notably, ligand-activated GPCRs were still able to recruit  $\beta$ -arrestins in the absence of functional G proteins (Grundmann et al., 2018). These

observations led to the hypothesis that  $\beta$ -arrestins primarily act as modulators ('rheostats') of GPCR-mediated ERK1/2 activation by regulating the intensity and temporal and spatial pattern of ERK1/2 signaling (Gutkind and Kostenis, 2018). In a subsequent study, Luttrell et al. (Luttrell et al., 2018) reported that independently generated  $\beta$ arr1/2 knockout (KO) clonal HEK293 cell lines showed variable ERK1/2 responses after stimulation of the  $\beta$ 2-AR and other GPCRs including the  $\beta$ 1-AR and the V2 vasopressin and follicle stimulating hormone receptors. This finding, complemented by  $\beta$ arr1/2 siRNA knockdown and overexpression studies, raised the possibility that clonal variation and potential 'rewiring' of intracellular signaling pathways caused by the lack of specific G $\alpha$  or arrestin subtypes may affect the outcome of ERK1/2 signaling assays (Luttrell et al., 2018). Further studies are needed to shed more light on these seemingly discrepant findings. Importantly, such studies should focus on cell types other than HEK293 and include the analysis of signaling pathways in vivo.

### III. Tools for Studying Arrestin Function and Physiology

In the following, we will briefly review strategies that are most commonly employed to explore the cellular and biological functions of  $\beta$ arr1 and  $\beta$ arr2 in vitro and in vivo.

#### *A. Knockdown or Knockout of Arrestin Expression*

The knockdown of  $\beta$ arr1 and  $\beta$ arr2 expression via siRNA technology in cultured cells has provided important insights into the cellular function of these two signaling proteins. However, the use of siRNA or related tools usually does not lead to a complete suppression of protein expression, making negative data difficult to interpret. More recently, CRISPR/Cas9 technology

has been employed to generate cell lines that completely lack  $\beta$ arr1 and/or  $\beta$ arr2 (Grundmann et al., 2018; Luttrell et al., 2018; O'Hayre et al., 2017). A potential caveat associated with the use of this approach is that it may lead to the inadvertent selection of atypical cells that do not require the targeted protein for survival (Gurevich and Gurevich, 2020; Luttrell et al., 2018).

In vivo studies with whole body  $\beta$ arr1 and  $\beta$ arr2 KO mice have demonstrated that the two  $\beta$ -arrestins play critical roles in regulating numerous physiological functions, including key activities of the CNS and many important functions of peripheral tissues and organs (Porter-Stransky and Weinshenker, 2017; Schmid and Bohn, 2009; Zhao and Pei, 2013). One disadvantage associated with the use of whole body KO mice is that this approach does not provide clear information about the specific cell types and potential cellular mechanisms underlying the observed phenotypes. Moreover, the whole body  $\beta$ -arrestin KO mice that have been analyzed in the past lack  $\beta$ arr1 or  $\beta$ arr2 throughout development, raising the possibility that at least some of the observed phenotypes may be affected by compensational developmental changes (Kovacs et al., 2009; Philipp et al., 2013). It should also be noted that the first whole body  $\beta$ arr1 (Conner et al., 1997) and  $\beta$ arr2 (Bohn et al., 1999) KO mice that were generated had a mixed genetic background which can affect the outcome of mouse phenotyping studies (Gerlai, 1996).

The recent development of floxed  $\beta$ arr1 or  $\beta$ arr2 mutant mice has greatly advanced our knowledge about the in vivo functions of the two  $\beta$ -arrestins in specific cell types or tissues (Kim et al., 2018; Urs et al., 2016). By crossing floxed  $\beta$ arr1 or  $\beta$ arr2 mice with specific Cre driver lines, it has been possible to generate mice that lack  $\beta$ arr1 or  $\beta$ arr2 only in distinct cell types (Pydi et al., 2022). In many cases, these mutations were induced in adult animals by using Cre

driver lines in which Cre activity can be stimulated in a tamoxifen-dependent fashion (Pydi et al., 2022).

### *B. Arrestin-Biased GPCR Ligands*

During the past two decades, GPCR ligands have been identified that are unable or impaired in their ability to promote receptor-mediated G protein activation but can recruit  $\beta$ -arrestins and initiate arrestin-dependent signaling with high efficacy (Gurevich and Gurevich, 2020; Luttrell et al., 2015; Peterson and Luttrell, 2017; Seyedabadi et al., 2019; Smith et al., 2018). These so-called arrestin-based ligands are widely used to explore mechanisms of arrestin-mediated signaling in vitro and in vivo. The in vivo use of these agents has led to important novel findings regarding the physiological and pathophysiological roles of various arrestin-regulated signaling pathways (Peterson and Luttrell, 2017; Smith et al., 2018). In some cases, arrestin-biased ligands have been shown to mimic the (potential) therapeutic effects of GPCR agonists, while ligand-induced G protein activation was associated with unwanted side effects (Luttrell et al., 2015; Peterson and Luttrell, 2017; Smith et al., 2018). GPCR ligands that preferentially activate G proteins but are impaired in their ability to recruit  $\beta$ -arrestins are referred to as G protein-biased agonists (Luttrell et al., 2015; Peterson and Luttrell, 2017; Smith et al., 2018). Several studies suggest that the use of this class of GPCR ligands may offer therapeutic advantage under conditions where GPCR-mediated arrestin recruitment and/or signaling may cause unwanted side effects. Recent studies have shown that GPCRs are highly dynamic proteins and that structurally different GPCR ligands have the potential to preferentially stabilize distinct GPCR conformations (Smith et al., 2018; Wingler and Lefkowitz, 2020). The conformational heterogeneity of GPCRs thus provides a structural basis for the identification of

arrestin- and G protein-biased GPCR ligands. Examples for the potential therapeutic usefulness of arrestin- and G protein-biased GPCR agonists are given throughout this review.

### *C. Caveats Associated with the Use of Arrestin-Biased GPCR Ligands*

Several caveats must be considered when interpreting the outcome of studies involving the use of biased GPCR agonists. For example, the expression levels and localization of GPCRs and downstream effector and regulatory proteins may vary in different cell types and may be altered under specific physiological or pathophysiological conditions (Seyedabadi et al., 2019). Moreover, it is important to test the potential clinical efficacy of a particular biased GPCR ligand in an experimental setting that closely mimics the clinical condition for which this drug is being developed (Seyedabadi et al., 2019). Also, many published studies reported ligand bias for  $\beta$ -arrestins simply based on GPCR-arrestin recruitment assays without exploring arrestin-dependent changes in intracellular signaling (Gurevich and Gurevich, 2020). Such studies do not provide convincing information regarding ligand-dependent biased signaling. As discussed in detail recently (Gurevich and Gurevich, 2020), ligand-dependent changes in the kinetics of GPCR internalization and trafficking can complicate the identification of ligands that show intrinsic bias for facilitating GPCR-dependent arrestin signaling. However, to the best of our knowledge, no unambiguous readout for arrestin-dependent signaling currently exists to assess arrestin activity in signaling assays for screening purposes (Gurevich and Gurevich, 2020). The development of such assays is complicated by the multitude of signaling pathways modulated by both G proteins and  $\beta$ -arrestins, as well as by overlapping activities of these two signaling arms.

### *D. Arrestin-Biased GPCRs*



Interestingly, mutant versions of the angiotensin II AT<sub>1</sub> receptor (AT<sub>1</sub>R) (Wei et al., 2003) and the  $\beta_2$ -adrenergic (Shenoy et al., 2006) receptor have been described which, following agonist binding, fail to activate heterotrimeric G proteins but can recruit  $\beta$ -arrestins and stimulate ERK in a  $\beta$ -arrestin-dependent fashion. More recently, Nakajima et al. (Nakajima and Wess, 2012) developed an M<sub>3</sub> muscarinic receptor-based designer GPCR (DREADD; Designer Receptor Exclusively Activated by a Designer Drug) that contained a point mutation within the “DRY” motif of the receptor. This point mutation disrupted receptor/G protein coupling but still allowed recruitment of  $\beta$ -arrestins by the receptor in the presence of clozapine-N-oxide (CNO), a DREADD agonist (Urban and Roth, 2015). CNO stimulation of mouse  $\beta$ -cells expressing the arrestin-biased designer receptor resulted in a significant increase in insulin release, implicating arrestin-dependent pathways in the regulation of insulin secretion (Nakajima and Wess, 2012). However, CNO treatment of mice expressing this new designer receptor in hepatocytes and other metabolically important cell types in vivo has not resulted in significant phenotypic changes so far (J. Wess, unpublished data).

Similarly, more recent studies also generated mutant versions of other GPCRs, including the D<sub>2</sub> dopamine receptor (Donthamsetti et al., 2020; Peterson et al., 2015), that show a strong bias for the recruitment of  $\beta$ -arrestins. In vivo studies with this new class of receptors should provide important novel insights into the physiological relevance of  $\beta$ -arrestin-dependent signaling cascades. Specific examples for this approach are given below under “Psychoactive Drugs”.

### *E. Dominant Negative $\beta$ -Arrestin Mutants*

Dominant-negative mutants of  $\beta$ arr1 and  $\beta$ arr2 represent useful tools to selectively interfere with distinct  $\beta$ -arrestin functions. An early study (Luttrell et al., 1999) reported the generation of

$\beta$ arr1 mutants, impaired either in c-Src binding or their ability to target GPCRs to clathrin-coated pits, that act as dominant negative inhibitors of  $\beta$ 2-AR-mediated stimulation of ERK1/2. More recently, dominant negative mutant versions of  $\beta$ arr1 and  $\beta$ arr2 or minigene fragments of  $\beta$ arr1 have been used to explore mechanisms underlying  $\beta$ -arrestin-dependent GPCR endocytosis (Ghosh et al., 2017; Kang et al., 2014). Interestingly, a  $\beta$ arr2 mutant containing a series of alanine substitutions of key receptor-binding residues (“KNC mutant”) displays dominant negative activity, competitively decreasing JNK activation by wild-type (WT)  $\beta$ arr2 (Breitman et al., 2012). In addition, introduction of a single point mutation (R307A) into  $\beta$ arr1 is able to prevent  $\beta$ arr1-dependent ERK1/2 activation by selectively reducing  $\beta$ arr1 binding to c-Raf1 (Coffa et al., 2011). A related study demonstrated that a minigene fragment of  $\beta$ arr1 (residues 25–161) interferes with  $\beta$ arr1 binding to STAM1 (signal transducing adaptor molecule 1) (Malik and Marchese, 2010). A subsequent study using this minigene fragment as a tool showed that formation of the  $\beta$ arr1–STAM1 complex is essential for CXCR4-dependent activation of focal adhesion kinase and chemotaxis (Alekhina and Marchese, 2016; Zhuo et al., 2020). These findings indicate that it is feasible to design dominant negative  $\beta$ -arrestin mutants that selectively suppress specific  $\beta$ -arrestin functions.

#### *F. $\beta$ -Arrestins with Altered GPCR Binding Affinity and/or Selectivity*

Following activation by agonist ligands, phosphorylated GPCRs are able to bind  $\beta$ -arrestins with high affinity via destabilization of two key interactions that keep  $\beta$ -arrestins in their basal state: the polar core and the three-element interaction between  $\beta$ -strand I,  $\beta$ -strand XX of the C-terminus, and  $\alpha$ -helix I (Gurevich and Gurevich, 2019a; Karnam et al., 2021) (Fig. 1). These intramolecular interactions in  $\beta$ arr1 and  $\beta$ arr2 can be destabilized by mutations, yielding mutant

$\beta$ -arrestins that bind active phosphorylated and even unphosphorylated GPCRs more readily than WT  $\beta$ -arrestins (Gurevich and Gurevich, 2019a; Karnam et al., 2021). Gain-of-function mutations that can occur in numerous GPCRs are known to cause various human disorders (Arang and Gutkind, 2020; Schöneberg et al., 2004; Stoy and Gurevich, 2015). Arrestin mutants that can bind to overactive receptors with greater affinity than to WT receptors have the potential to suppress excessive G protein-mediated signaling by disease-causing mutant GPCRs (Samaranayake et al., 2018; Song et al., 2009). In particular, arrestin mutants that do not require GPCR phosphorylation for tight binding to the receptor can compensate for defects in receptor phosphorylation. For example, structure-function studies identified several “enhanced” mutant versions of arrestin-1 that bind all active forms of rhodopsin more readily and interact with high affinity with active unphosphorylated rhodopsin. One of these arrestin-1 mutants was able to partially compensate for defects of rhodopsin phosphorylation in vivo, improving photoreceptor function and survival in mice (Samaranayake et al., 2018; Song et al., 2009).

Enhanced phosphorylation-independent mutants of  $\beta$ arr1 and  $\beta$ arr2 have also been constructed and characterized in vitro (Cerver et al., 2002; Gurevich et al., 1997; Kovoov et al., 1999; Pan et al., 2003), but their therapeutic potential has yet to be tested. As discussed earlier,  $\beta$ arr1 and  $\beta$ arr2 interact with a very large number of activated GPCRs. Mutant versions of  $\beta$ arr2 that show a certain degree of GPCR binding selectivity have also been described (Gimenez et al., 2014; Gimenez et al., 2012). This observation may stimulate the development of novel, GPCR subtype-biased  $\beta$ -arrestins that may prove useful both as novel research tools and also for therapeutic purposes. It is likely that the recently published high-resolution structures of GPCR-arrestin-1/ $\beta$ arr1 complexes (Bous et al., 2022; Cao et al., 2022; Huang et al., 2020; Kang et al.,

2015; Lee et al., 2020; Staus et al., 2020; Yin et al., 2019; Zhou et al., 2017) will promote the rational design of such novel classes of mutant  $\beta$ -arrestins.

### *G. $\beta$ -Arrestin Mutants with Altered Signaling Properties*

The two  $\beta$ -arrestins interact, directly or indirectly, with a very large number of effector proteins (e.g., (Xiao et al., 2007)), thus regulating numerous intracellular signaling pathways. Several mutant versions of  $\beta$ arr1 and  $\beta$ arr2 have been identified which selectively interfere with distinct arrestin/effector protein interactions. For example,  $\beta$ arr1 or  $\beta$ arr2 mutants have been described that fail to interact with distinct effector proteins, including MEK1 (Meng et al., 2009) and c-Raf1 (Coffa et al., 2011), or do not facilitate JNK3 activation (Breitman et al., 2012). ERK1/2 activation usually promotes cell proliferation, whereas activated JNKs cause anti-proliferative and sometimes pro-apoptotic effects. For this reason, these  $\beta$ -arrestins mutants may become therapeutically useful in disorders associated with excessive proliferation (e.g., cancer) or excessive cell death including neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. Since methods of targeted gene delivery are being rapidly developed and improved (Alnasser, 2021), the cell-type specific expression of certain  $\beta$ -arrestin mutants for the treatment of life-threatening diseases may become feasible at some point in the future.

### *H. $\beta$ -Arrestin-Derived Peptides*

Arrestin-derived peptides also represent useful tools for modulating arrestin function in a more targeted function. For example, a cell-permeable 25 amino acid  $\beta$ arr1 peptide encompassing the MEK1-binding site can block  $\beta$ arr1-MEK1 interactions and inhibit ERK1/2 phosphorylation (Meng et al., 2009). Recently, short  $\beta$ arr2 peptides have been developed that

promote activation of the ASK1-MKK4/7-JNK3 signaling cascade (Perry-Hauser et al., 2022b; Zhan et al., 2016). Since short peptides are rapidly degraded by cytoplasmic exopeptidases, these peptides were fused to the Venus protein to enhance their stability (Perry-Hauser et al., 2022b; Zhan et al., 2016). The  $\beta$ arr2 peptides can be considered ‘mono-functional’ since they lack most receptor-binding residues and are unlikely to interfere with any other  $\beta$ arr2 functions. As discussed in the previous paragraph, activation of JNKs has the potential to suppress excessive proliferation or induce apoptosis of cancer cells (Bubici and Papa, 2014). Thus, these  $\beta$ arr2-based peptides capable of activating JNK signaling should be of considerable translational interest.

In sum, recent studies have demonstrated the feasibility of creating arrestin-based tools for targeted manipulation of cell signaling and, potentially, for therapeutic purposes. However, given the remarkable multi-functionality of  $\beta$ arr1 and  $\beta$ arr2, the development and further evaluation of these agents for therapeutic utility represents a daunting task. Most importantly, future work in this area requires the identification of arrestin binding sites for numerous other signaling proteins known as  $\beta$ -arrestin interaction partners. It is likely that such studies will lead to the development of novel tools that are able to alter arrestin-dependent functions in a more targeted fashion, a prerequisite for the potential evaluation of arrestin-based therapeutic strategies.

#### **IV. Role of $\beta$ -Arrestins in Modulating Key Functions of the CNS**

Neurotransmission via the chemical synapses is essential for the functioning of the central nervous system (CNS). The signals in the synapse are carried by the neurotransmitter molecules that interact with plasma membrane receptors, initiating a cascade of signaling events eventually

resulting in a specific neural response. Most neurotransmitter receptors belong to the GPCR superfamily, including, for example, all dopamine and opioid receptor subtypes. Since  $\beta$ -arrestins are critical regulators of GPCR function, it is not surprising that  $\beta$ -arrestins make important contributions to many neural processes.

#### *A. Opioid Receptor-Mediated Analgesia*

Acute and chronic pain has emerged as a major societal challenge (Davis et al., 2020). The most commonly used drugs endowed with superior analgesic activity target GPCRs of the opioid receptor family which consists of three major subtypes,  $\mu$ ,  $\kappa$ , and  $\delta$  (Fig. 2) (Stein, 2016). These receptors, which preferentially interact with G proteins of the  $G_{i/o}$  family, are widely expressed in the CNS and are also present in various peripheral tissues (Stein, 2016).

*1.  $\mu$ -Opioid Receptors.* The most frequently used agents to treat moderate to severe pain are  $\mu$ -opioid receptor (MOR) agonists, including morphine and oxycodone. However, the use of morphine and its derivatives causes major side effects, including respiratory depression, constipation, tolerance, and addiction (Fig. 2B) (Darcq and Kieffer, 2018). The severity of the side effects associated with the use of MOR agonists is underscored by the staggering loss of life resulting from the current opioid epidemic in the United States (about ~75,000 deaths per year in 2020/2021; [https://www.cdc.gov/nchs/pressroom/nchs\\_press\\_releases/2021/20211117.htm](https://www.cdc.gov/nchs/pressroom/nchs_press_releases/2021/20211117.htm)). For this reason, novel classes of MOR agonists endowed with an improved therapeutic window are urgently needed.

Following the generation of whole body  $\beta$ arr2 KO mice, Caron and coworkers demonstrated that  $\beta$ arr2 deficiency led to a pronounced potentiation and prolongation of the

analgesic effects of morphine (Bohn et al., 1999) and that tolerance did not occur after chronic morphine treatment of  $\beta$ arr2 KO mice (Bohn et al., 2000). More recently, Manglik et al. (Manglik et al., 2016) reported the development of a novel, G protein-biased MOR agonist, PZM21, that shows no structural similarity to known opioid drugs. This agent displays strong analgesic activity but is devoid of many of the side effects associated with the use of morphine-like analgesics (Manglik et al., 2016). Based on these findings, many academic and industry laboratories focused on developing clinically useful MOR agonists that promote MOR-mediated activation of G proteins but do not trigger  $\beta$ -arrestin recruitment to the receptor. These efforts led to the development of novel G protein-biased MOR agonists which, as was hoped, retained strong analgesic activity but displayed an improved side effect profile, as compared to morphine (Schmid et al., 2017). For example, chronic treatment of mice with one of these compounds (SR-17018) did not cause antinociceptive tolerance or MOR desensitization, effects typically observed after chronic administration of morphine (Grim et al., 2020). Similarly, in vivo studies in rodents showed that a G protein-biased MOR agonist, TRV130 (alternative name: oliceridine) had strong analgesic activity but was less likely than morphine to cause respiratory depression, constipation, or tolerance upon chronic administration (Altarifi et al., 2017; DeWire et al., 2013). Following the successful completion of a series of clinical studies, oliceridine was approved by the FDA in 2020 for intravenous use in moderate to severe pain in adults (Azzam and Lambert, 2022). As reviewed recently (Azzam and Lambert, 2022), oliceridine possesses potent analgesic activity but appears to have less abuse potential and an improved side effect profile, as compared to traditional opioid drugs.

In contrast to the findings that led to the successful development of oliceridine as a novel analgesic agent, two recent studies using independently generated  $\beta$ arr2 KO mice failed to

reproduce the original result that  $\beta$ arr2 mediates respiratory depression and constipation caused by morphine or other opioids (Bachmutsky et al., 2021; Kliewer et al., 2020). Moreover, recent evidence suggests that effective morphine-induced recruitment of  $\beta$ arr2 by a mutant MOR can improve morphine analgesia without exacerbating respiratory depression (He et al., 2021). One possible reason for these discrepant findings is that the analyzed  $\beta$ arr2 KO strains differed in their genetic background. The  $\beta$ arr2 KO mice analyzed by Caron and coworkers had a mixed genetic background (129/SvJ x C57BL/6) (Bohn et al., 2000; Bohn et al., 1999). In contrast, Kliewer et al. (Kliewer et al., 2020) analyzed  $\beta$ arr2 KO mice that had been extensively backcrossed to the C57BL/6J background. In fact, previous work has demonstrated considerable variation among different mouse strains in opioid-induced antinociception, respiratory depression, and tolerance Kelly et al. (2023). Recent data link the beneficial pharmacological profile of oliceridine or other G protein-biased MOR agonists to the partial agonist properties of these compounds (Azevedo Neto et al., 2020; Gillis et al., 2020a; Gillis et al., 2020b; Kelly et al., 2023; Singleton et al., 2021). However, as discussed recently, partial agonist activity alone cannot account for the improved side effect profiles of the new class of MOR agonists described above (Stahl and Bohn, 2022).

Clearly, the recently developed novel MOR agonists display an improved therapeutic window and expand the spectrum of clinically useful drugs that can suppress severe pain. However, additional studies are needed to explore to which extent changes in  $\beta$ -arrestin recruitment or signaling (or the lack thereof) affect the pharmacological profile of this new class of analgesic agents.



2. *κ-Opioid Receptors*. Similar to MOR,  $\kappa$ -opioid receptors (KOR) are widely expressed in the CNS and mediate analgesia following agonist activation (French and van Rijn, 2022; Mores et al., 2019). KOR agonists are also useful for the treatment of intractable itch (Bohn and Aubé, 2017). Importantly, KOR agonists do not induce respiratory depression and lack abuse potential, making them an attractive alternative to MOR agonists to treat moderate to severe pain (French and van Rijn, 2022; Mores et al., 2019). However, KOR agonists can induce central side effects such as sedation, lack of motor coordination, and dysphoria, which limit the utility of these agents as analgesic drugs (Fig. 2B) (French and van Rijn, 2022; Mores et al., 2019).

Several reports, including studies with  $\beta$ arr2 KO mice, suggest that  $\beta$ arr2 signaling may play a role in KOR-mediated dysphoria (Chavkin et al., 2014). In contrast, the anti-pruritic and analgesic effects of KOR agonists are not affected in  $\beta$ arr2 KO mice (Morgenweck et al., 2015; White et al., 2015). Based on these findings, several laboratories set out to develop G protein-biased KOR agonists (Bohn and Aubé, 2017; Faouzi et al., 2020; Mores et al., 2019) with reduced side effects such as dysphoria, sedation, and other KOR-mediated central side effects. As reviewed recently (Bohn and Aubé, 2017), the outcome of many animal studies are consistent with the concept that KOR agonists which can efficiently activate G proteins but are impaired in the ability to recruit  $\beta$ -arrestins are endowed with an increased therapeutic window. It remains to be seen whether these preclinical studies will eventually lead to the development of G protein-biased KOR agonists that will prove clinically useful as novel analgesic and antipruritic drugs.

3. *δ-Opioid Receptors*.  $\delta$ -Opioid receptor (DOR) agonists are a poor substitute for MOR agonists for the treatment of acute pain, but are highly efficacious in chronic pain models (Quirion et al., 2020). Importantly, the use of DOR agonists is not associated with significant abuse potential or

severe side effects (Quirion et al., 2020). However, the use of DOR agonists in various animal models is generally associated with the development of significant analgesic tolerance upon repeated drug administration (Pradhan et al., 2011). DOR activity is regulated by interactions of the activated receptor with both  $\beta$ -arrestins (see, for example: (Pradhan et al., 2016)). Studies with  $\beta$ -arrestin KO mice indicate that agonist-activated DORs interact with  $\beta$ -arrestins in a ligand-specific manner (Pradhan et al., 2016; Vicente-Sanchez et al., 2018a). Interestingly, the tolerance induced by DOR agonists that induce DOR internalization with high efficacy has been linked to DOR recruitment of  $\beta$ arr1 (Vicente-Sanchez et al., 2018b). Based on these findings, the development of DOR agonists that induce DOR conformations that are less likely to interact with  $\beta$ arr1 represents an attractive goal. Such agents may offer the advantage that they can suppress chronic pain in humans but are less prone to cause tolerance upon repeated administration.

## *B. Dopaminergic Signaling*

*1. Schizophrenia.* Most antipsychotic drugs are thought to exert their therapeutic actions primarily by blocking D<sub>2</sub>-class dopamine (DA) receptors, in agreement with the concept that enhanced dopaminergic neurotransmission plays a central role in the pathophysiology of schizophrenia (Seeman, 2021). Pharmacological studies have shown that activation of D<sub>2</sub>-type DA receptors impairs central Akt activity, resulting in enhanced glycogen synthase kinase-3 (GSK3) signaling in the striatum (Beaulieu et al., 2009). Studies with  $\beta$ arr2 KO mice demonstrated that  $\beta$ arr2 plays an important role in regulating the activity of this signaling cascade (Beaulieu et al., 2009). Specifically, activation of D<sub>2</sub>-class DA receptors stimulates the formation of a protein complex containing Akt,  $\beta$ arr2, and protein phosphatase 2A (PP2A) (Beaulieu et al., 2005). This multiprotein complex is predicted to facilitate DA-induced

dephosphorylation (inactivation) of Akt (Beaulieu et al., 2005). Various lines of evidence indicate that this signaling complex is involved in regulating several important DA-dependent behaviors, including locomotor hyperactivity, responsiveness to psychostimulants (e.g. amphetamines), and amphetamine (AMPH)-induced disruption of sensorimotor gating, an animal model of psychosis commonly used to test drugs for potential antipsychotic activity (Beaulieu et al., 2009).

The Akt/GSK3 signaling cascade is also involved in mediating the therapeutic effects of lithium (Beaulieu et al., 2009), a drug commonly used for the treatment of bipolar disorder (also known as manic depression) (Volkmann et al., 2020). Interestingly,  $\beta$ arr2 KO mice are unresponsive to both acute and chronic treatment with lithium in various behavioral and biochemical tests. For example, Caron and coworkers demonstrated that lithium is unable to stimulate Akt activity in  $\beta$ arr2 KO mice, in contrast to findings obtained with WT mice (Beaulieu et al., 2008). Mechanistic studies showed that lithium disrupts the formation of the Akt/ $\beta$ arr2/PP2A complex both in vitro and in vivo (Beaulieu et al., 2008), suggesting that at least some of the behavioral effects of lithium are mediated via this mechanism.

Besides enhanced striatal DA release, schizophrenia is also associated with reduced cortical DA tone (Slifstein et al., 2015). Current antipsychotic drugs primarily act by reducing elevated DA signaling in the striatum. A recent study (Urs et al., 2016) tested the hypothesis that  $\beta$ arr2-biased  $D_2$  receptor partial agonists, including UNC9994, may be able to improve cortical hypodopaminergia. Studies with mutant mice lacking  $\beta$ arr2 in specific neuronal subpopulations demonstrated that the antipsychotic-like effects of UNC9994A are due to a combination of striatal antagonism and cortical agonism of  $D_2$  receptor- $\beta$ arr2 signaling (Urs et al., 2016). These

findings suggest that  $\beta$ arr2-biased  $D_2$  receptor ligands could prove beneficial as a novel class of antipsychotic drugs.

2. *Psychostimulants*. Psychostimulant drugs such as cocaine and AMPH enhance striatal dopaminergic neurotransmission, causing stimulation of striatal DA receptors, and as a result, increased locomotor activity (Kohno et al., 2022). Repeated administration of these agents leads to a further stimulation of locomotor activity, a phenomenon referred to as behavioral sensitization (Steketee and Kalivas, 2011). AMPH-induced behavioral sensitization (augmentation of locomotor activity) is considered a useful animal model to explore the neural basis of drug addiction (Steketee and Kalivas, 2011).

Recent work demonstrated that  $\beta$ arr2 KO mice are deficient in AMPH-induced locomotor sensitization (Zurkovsky et al., 2017), suggesting that  $\beta$ arr2 plays a key role in facilitating this behavior. The data presented in this study suggest that  $\beta$ arr2 regulates AMPH-induced locomotor sensitization via  $\beta$ arr2-dependent cellular signaling rather than  $\beta$ arr2-mediated DA receptor desensitization. The precise neuronal and molecular mechanisms underlying this type of regulation remain to be explored in future studies.

Most drugs of abuse, including psychostimulants and opioids, stimulate DA neurotransmission in the nucleus accumbens by activating  $D_1$ - and  $D_2$ -like DA receptors expressed by medium spiny neurons (MSNs) (Pierce and Kumaresan, 2006). A recent study demonstrated that mice selectively lacking  $\beta$ arr1 in  $D_1$  receptor-expressing cells showed similar behavioral responses as control mice following treatment with cocaine or morphine (Porter-Stransky et al., 2020). In contrast, mice lacking  $\beta$ arr2 in  $D_2$  receptor-expressing neurons showed significantly reduced hyperlocomotor activity in response to both drugs, attenuated locomotor

sensitization in response to cocaine, and blunted cocaine-seeking behavior (Porter-Stransky et al., 2020). Electrophysiological data indicated that the lack of  $\beta$ arr2 impaired the ability of DA to inhibit D<sub>2</sub>-receptor-expressing MSNs of the nucleus accumbens (Porter-Stransky et al., 2020). These findings indicate that  $\beta$ arr2 plays a key role in the excitability of this neuronal population, suggesting that this deficit may contribute to the behavioral changes observed after psychostimulant treatment of mice lacking  $\beta$ arr2 in this subclass of neurons. However, it should be noted that the  $\beta$ arr2 mutant mice used in this study (Porter-Stransky et al., 2020) lacked  $\beta$ arr2 in all D<sub>2</sub> (or D<sub>1</sub>) receptor-expressing neurons, raising the possibility that other neuronal subpopulations (e.g. certain striatal neurons) may also be involved in the observed phenotypes.

Interestingly, the Caron laboratory developed mutant D<sub>2</sub> DA receptors that were biased for either G protein or  $\beta$ -arrestin interactions (Peterson et al., 2015). Expression of the arrestin-biased designer receptor in mouse GABAergic MSNs of the striatum resulted in a pronounced augmentation in locomotor activity in response to AMPH, whereas overexpression of the G protein-biased D<sub>2</sub> mutant receptor in the same set of neurons had little effect on this AMPH response (Peterson et al., 2015). One major caveat associated with this approach is that the functions of the D<sub>2</sub> designer receptors are explored in the presence of endogenous D<sub>2</sub> receptors which may affect the nature of the phenotypes observed with the mutant mice. Nevertheless, these newly developed designer GPCRs represent excellent novel tools to explore the relative contributions of G protein and arrestin signaling pathways to the physiological and pathophysiological functions of the D<sub>2</sub> DA receptor.

In a related study, an arrestin-biased D<sub>2</sub> designer receptor was expressed in “indirect pathway” medium spiny neurons (iMSNs) of the nucleus accumbens of whole body D<sub>2</sub> receptor KO mice (Donthamsetti et al., 2020). Strikingly, expression of this mutant receptor in this

neuronal population resulted in normal (WT-like) locomotor activity and cocaine-induced locomotor activity (Donthamsetti et al., 2020). In contrast, the reward effect of cocaine could only be restored by expression of the WT D<sub>2</sub> receptor. These data support the concept that D<sub>2</sub>-receptor mediated  $\beta$ -arrestin recruitment can modulate locomotor activity without simultaneous activation of G proteins.

Using a similar strategy as the Javitch laboratory (Donthamsetti et al., 2020), Caron and colleagues expressed  $\beta$ -arrestin- or G protein-biased mutant D<sub>2</sub> receptors in D<sub>2</sub> receptor-deficient iMSNs of the dorsal (caudate–putamen) and ventral striatum (nucleus accumbens) (Rose et al., 2018). Behavioral studies indicated that coordinated G protein and arrestin actions were required to restore proper basal locomotion and WT-like AMPH- or cocaine-induced hyperlocomotion in these mutant mice (Rose et al., 2018). These data suggest that D<sub>2</sub> receptor-dependent control of locomotor activity relies on the proper balance between G protein and  $\beta$ -arrestin activities in iMSNs. Possible reasons for the seemingly discrepant findings by the Javitch and Caron laboratories (Donthamsetti et al., 2020; Rose et al., 2018) may be differences in receptor expression levels, coupling efficacy of the various designer D<sub>2</sub> receptors, the type of targeted neuronal subpopulations, or other changes in experimental conditions that remain to be explored.

### *C. Learning, Memory, and Mood*

Relatively little is known about the potential roles of  $\beta$ -arrestins in regulating cognitive and memory functions.

*1. Memory Reconsolidation.* Behavioral studies demonstrated that  $\beta$ arr2 KO mice show deficits in memory reconsolidation (Liu et al., 2015), a process that enhances, updates, or reduces a

previously acquired memory after recall (Lee et al., 2017). Studies with various  $\beta$ -AR antagonists including carvedilol, an agent that retains the ability to stimulate  $\beta$ -arrestin-dependent signaling (Wisler et al., 2007), suggested that  $\beta_1$ -AR/ $\beta$ arr2/ERK signaling module plays a key role in mediating memory reconsolidation (Liu et al., 2015). Strikingly, selective expression of  $\beta$ arr2 in the entorhinal cortex of  $\beta$ arr2 KO mice greatly improved impaired memory reconsolidation in an object recognition paradigm (Liu et al., 2015). The outcome of this study is of considerable clinical relevance since disruption of memory reconsolidation can potentially erase pathophysiological memories including symptoms associated with posttraumatic stress disorder. For this reason,  $\beta$ -AR antagonists that can selectively interfere with  $\beta$ -AR-mediated arrestin recruitment/signaling may prove more efficacious and cause fewer side effects than propranolol and other conventional  $\beta$ -AR blockers in the treatment of posttraumatic stress disorder and related pathophysiological conditions (Liu et al., 2015).

2. *Working Memory.* DA modulates working memory largely via activation of the cortical  $D_1$  receptors, and  $D_1$  receptor agonists have been shown to improve cognitive performance in a dose-dependent manner (Arnsten et al., 2017; Arnsten et al., 2015; Wang et al., 2019). Recently, Yang et al. (Yang et al., 2021) identified two  $D_1$  receptor-selective compounds (2MDHX and CY208,243) that stimulated G protein signaling with similar intrinsic activity, but showed marked differences in their ability to recruit  $\beta$ -arrestins (2MDHX > CY208,243). Behavioral studies showed that treatment with 2MDHX resulted in a slight improvement in working memory (decrease in decision-making time), as compared to CY208,243. 2MDHX administration also led to greater improvements at the electrophysiological level (Yang et al., 2021). To strengthen the concept that  $\beta$ -arrestins play a role in mediating the beneficial cognitive

effects of 2MDHX, it will be important to study the activity of 2MDHX in  $\beta$ -arrestin mutant mice.

*3. Metabotropic Glutamate Receptors and Cognition.* Group I metabotropic glutamate receptors, mGluR1 and mGluR5, are widely expressed at central excitatory synapses (Gregory and Goudet, 2021) where they mediate changes in synaptic plasticity which are closely linked to memory formation (Crupi et al., 2019; Ménard and Quirion, 2012). For example, mGluRs mediate intermediate-term potentiation of excitatory synapses in CA3 hippocampal neurons (Frausto et al., 2011). A recent study demonstrated that this form of synaptic plasticity is absent in  $\beta$ arr2 KO mice but preserved in  $\beta$ arr1 KO mice (Eng et al., 2016). Electrophysiological studies demonstrated that mGluR-dependent depression of synaptic transmission in CA1 pyramidal neurons was also dependent on the presence of  $\beta$ arr2 (Eng et al., 2016). In contrast, classical long-term potentiation of the mossy fibers-CA3 synapses remained unaffected by the lack of  $\beta$ arr2. Immunoprecipitation studies indicated that  $\beta$ arr2 can associate with both mGluR1 and mGluR5 in the mouse hippocampus. These data suggest that  $\beta$ arr2 plays a key role in mediating various forms of hippocampal synaptic plasticity in response to mGluR1/5 activation, most likely by facilitating c-Src and ERK1/2 signaling (Eng et al., 2016).

The function of mGluR5 is critically involved in the pathology of fragile X syndrome (FXS), the most common form of heritable mental retardation and the leading identified cause of autism (Stoppel et al., 2021). FXS is caused by the transcriptional silencing of the gene encoding fragile X mental retardation protein. However, many pathological features seem to result from the hyperactivity of mGluR5 receptors (Stoppel et al., 2021). At the neural level, FXS mouse models show enhanced mGluR5-dependent LTD (Dölen and Bear, 2008) which requires the



presence of  $\beta$ arr2 (Eng et al., 2016) and enhanced protein synthesis (Dölen and Bear, 2008; Osterweil et al., 2010). The increase in mGluR5-dependent protein synthesis was absent in mice with heterozygous deletion of  $\beta$ arr2, suggesting that  $\beta$ arr2 acts as a mediator of mGluR5 signaling to protein translation (Stoppel et al., 2017). Importantly, reducing  $\beta$ arr2 expression levels reversed the behavioral and synaptic deficits in a mouse model of FXS without affecting G protein signaling or causing psychosis-like effects (Stoppel et al., 2017). These findings are of considerable translational relevance for the development of novel drugs useful for the treatment of FXS.

*4. Muscarinic Receptor-Mediated Cognitive Improvements.* Interestingly, a knockin mouse strain expressing a phosphorylation-deficient version of the  $M_3$  muscarinic receptor showed deficits in fear conditioning which requires hippocampus-dependent learning and memory (Poulin et al., 2010). The mutant  $M_3$  receptor was able to activate  $G_{q/11}$  proteins normally but failed to recruit  $\beta$ -arrestins, suggesting that  $\beta$ -arrestin-dependent pathways play a role in the cognition-enhancing effects of  $M_3$  receptor signaling, at least under certain experimental conditions (Poulin et al., 2010).

In a related study, Scarpa et al. (Scarpa et al., 2021) analyzed knockin mice expressing a phosphorylation-deficient version of the  $M_1$  muscarinic receptor. This mutant receptor couples normally to  $G_{q/11}$  but is deficient in  $\beta$ -arrestin recruitment (Bradley et al., 2020). Interestingly, the authors found that mouse prion disease progresses more rapidly in the  $M_1$  receptor mutant mice as compared to WT littermates (Scarpa et al., 2021). Mouse prion disease, a progressive terminal neurodegenerative disease, displays many of the hallmarks of human Alzheimer's disease (AD) (Mallucci et al., 2003). Based on these observations, muscarinic agonists that promote  $M_1$

receptor phosphorylation/ $\beta$ -arrestin-dependent signaling are predicted to be endowed with neuroprotective properties and may be able to slow down the progression of neurodegenerative diseases such as AD (Scarpa et al., 2021).

*5. Potential Role of  $\beta$ -Arrestins in Tauopathies.* The  $\beta$ 2-AR and the metabotropic glutamate receptor 2 (mGluR2) have been shown to mediate the hyperphosphorylation of tau, a key feature of AD (see (Woo et al., 2022) and references therein). A recent study reported that  $\beta$ -arrestins are required for the ability of these two GPCRs to promote the abundance of pathogenic tau (Woo et al., 2022). Interestingly, enhanced levels of  $\beta$ arr1 promoted the accumulation of pathogenic tau, whereas genetic reduction of  $\beta$ arr1 expression alleviated tauopathy and certain cognitive deficits in mice (Woo et al., 2022). Mechanistic data indicated that  $\beta$ arr1 causes tauopathy by various molecular mechanisms including the dissociation of tau from microtubules (Woo et al., 2022). These new data suggest that strategies aimed at decreasing  $\beta$ arr1 expression levels or  $\beta$ arr1 activity may become clinically relevant for the treatment of tauopathies such as AD.

*6. Drug Addiction and Associative Learning.* Drug addiction involves associative learning, a process that attributes excessive motivational value to distinct stimuli or environments that are paired with drug use (Meyer et al., 2016). The infralimbic prefrontal cortex (IL-PFC) is thought to play a key role in extinction learning and the attenuation of the original associative memory, reducing the craving for drugs of abuse such as cocaine (Huang et al., 2018a). A recent study reported that infusion of propranolol, a non-biased  $\beta$ -AR blocker, into the IL-PFC interfered with extinction learning of cocaine-induced conditioned place preference (CPP memory) in mice (Huang et al., 2018a). This effect was not observed after infusion of carvedilol, a  $\beta$ -AR blocker

that is able to stimulate arrestin-dependent signaling (Wisler et al., 2007). Moreover, the lack of  $\beta$ arr2 in IL-PFC excitatory neurons disrupted extinction learning of cocaine-CPP, while overexpression of  $\beta$ arr2 in IL-PFC facilitated this behavior (Huang et al., 2018a).  $\beta$ arr2 knockdown in mouse IL-PFC excitatory neurons also interfered with extinction learning of cocaine self-administration memory. These findings suggest that a  $\beta$ -AR/ $\beta$ arr2 signaling pathway that is operative in IL-PFC excitatory neurons is required for extinction learning of cocaine-associated memories.

Recently, the Caron lab reported that SBI-553, a small molecule binding to the neurotensin receptor 1 (NTSR1), functions as a  $\beta$ -arrestin-biased allosteric NTSR1 agonist and promotes  $\beta$ -arrestin recruitment to the neurotensin-occupied NTSR1 while inhibiting G protein coupling (Slosky et al., 2020). Importantly, SBI-553 demonstrated efficacy in various animal models of psychostimulant abuse but did not cause the side effects typically observed after administration of non-biased NTSR1 agonists (Slosky et al., 2020). Clearly, these observations are of considerable clinical relevance for the development of novel approaches toward the treatment of drug addiction.

*7. Anxiety Disorders.* Aberrant stimulation of neural circuits that regulate anxiety and fear behaviors can cause psychiatric disorders, including post-traumatic stress disorder and various phobias (Tovote et al., 2015). Anxiety and fear are complex behaviors that are under the control of several neurotransmitter systems (Chen et al., 2022; Hare and Duman, 2020; Tovote et al., 2015). In agreement with this concept, drugs targeting multiple GPCRs can modulate the expression of fear and anxiety (de la Mora et al., 2010; French and van Rijn, 2022; Kindt et al., 2009; Mores et al., 2019; Takahashi, 2001). For example, DOR agonists can reduce anxiety and

fear expression (Saitoh et al., 2004; Sugiyama et al., 2019; Yamada et al., 2019). Interestingly, treatment of mice with a  $\beta$ -arrestin-biased DOR agonist (SNC80) (Chiang et al., 2016) resulted in reduced anxiety-like and fear-related behaviors (Ko et al., 2021). This effect was absent in  $\beta$ arr2 KO mice, indicative of a central role of  $\beta$ arr2 in mediating these behaviors (Ko et al., 2021). Moreover, the SNC80-induced activation of ERK1/2 in limbic brain structures was not observed in  $\beta$ arr2 KO mice. Studies with a MEK1/2 inhibitor indicated that ERK1/2 signaling is required for SNC80-mediated anxiolysis (Ko et al., 2021). In contrast, additional studies with G protein- or arrestin-biased DOR agonists and  $\beta$ arr1 and  $\beta$ arr2 KO mice demonstrated that both  $\beta$ -arrestins are involved in regulating fear-related behaviors (Ko et al., 2021). These findings may guide the development of more efficacious drugs useful for the treatment of fear and anxiety disorders. As mentioned earlier, G protein-biased DOR agonists are currently being developed as novel analgesic drugs (Quirion et al., 2020). The study by the van Rijn lab (Ko et al., 2021) therefore raises the caveat that the use such agents for the treatment of pain could affect neuronal circuits involved in anxiety- and fear-related behaviors.

*8. Alcohol Intake and Depression.* DOR agonists endowed with analgesic activity are also being developed for the treatment of depression and alcohol dependence (Chu Sin Chung and Kieffer, 2013; Pradhan et al., 2011). Studies with DOR agonists differing in their efficacy to recruit  $\beta$ -arrestins showed that the efficacy of these drugs to increase the intake of alcohol positively correlated with their efficacy to recruit  $\beta$ -arrestins (Chiang et al., 2016). Interestingly, the ability of SNC80, a DOR agonist that can recruit  $\beta$ -arrestins with high efficacy, to enhance alcohol intake was abolished in  $\beta$ arr2 KO mice. In contrast, TAN67, a G protein-biased DOR agonist, suppressed alcohol intake in a  $\beta$ -arrestin-independent fashion. Both SNC80 and TAN67

displayed anti-depressive properties in the forced swimming paradigm, in agreement with previous results (Saitoh et al., 2004; Saitoh and Yamada, 2012). The antidepressive effect of SNC80 but not that of TAN67 was significantly attenuated in  $\beta$ arr2 KO mice (Chiang et al., 2016). Interestingly, the antidepressant effects of fluoxetine, a selective serotonin reuptake inhibitor, were also reduced in  $\beta$ arr2 KO mice (David et al., 2009). The finding that DOR agonists that are able to recruit  $\beta$ arr2 with high efficacy can promote alcohol intake suggest that drug development efforts should focus on DOR agonists that are unable to recruit  $\beta$ arr2 efficiently.

9. *Caveats.* In most studies reviewed above, the precise molecular and cellular mechanisms through which  $\beta$ -arrestins modulate the actions of neurotropic drugs remain incompletely understood. One complicating factor is that receptor agonists or mutant GPCRs biased towards G protein- or  $\beta$ -arrestin-mediated signaling affect both signaling branches via arrestin recruitment. This could be one of the reasons for the seemingly inconsistent results observed in related studies. Thus, a receptor biased towards G proteins is likely to induce exaggerated G protein signaling, which may involve altered receptor trafficking and recycling patterns. Similarly, an arrestin-biased receptor may cause specific signaling and/or behavioral effects not only via arrestin-dependent signaling but also via altered G protein signaling due to impaired G protein recruitment. The role of  $\beta$ -arrestins in GPCR recycling and resensitization is also often overlooked. Similar caveats apply to the use of biased GPCR ligands.

Interestingly, a recent study with  $\beta$ arr1/ $\beta$ arr2 double mutant mice revealed a complex interplay between the two arrestin isoforms regarding the behavioral effects of AMPH (Zurkovsky et al., 2017). It remains to be explored whether this finding is of more general

relevance for  $\beta$ -arrestin-modulated actions of neurotropic drugs. The potential roles of  $\beta$ arr1, which is highly expressed in most neuronal subpopulations (Gurevich et al., 2002), in the behavioral and/or therapeutic effects of psychotropic drugs remain largely unexplored.

## V. Modulation of Cardiovascular Functions by $\beta$ -Arrestins

The potential roles of  $\beta$ -arrestins in regulating various cardiovascular functions have been reviewed in detail recently (Jiang et al., 2022; Lino and Barreto-Chaves, 2022; Lymperopoulos, 2018). In the following, we will only briefly discuss the therapeutic potential of arrestin-biased angiotensin II AT<sub>1</sub> receptor (AT<sub>1</sub>R) agonists. Angiotensin II regulates cardiovascular functions primarily via activation of AT<sub>1</sub>Rs (Balakumar and Jagadeesh, 2014; Lino and Barreto-Chaves, 2022). Abnormal signaling by AT<sub>1</sub>R is involved in multiple cardiovascular pathologies including hypertension and heart failure, as well as various other pathophysiological conditions (Balakumar and Jagadeesh, 2014; Karnik et al., 2015). Various lines of evidence suggest that arrestin signaling mediates the beneficial effects of AT<sub>1</sub>R activation on cardiac contractility while limiting the deleterious effects of excessive adrenergic and AT<sub>1</sub>R stimulation leading to heart failure (Capote et al., 2021; Lino and Barreto-Chaves, 2022; Monasky et al., 2013; Ryba et al., 2017). For this reason, arrestin-biased AT<sub>1</sub>R agonists are predicted to have considerable potential for the treatment of various cardiovascular disorders.

In a recent study, an arrestin-biased AT<sub>1</sub>R agonist, TRV027, was administered in a phase IIb clinical trial to patients with acute heart failure (Pang et al., 2017). Disappointingly, TRV027 did not lead to any clinically significant improvements at any of the three doses tested (Pang et al., 2017). Possible reasons for the negative outcome of this study have been discussed recently

(Lino and Barreto-Chaves, 2022). Given the vast amount of encouraging pre-clinical and clinical data that have been obtained with arrestin-biased AT1R agonists (Capote et al., 2021; Lino and Barreto-Chaves, 2022; Monasky et al., 2013; Ryba et al., 2017), other members of this drug family endowed with different pharmacokinetic and pharmacodynamic properties may prove clinically beneficial in different patient cohorts suffering from heart failure and other cardiovascular conditions.

## **VI. Cancer and $\beta$ -Arrestins**

### *A. General Comments*

Many studies have shown that altered GPCR signaling can contribute to cancer progression at multiple levels (Arang and Gutkind, 2020). Since  $\beta$ arr1 and  $\beta$ arr2 are important regulators of GPCR activity, it is not surprising that these two proteins have been implicated in the pathogenesis of various types of cancer (Aamna et al., 2022; Bagnato and Rosanò, 2019; Shukla and Dwivedi-Agnihotri, 2020; Tian et al., 2022) (Fig. 3).

Moreover,  $\beta$ -arrestins can modulate various signaling pathways involved in cell proliferation, carcinogenesis, and metastasis (Peterson and Luttrell, 2017). As reviewed recently (Peterson and Luttrell, 2017), Ras-mediated activation of mitogenic ERK1/2 signaling, transactivation of EGF receptors, and cytoskeletal rearrangements represent three major cellular pathways or mechanisms through which  $\beta$ -arrestins can promote cell cycle progression. In line with these findings, many studies have shown that  $\beta$ -arrestins play a role in cancer initiation and progression (Aamna et al., 2022; Bagnato and Rosanò, 2019; Shukla and Dwivedi-Agnihotri, 2020; Tian et al., 2022).

In the following, we will briefly review several recent studies in the  $\beta$ -arrestin/cancer field. Cancer is a highly heterogeneous disease that is endowed with distinct molecular signatures depending on tumor stage and initial site of formation (Dagogo-Jack and Shaw, 2018). This heterogeneity, combined with the various different in vivo and in vitro tumor models used by different laboratories, may explain that the two  $\beta$ -arrestins can both promote or inhibit cancer formation and progression under specific experimental conditions (see below).

## *B. Roles of $\beta$ arr1 in Carcinogenesis*

*1. Ovarian Cancer.* Activation of the two endothelin receptor (ETR) subtypes (ET<sub>A</sub> and ET<sub>B</sub>) can promote tumorigenesis and metastatic progression (Tocci et al., 2019). Accumulating evidence suggests that ETR/ $\beta$ arr1 signaling plays an important role in mediating these processes. In ovarian cancer, for example, the ETR/ $\beta$ arr1 signaling module stimulates cellular processes, including changes in gene transcription, that promote tumorigenesis, suggesting that strategies aimed at interfering with ETR/ $\beta$ arr1 signaling may prove useful for the treatment of this type of cancer (Tocci et al., 2019). A related study reported that hMENA, a member of the actin-regulatory protein ENA/VASP family, can bind to  $\beta$ arr1 and that this interaction is required for ETR<sub>A</sub>-mediated invadopodial function during the progression of serous ovarian cancer (SOC) (Di Modugno et al., 2018). Interestingly, ligand activation of ETR<sub>A</sub> in cultured SOC cells promoted the binding of  $\beta$ arr1 to hMENA/hMENA $\Delta$ v6, resulting in the activation of RhoC, cortactin, and other cellular pathways, triggering the maturation of invadopodia and the spread of SOC cells (Di Modugno et al., 2018). Moreover, treatment with an ETR<sub>A</sub> antagonist interfered with the formation of the  $\beta$ arr1/hMENA complex, resulting in impaired invadopodial maturation



(Di Modugno et al., 2018). This finding suggests that ETR<sub>A</sub> antagonists may prove clinically useful to prevent the progression of ovarian cancer.

*2. Prostate Cancer.* Several reports suggest that  $\beta$ arr1 signaling may also play a role in the pathogenesis of prostate cancer (see, for example, (Zecchini et al., 2014)). A recent study demonstrated that  $\beta$ arr1 can promote cell growth by inhibiting the expression of FOXO3a in prostate cancer cells in vitro and in vivo (Kong et al., 2018). Mechanistic data suggested that  $\beta$ arr1 inhibits the transcriptional activity of FOXO3a via Akt- and ERK1/2-dependent pathways and stimulates the degradation of FOXO3a via MDM2-mediated ubiquitination (Kong et al., 2018). These findings support the concept that strategies aimed at suppressing the expression and/or activity of  $\beta$ arr1 may lead to novel anticancer drugs.

Androgen receptor (AR)-mediated signaling drives all stages of prostate cancer, including castration-resistant prostate cancer (CRPC), the lethal and drug-resistant form of the disease (Feng and He, 2019). Interestingly, a recent study showed that the expression of  $\beta$ arr1 is upregulated in CRPC and that nuclear  $\beta$ arr1 promotes prostate cancer cell migration and invasion in vitro and stimulates prostate tumor growth in vivo (Purayil et al., 2021). Mechanistic data suggest that these effects are mediated by a  $\beta$ arr1/ $\beta$ -catenin complex that controls the expression of AR-regulated genes that drive CRPC (Purayil et al., 2021). Approaches capable of inhibiting the formation of this nuclear complex may lead to new drugs useful for the treatment of CRPC.

Upregulation of  $\beta$ arr1 expression in human prostate cancer, including CRPC, is also positively correlated with increased expression and function of the glucocorticoid receptor (GR) (Purayil and Daaka, 2022). Biochemical studies have shown that  $\beta$ arr1 forms a complex with GR

in the nucleus of CRPC cells and that downregulation of  $\beta$ arr1 expression inhibits GR function and CRPC growth and invasion both in vitro and in vivo (Purayil and Daaka, 2022). These data suggest that the  $\beta$ arr1/GR complex represents a potential target for the treatment CRPC.

3. *Breast Cancer*. An early study (Lundgren et al., 2011) demonstrated the potential importance of  $\beta$ arr1 as a prognostic and treatment-predictive marker in breast cancer. A related study reported that  $\beta$ arr1 levels are reduced, while  $\beta$ arr2 levels are elevated during breast cancer progression and that these changes in  $\beta$ -arrestin expression levels correlate with a poor clinical outcome (Michal et al., 2011). Moreover, Shenoy et al. (Shenoy et al., 2012) showed that  $\beta$ arr1 can interact with hypoxia-induced factor-1 $\alpha$  (HIF-1 $\alpha$ ) in breast carcinoma cells and that this interaction is critical for HIF-1 $\alpha$ -dependent expression of vascular endothelial growth factor-A (VEGF-A). Interestingly,  $\beta$ arr1 and VEGF-A expression levels were positively correlated in metastatic human breast cancer tissues, suggesting that  $\beta$ arr1 modulates gene transcription under hypoxic conditions to promote breast cancer cell growth in a VEGF-dependent fashion (Shenoy et al., 2012).

A recent study demonstrated that suppression of the expression of miR-374a-5p, a miRNA upregulated in triple negative breast cancer (TNBC), reduced tumor progression and growth in TNBC cell lines (Son et al., 2019). Moreover, inhibition of miR-374a-5p expression resulted in increased  $\beta$ arr1 expression in TNBC cell lines and in xenograft mouse models. Additional studies demonstrated that overexpression of  $\beta$ arr1 inhibited the growth and migration of TNBC cell lines in an AMPK-dependent fashion (Son et al., 2019), suggesting that  $\beta$ arr1 acts as a tumor suppressor in TNBC. In contrast to this finding, several other studies reported that  $\beta$ arr1 expression is upregulated in different types of cancer (Purayil and Daaka, 2018; Yang et al.,

2015; Zecchini et al., 2014). These data suggest that the ability of  $\beta$ arr1 to promote or suppress cancer cell growth and metastasis may depend on the type of cancer under investigation.

Consistent with the findings by Son et al. (Son et al., 2019), a recent study demonstrated that knockdown of  $\beta$ arr1 or  $\beta$ arr2 expression in TNBC cell lines stimulated cell proliferation and invasion, whereas overexpression of  $\beta$ arr1 or  $\beta$ arr2 inhibited these processes (Bostanabad et al., 2021). Overexpression of the two  $\beta$ -arrestins resulted in cell cycle S-phase arrest and the altered expression of many cell cycle genes (Bostanabad et al., 2021). The authors also reported a positive correlation between low  $\beta$ arr1 expression levels and poorer prognosis in breast cancer patients (Bostanabad et al., 2021). These data support the concept that  $\beta$ arr1 functions as a tumor suppression in TNBC.

*4. Leukemia.* Human telomerase reverse transcriptase (hTERT) is highly expressed in many malignant tumors including certain types of leukemia (Leão et al., 2018; Liu et al., 2017a). Interestingly, knockdown of  $\beta$ arr1 expression promoted cell senescence in cells mimicking a certain subtype of leukemia-initiating cells (B-ALL LICs) in vivo and in vitro (Liu et al., 2017a). Mechanistic data indicated that  $\beta$ arr1 stimulates hTERT transcription by facilitating the binding of P300-Sp1 to the hTERT promoter (Liu et al., 2017a). Moreover, elevated  $\beta$ arr1 levels in senile leukemia-initiating cells from acute lymphoblastic leukemia patients were predictive of poor prognosis (Liu et al., 2017a). These findings suggest that  $\beta$ arr1 acts as tumor-promoting factor in certain forms of leukemia.

Activating mutations of NOTCH1, a transmembrane receptor linked to many signaling pathways involved in tumorigenesis (Gharaibeh et al., 2020), are observed in most cases of T-cell acute lymphoblastic leukemia (T-ALL) (Liu et al., 2017b), the most common type of

childhood cancer. A recent study reported that overexpression of  $\beta$ arr1 inhibited the progression of T-ALL in vivo and in vitro (Shu et al., 2020). RNA-seq data suggested that  $\beta$ arr1 probably exerts its tumor-suppressive activity, at least in part, by reducing the expression of genes associated with the NOTCH1 signaling pathway (Shu et al., 2020). Additional data obtained with cultured cells indicated that  $\beta$ arr1 is also able to promote the degradation of NOTCH1 by facilitating NOTCH1 ubiquitination (Shu et al., 2020). These findings establish  $\beta$ arr1 as a tumor suppressor in T-ALL. In contrast, as reviewed in this section, many recent studies have shown that  $\beta$ arr1 signaling contributes to the formation and progression of various other types of cancer.

*5. Medulloblastoma.* Medulloblastoma (MB) is the most common pediatric malignant brain tumor (Massimino et al., 2011). In about one quarter of patients, MB is driven by aberrant sonic-hedgehog/Gli signaling (SHH-MB) (Miele et al., 2017). A recent study (Miele et al., 2017) using a mouse model of SHH-MB reported the downregulation of  $\beta$ arr1 in cancer stem cells derived from SHH-MB. Interestingly, the expression of miR-326, which is localized to the first intron of the mouse  $\beta$ arr1 gene, was also significantly reduced in these cells (Miele et al., 2017).

Additional studies showed that miR-326 suppresses sonic-hedgehog/Gli signaling by targeting various components of this signaling cascade and that  $\beta$ arr1 interferes with Gli1 transcriptional activity by facilitating p300-dependent Gli1 acetylation, resulting in a further impairment of the sonic-hedgehog/Gli signaling module (Miele et al., 2017). These new findings suggest that  $\beta$ arr1 represents a potential target for the treatment of SHH-MB.

*6. Glioblastoma.* Glioblastoma is the most common type of malignant brain tumor among adults (Tan et al., 2020). Several studies have demonstrated that neurokinin-1 receptor (NK1R) agonists

promote glioblastoma cell proliferation and that NK1R antagonists can slow down glioma cell growth (for references, see (Zhang et al., 2017)). Knockdown of  $\beta$ arr1 expression in human glioblastoma cells inhibited NK1R-mediated cell proliferation and caused G<sub>2</sub>/M phase cell cycle arrest, associated with the downregulation of several genes involved in cell cycle progression. In glioblastoma cells, NK1R activation resulted in a prolonged phosphorylation of ERK1/2 and Akt in an  $\beta$ arr1-dependent fashion (Zhang et al., 2017). This response was absent in glioblastoma cells with reduced  $\beta$ arr1 expression, suggesting that inhibition of these signaling pathways contribute to impaired cell cycle progression and cell proliferation. Interestingly, following knockdown of  $\beta$ arr1 expression, glioblastoma cells showed increased sensitivity to treatment with NK1R antagonists (Zhang et al., 2017), suggesting that inhibition of NK1R-dependent  $\beta$ arr1-signaling may prove beneficial for the therapy of glioblastoma.

*7. Lung Cancer.* The G protein–coupled bile acid receptor (GPBAR) regulates many important physiological and pathophysiological functions including carcinogenesis (Jia et al., 2018). The GPBAR is highly expressed in non-small cell lung cancer (NSCLC), and a positive correlation exists between GPBAR expression levels and the clinical progression of NSCLC (Liu et al., 2018). Ma et al. (Ma et al., 2022) recently identified a novel GPBAR agonist, R399, that preferentially promotes  $\beta$ arr1 signaling. Studies with NSCLC cells showed that R399 stimulated YAP signaling and cell proliferation in a  $\beta$ arr1-dependent fashion (Ma et al., 2022). In contrast, treatment of NSCLC cells with a G protein-biased GPBAR agonist, INT-777, interfered with YAP signaling, inhibited cell proliferation, and induced apoptosis (Ma et al., 2022). These new data are highly relevant for the design of novel anticancer agents targeting the GPBAR.

A recent study reported that PLEKHH2, a member of the pleckstrin homology domain-containing family H, is also highly expressed in NSCLC (Wang et al., 2022b). Studies with NSCLC cells showed that high expression of PLEKHH2 facilitated cell proliferation, migration, and invasion (Wang et al., 2022b). Biochemical studies showed that PLEKHH2 binds to  $\beta$ arr1 through its FERM domain, resulting in the activation of the FAK/PI3K/AKT signaling cascade and the stimulation of NSCLC cell proliferation, migration, and invasion (Wang et al., 2022b). These findings indicate that  $\beta$ arr1 can modulate the progression of NSCLC via multiple cellular mechanisms that could be targeted for therapeutic purposes.

### *C. Roles of $\beta$ arr2 in Carcinogenesis*

*1. Breast Cancer.* Previous work has demonstrated that  $\beta$ arr2 can be covalently modified via SUMOylation (Wyatt et al., 2011; Xiao et al., 2015). Recent data (Dong et al., 2020) showed that a SUMOylation-deficient mutant version of  $\beta$ arr2 slowed the migration of breast cancer cells but had little effect on cell proliferation. This effect was accompanied by changes in the expression levels of a series of metabolically important genes, suggesting that  $\beta$ arr2 SUMOylation represents an important factor regulating breast cancer progression (Dong et al., 2020).

A recent study reported that  $\beta$ arr2 plays an important role in kisspeptin receptor (GPR54)-dependent formation of invadopodia in breast cancer cells (Goertzen et al., 2016). Invadopodia are actin-rich protrusions of the plasma membrane involved in cancer invasiveness and metastasis (Linder et al., 2022). Specifically, agonist stimulation of kisspeptin receptors stimulated the formation of invadopodia via an  $\beta$ arr2- and ERK1/2-dependent mechanism

(Goertzen et al., 2016). These data suggest that inhibition of  $\beta$ arr2 binding to kisspeptin receptors may lead to the development of novel anti-cancer drugs.

The Iverson lab (Perry et al., 2019) recently demonstrated that  $\beta$ arr2 can directly interact with the kinase domain of maternal embryonic leucine zipper kinase (MELK). The expression of MELK, a serine/threonine kinase known to play an important role in cell cycle regulation and proliferation, is increased in various cancer cells including breast cancer cells (Ganguly et al., 2015). Strikingly, co-expression of  $\beta$ arr2 and MELK led to a significant decrease in the number of cells in the S-phase, indicating that  $\beta$ arr2 can interfere with cell proliferation via this mechanism (Perry et al., 2019). More detailed mechanistic studies are needed to further explore how  $\beta$ arr2 affects MELK function and cellular localization where it exerts its antiproliferative activity.

*2. Leukemia.* Chronic myelogenous leukemia (CML) is a myeloproliferative form of cancer that is defined by the unrestrained proliferation of pluripotent bone marrow stem cells (Thompson et al., 2015). Studies with  $\beta$ -arrestin KO mice demonstrated that  $\beta$ arr2 is essential for the progression of CML, most likely due to its ability to stabilize  $\beta$ -catenin, thus promoting Wnt/ $\beta$ -catenin signaling (Fereshteh et al., 2012). This observation raises the possibility that  $\beta$ arr2 also plays a role in the pathogenesis of other solid cancers characterized by aberrant Wnt signaling (Fereshteh et al., 2012).

Primary myelofibrosis (PMF), which is closely related to CML, represents another form of chronic leukemia characterized by myeloproliferation and bone marrow fibrosis (Abdel-Wahab and Levine, 2009). Studies with a mouse model of PMF showed that conditional deletion of

$\beta$ arr2 from established PMF interferes with the progression of this disease, possibly due to the antiapoptotic properties of  $\beta$ arr2 (Rein et al., 2017).

*3. Ovarian Cancer.* Ovarian cancer is the most fatal gynecologic cancer (Stewart et al., 2019). It is often referred to as the ‘silent killer’, since it is frequently not diagnosed until it has progressed to advanced stages (Stewart et al., 2019). A recent study reported a positive correlation between cytoplasmic  $\beta$ arr2 expression levels in ovarian cancer samples and reduced overall survival (Czogalla et al., 2020). In agreement with this observation,  $\beta$ arr2 overexpression enhanced the viability of an ovarian cancer cell line (A2780) in vitro (Czogalla et al., 2020). These findings suggest that  $\beta$ arr2 expression levels in ovarian cancer are of potential prognostic value and that strategies that can interfere with  $\beta$ arr2 expression or activity may have potential in this type of cancer.

*4. Colorectal Cancer.* Colorectal cancer (CRC) is among the most frequent types of cancer and most common causes of cancer death worldwide (Favoriti et al., 2016). 5-Fluorouracil (5-FU) represents the first-line treatment for CRC (Vodenkova et al., 2020). A recent study found that  $\beta$ arr2 levels were increased in CRC tissues compared with normal colon tissues (Ren et al., 2018). In vitro studies with CRC cell lines showed that knockdown of  $\beta$ arr2 expression resulted in a decrease in 5-FU-induced apoptosis, while overexpression of  $\beta$ arr2 promoted cancer cell apoptosis (Ren et al., 2018). Moreover, following 5-FU treatment, downregulation of  $\beta$ arr2 expression led to reduced levels of pro-apoptotic proteins (cleaved caspase-3 and Bax) and increased expression of Bcl-2, an anti-apoptotic protein (Ren et al., 2018). These data indicate



that  $\beta$ arr2 plays a key role in mediating 5-FU-stimulated apoptotic responses in CRC, a finding that is of considerable translational relevance.

5. *Medulloblastoma*. Suppressor of Fused (SuFu) is a highly conserved protein that functions as an inhibitor of the hedgehog (HH) signaling pathway, a major determinant of cell differentiation and proliferation (Huang et al., 2018b). Mutations in the human SuFu gene predispose to sonic hedgehog medulloblastoma (Shh-MB) (Guerrini-Rousseau et al., 2018). A recent study reported that a complex between Itch, a E3 ubiquitin ligase, and  $\beta$ arr2 promotes the ubiquitination of SuFu, resulting in impaired HH signaling (Infante et al., 2018). This observation indicates that  $\beta$ arr2 is an important regulator of the tumor suppressor functions of SuFu, suggesting that  $\beta$ arr2 may represent a potential target for the treatment of medulloblastoma.

6. *Kidney Cancer*. Kidney cancer is among the most common cancers worldwide (Scelo and Larose, 2018). Most kidney cancers represent renal cell carcinomas (RCC). Masannat et al. (Masannat et al., 2018) recently reported that *ARRB2* (gene encoding human  $\beta$ arr2) transcript levels are increased in RCC and that a positive correlation exists between *ARRB2* expression levels and patient survival rate. Deletion of *ARRB2* inhibited the rate of RCC cell proliferation and migration both in vitro and in vivo (Masannat et al., 2018). Additional studies showed that  $\beta$ arr2 regulates RCC cell cycle progression through c-Src activation and cyclin A expression (Masannat et al., 2018). In sum,  $\beta$ arr2 regulates the progression of RCC and represents a potential target for new drugs targeting this type of cancer.

7. *Glioblastoma*. High expression levels of  $\beta$ arr2 correlate with reduced tumorigenesis in glioblastoma and increased survival probability in glioblastoma patients (Bae et al., 2021).

Recent data suggest that  $\beta$ arr2 promotes the degradation of HIF-1 $\alpha$ , the master regulator of the body's response to low oxygen concentrations (Bae et al., 2021). Functional studies showed that  $\beta$ arr2 interacts with HIF-1 $\alpha$  and stimulates its proteasomal degradation by recruiting PHD2 and pVHL, two key factors intimately involved in ubiquitin-dependent HIF-1 $\alpha$  degradation (Bae et al., 2021). Overexpression of  $\beta$ arr2 in human glioblastoma cells resulted in impaired HIF-1 $\alpha$  signaling, tumor growth, and angiogenesis (Bae et al., 2021), supporting the concept that  $\beta$ arr2 can regulate the stability of HIF-1 $\alpha$  in certain human cancers such as glioblastoma, a finding that is of potential translational relevance.

#### *D. Roles of $\beta$ arr1/ $\beta$ arr2 in Carcinogenesis*

1. *Atypical Chemokine Receptors*. Chemokines are involved in the pathophysiology of various cancers, due to their ability to modulate cell migration and proliferation (Caronni et al., 2016; Liu et al., 2020). Recent reports have implicated atypical chemokine receptors (ACKRs) in tumor initiation and metastasis (Lokeshwar et al., 2020; Mollica Poeta et al., 2019; Sjöberg et al., 2020; Torphy et al., 2022). ACKRs, following the binding of distinct chemokine ligands, are unable to initiate classical G protein-mediated signaling (Nibbs and Graham, 2013; Torphy et al., 2022). In agreement with this notion, ligand activation of the atypical chemokine receptor 3 (ACKR3) stimulates arrestin-mediated pathways, without activating heterotrimeric G proteins (Gustavsson et al., 2017; Rajagopal et al., 2010a). The expression of ACKR3 protein or mRNA is increased in numerous cancers (Sjöberg et al., 2020). Because of this finding, this chemokine receptor subtype is considered a potential target for novel anti-cancer drugs (Sjöberg et al.,

2020). ACKR3 activation can lead to the stimulation of ERK1/2, Akt, and other pathways able to promote tumor formation in a  $\beta$ -arrestin-dependent fashion (Sjöberg et al., 2020), suggesting that inhibition of the ACKR3/ $\beta$ -arrestin module might prove beneficial clinically in certain types of cancer.

*2. Bladder Cancer.* A recent study reported that specimens from human bladder cancer show increased  $\beta$ arr1 but reduced  $\beta$ arr2 expression, as compared with specimens from normal bladder tissues (Kallifatidis et al., 2019). Both changes in  $\beta$ -arrestin gene expression positively correlated with bladder cancer metastasis. Knockdown of  $\beta$ arr2 expression in bladder cancer cell lines resulted in an increase in cancer stem cell markers, while overexpression of  $\beta$ arr2 had the opposite effect (Kallifatidis et al., 2019). In contrast, deletion of the  $\beta$ arr1 gene in bladder cancer cells resulted in reduced expression of several cancer stem cell markers. The authors also presented data suggesting that  $\beta$ arr1/2 expression levels can be predictive of the response to chemotherapy in bladder cancer (Kallifatidis et al., 2019). These findings suggest that  $\beta$ arr1 and  $\beta$ arr2 can affect bladder cancer progression in an opposing fashion, most likely by interacting with different sets of cellular signaling proteins.

*3. Leukemia.* Chronic lymphoproliferative disorder of natural killer cells (CLPD-NK) is a form of leukemia defined by the clonal expansion of natural killer (NK) cells. Baer et al. (Baer et al., 2022) recently reported that somatic mutations in a chemokine gene (*CCL22*) are found in many cases of CLPD-NK. These mutations interfered with the ability of CCL22 to promote the internalization of the CCR4 chemokine receptor subtype, due to impaired  $\beta$ -arrestin recruitment (Baer et al., 2022). This deficit resulted in increased cell chemotaxis and enhanced NK cell

proliferation in vitro and in vivo, indicating that impaired chemokine receptor (CCR4)-mediated  $\beta$ -arrestin recruitment can promote tumor formation.

*4. Melanoma.* The incidence of malignant melanoma has increased dramatically over the past few decades (Lopes et al., 2022). Interestingly, a recent study reported that the expression of the oxytocin receptor (OTR) was increased in malignant melanoma (Ji et al., 2019). In vitro and in vivo studies showed that ligand-dependent OTR activation facilitated melanoma cell migration, invasion, and angiogenesis via a  $\beta$ arr2-dependent ERK-vascular endothelial growth factor (VEGF)-matrix metalloproteinase-2 (MMP-2) signaling network (Ji et al., 2019). These findings raise the possibility that OTR-mediated  $\beta$ arr2 recruitment may play a role in the pathophysiology of at least certain forms of melanoma.

A related study reported that the C-X-C chemokine receptor 7 (CXCR7) was the most highly expressed chemokine receptor in mouse melanoma cell lines and that CXCR7 expression levels were positively correlated with melanoma progression in human melanoma samples (Xu et al., 2019). Overexpression of CXCR7 stimulated melanoma proliferation in vitro and in vivo, whereas inactivation of the CXCR7 gene resulted in opposite effects (Xu et al., 2019). Additional studies showed that CXCR7-mediated stimulation of melanoma cell proliferation required  $\beta$ arr2-dependent activation of Src (Xu et al., 2019). CXCR7 activation also promoted the secretion of VEGF and melanoma angiogenesis via up-regulation of the expression of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ). These findings suggest that the CXCR7 receptor subtype represents a potential target for novel anticancer drugs.

Several studies have shown that the apelin receptor is overexpressed in different types of cancer including melanoma (Wysocka et al., 2018). Loss-of-function mutations in the apelin

receptor are linked to impaired CD8<sup>+</sup> T cell cytotoxicity, reduced interferon- $\gamma$  (IFN- $\gamma$ ) signaling in tumor cells, and decreased efficacy of cancer immunotherapies (Patel et al., 2017). Liu et al. (Liu et al., 2022) recently reported that activation of the apelin receptor in melanoma cells stimulates IFN- $\gamma$  signaling via recruitment of  $\beta$ arr1 and that  $\beta$ arr1 binding to STAT1 results in impaired STAT1 phosphorylation and reduced IFN- $\gamma$  signaling (Liu et al., 2022). These data suggest that the effectiveness of melanoma immunotherapies may depend on the activity of the apelin receptor/ $\beta$ arr1 signaling module.

### *E. Conclusions.*

The studies summarized above indicate that  $\beta$ arr1 and  $\beta$ arr2 play important roles in regulating the formation and progression of many different types of cancer. Depending on the specific tumor models employed, these effects can be stimulatory or inhibitory in nature. Clearly, the outcome of this work strongly suggests that it should be possible to target  $\beta$ -arrestins and the signaling pathways through which they regulate cell proliferation and survival for the development of novel anti-cancer drugs.

## **VII. Regulation of Metabolic Functions by $\beta$ -Arrestins**

During the past decades, the prevalence of type 2 diabetes (T2D) has reached epidemic proportions worldwide, primarily resulting from changes in lifestyle that include the consumption of energy-dense refined food and reduced physical activity (Eizirik et al., 2020; Roden and Shulman, 2019). Although various drugs are in clinical use to reduce pathologically elevated blood glucose levels, the hallmark of T2D, the disease is still linked to a high degree of

morbidity and mortality (Eizirik et al., 2020; Roden and Shulman, 2019). It is likely that a better understanding of the cellular and molecular mechanisms that regulate the function of the various cell types involved in maintaining euglycemia will lead to more efficacious antidiabetic drugs with an improved side effect profile.

#### *A. Cell Type-Specific $\beta$ -Arrestin Mutant Mice as Novel Tools*

Whole body  $\beta$ arr1 and  $\beta$ arr2 KO mice exhibit major metabolic phenotypes, suggesting that the two  $\beta$ -arrestins regulate several important metabolic functions including glucose tolerance and insulin sensitivity (Zhao and Pei, 2013). Since the two  $\beta$ -arrestins are widely expressed throughout the body, the identification of specific cell types and signaling pathways responsible for the observed metabolic phenotypes remains a challenging task. The interpretation of the metabolic phenotypes displayed by whole body  $\beta$ -arrestin KO mice is further complicated by the fact that  $\beta$ -arrestins regulate important developmental functions (Kovacs et al., 2009; Philipp et al., 2013), raising the possibility that compensatory pathways affect glucose and energy homeostasis in adult  $\beta$ arr1 and  $\beta$ arr2 KO mice.

To overcome these difficulties, recent studies focused on generating and analyzing  $\beta$ -arrestin mutant mice that lack either  $\beta$ arr1 or  $\beta$ arr2 in specific cell types that are known to play key roles in regulating energy and glucose homeostasis (Pydi et al., 2022). These studies took advantage of the recent development of floxed  $\beta$ arr1 and  $\beta$ arr2 mutant mice (Kim et al., 2018; Urs et al., 2016). In mouse hepatocytes, myocytes, and pancreatic  $\beta$ -cells, the genes coding for  $\beta$ arr1 and  $\beta$ arr2 were inactivated in adult mice in a tamoxifen-dependent fashion by using Cre driver lines in which Cre activity is inducible by repeated tamoxifen injections (Pydi et al., 2022). In the following, we will briefly discuss the major metabolic phenotypes of these cell-type

specific  $\beta$ -arrestin KO mice, the underlying cellular and molecular mechanisms, and potential therapeutic targets suggested by the outcome of these studies.

## *B. Hepatocytes*

1.  *$\beta$ arr2*. Hepatocytes express many GPCRs including the glucagon receptor (GCGR) which is expressed at particularly high levels (Regard et al., 2008). Glucagon-mediated activation of hepatic GCGRs leads to the activation of the  $G_s$ /PKA signaling cascade, which triggers the stimulation of gluconeogenesis and glycogen breakdown and ultimately results in enhanced hepatic glucose production and output. While this signaling pathway plays a key role in maintaining euglycemia under fasting conditions, elevated hepatic GCGR signaling is thought to contribute to the pathophysiology of T2D and related metabolic disorders (Capozzi et al., 2022; D'Alessio, 2011).

Metabolic studies with mutant mice lacking  $\beta$ arr2 selectively in hepatocytes (hep- $\beta$ arr2 KO mice) demonstrated that hep- $\beta$ arr2 KO mice showed impaired glucose tolerance associated with enhanced hepatic GCGR signaling, both in vivo and in vitro (Zhu et al., 2017b). In agreement with this finding, glucagon treatment led to more pronounced elevations in blood glucose levels in hep- $\beta$ arr2 KO mice than in control littermates. Similarly, glucagon-stimulated cAMP levels and increases in hepatic glucose output were significantly augmented in primary hepatocytes lacking  $\beta$ arr2 (Zhu et al., 2017b), indicating that  $\beta$ arr2 functions as a negative regulator of GCGR signaling in hepatocytes (Fig. 4).

In vitro studies showed that glucagon treatment of primary hepatocytes prepared from control mice resulted in a marked decrease in the number of cell surface GCGRs (Zhu et al., 2017b). In striking contrast, this response was absent in primary hepatocytes lacking  $\beta$ arr2 (Zhu

et al., 2017b), clearly indicating that  $\beta$ arr2 is required for the internalization of hepatic GCGRs. These observations suggest that hepatic  $\beta$ arr2 functions to dampen GCGR signaling under physiological conditions by promoting GCGR internalization, a so-called canonical or conventional function of  $\beta$ arr2.

In contrast to hep- $\beta$ arr2 KO mice, mice overexpressing  $\beta$ arr2 selectively in hepatocytes (hep- $\beta$ arr2-OE mice) displayed significant improvements in glucose homeostasis, in particular when hep- $\beta$ arr2-OE mice were maintained on a calorie-rich high-fat diet (HFD) (Zhu et al., 2017b). Under these conditions, hep- $\beta$ arr2-OE mice showed reduced blood glucose levels and improved glucose tolerance, as compared to their control littermates. Additional studies demonstrated that these beneficial metabolic effects were due to reduced GCGR signaling caused by elevated hepatic  $\beta$ arr2 expression (Zhu et al., 2017b). These findings suggest that agents capable of enhancing the activity or expression of hepatic  $\beta$ arr2 may prove therapeutically beneficial under conditions of impaired glucose homeostasis.

2.  *$\beta$ arr1*. Mutant mice lacking  $\beta$ arr1 selectively in hepatocytes (hep- $\beta$ arr1 KO mice) did not display any significant metabolic phenotypes (Zhu et al., 2017b). This observation suggests that the physiological functions of  $\beta$ arr1 and  $\beta$ arr2 are not redundant in this cell type.

### C. Adipocytes

1.  *$\beta$ arr2*. The ongoing obesity epidemic is the major driver of the high prevalence of T2D in most parts of the world (Klein et al., 2022). Obesity is associated with the enlargement of adipocytes (adipocyte hypertrophy), inflammatory processes in fat tissue, and enhanced secretion of biologically active agents that have a negative impact on glucose homeostasis (Klein et al.,



2022). These factors, which include free fatty acids (FFAs) and pro-inflammatory cytokines, interfere with the ability of peripheral tissues to properly respond to insulin. This phenomenon, referred to as peripheral insulin resistance, represents a hallmark of T2D.

In mammals, adipose tissues contain two major types of adipocytes, white and brown adipocytes (Wang and Seale, 2016). White adipocytes primarily function to store excess calories in the form of triglycerides. In contrast, brown adipocytes consume nutrients such as FFAs and glucose to generate heat, in particular after activation of the sympathetic nervous system (SNS) (Wang and Seale, 2016). Interestingly, a third class of adipocytes, referred to as ‘beige adipocytes’, can arise from precursor cells in white adipose tissue (WAT) or through conversion of white adipocytes (Wang and Seale, 2016). Like brown adipocytes, beige adipocytes express uncoupling protein 1 (UCP1) and several other thermogenic genes and function to dissipate chemical energy stored in nutrients into heat.

To explore the role of  $\beta$ arr2 in regulating adipocyte function in vivo, Pydi et al. (Pydi et al., 2019) generated and analyzed mice that selectively lacked  $\beta$ arr2 in white adipocytes (adipo- $\beta$ arr2-KO mice). When adipo- $\beta$ arr2-KO mice were maintained on a HFD, the mutant mice gained significantly less body weight than their control littermates, due to reduced fat accumulation (Pydi et al., 2019). Moreover, the mutant mice were largely resistant to the metabolic deficits that are normally caused by the consumption of a HFD. Additional studies showed that these phenotypes resulted from an increase in energy expenditure caused by WAT  $\beta$ arr2 deficiency (Pydi et al., 2019). Consistent with this finding, the expression of UCP1 and other thermogenic genes was significantly increased in inguinal (subcutaneous) WAT (iWAT) lacking  $\beta$ arr2, indicating that  $\beta$ arr2 deficiency stimulates the beiging of WAT.

Adipocytes express numerous GPCRs that are linked to different functional classes of heterotrimeric G proteins (Ceddia and Collins, 2020). Since the activation of adipocyte  $\beta$ -ARs promotes lipolysis and the beiging of WAT (Collins, 2022), this class of receptors has been studied in considerable detail. The  $\beta_3$ -AR is the predominant  $\beta$ -AR subtype expressed by mouse adipocytes, and activation of this receptor subtype in vivo (e.g. by administration of CL316243, a selective  $\beta_3$ -AR agonist) promotes the beiging of WAT (Collins, 2022). Interestingly, CL316243 treatment of white adipocytes prepared from adipo- $\beta$ arr2 KO mice led to a more robust increase in cAMP levels, as compared to CL316243-treated control adipocytes (Pydi et al., 2019), suggesting that  $\beta$ arr2 acts as an inhibitor of  $\beta_3$ -AR signaling in WT adipocytes (Fig. 4). Thus, this  $\beta$ arr2 function is consistent with its conventional role as a terminator of GPCR G protein signaling.

CL316243 treatment of a cultured mouse adipocytes (3T3-F442A cells) resulted in the rapid disappearance of  $\beta_3$ -ARs from the cell surface (receptor internalization) (Pydi et al., 2019). Strikingly, siRNA-mediated knockdown of  $\beta$ arr2 expression in these cells completely blocked this CL316243 response (Pydi et al., 2019), strongly suggesting that the lack of  $\beta_3$ -AR internalization is responsible for the increase in  $\beta_3$ -AR signaling observed with adipo- $\beta$ arr2 KO mice.

The metabolic improvements observed with HFD adipo- $\beta$ arr2-KO mice were absent after treatment with propanolol, a  $\beta$ -AR blocker, or when the mutant mice were kept at thermoneutrality (30 °C). At thermoneutrality, the activity of the SNS is strongly attenuated. In addition, adipocyte-specific deletion of PRDM16, a master transcriptional coregulator required for the beiging of WAT (Wang and Seale, 2016), prevented all metabolic changes resulting from the lack of  $\beta$ arr2 in adipocytes. Taken together, these findings indicate that the metabolic

improvements displayed by the adipo- $\beta$ arr2-KO mice require the beiging of WAT which is mediated by enhanced G protein signaling via adipocyte  $\beta_3$ -ARs.

Human WAT cells also express  $\beta_3$ -ARs, although  $\beta_1$ - and  $\beta_2$ -AR are expressed at considerable higher levels (Collins, 2022). In any case, the data reported by Pydi et al. (Pydi et al., 2019) suggest that G protein-biased, selective  $\beta_3$ -AR agonists may prove clinically useful for the treatment of obesity and impaired glucose homeostasis.

2.  *$\beta$ arr1*. To explore the potential metabolic roles of  $\beta$ arr1 expressed by adipocytes, Pydi et al. (Pydi et al., 2020b) studied mutant mice lacking  $\beta$ arr1 selectively in adipocytes (adipo- $\beta$ arr1-KO mice). Unlike their HFD adipo- $\beta$ arr2-KO counterparts, HFD adipo- $\beta$ arr1-KO mice showed striking metabolic impairments including hyperglycemia, impaired glucose tolerance, and reduced insulin sensitivity. Also, in contrast to the HFD  $\beta$ arr2 mutant mice, the HFD adipo- $\beta$ arr1-KO mice did not display significant changes in body fat mass and total energy expenditure, as compared to their control littermates (Pydi et al., 2020b). Additional studies showed that mutant mice that overexpressed  $\beta$ arr1 in adipocytes were protected from HFD-induced metabolic deficits (Pydi et al., 2020b).

RNAseq and qRT-PCR studies showed that the expression levels of *Mstn* (gene encoding myostatin or short Mstn) and various other myogenic genes were significantly increased in brown adipose tissue (BAT), but not in WAT, of HFD adipo- $\beta$ arr1-KO mice. In contrast, the BAT expression levels of *Ucp1* and other thermogenic genes remained largely unaffected by the lack of  $\beta$ arr1. In agreement with the gene expression data, plasma Mstn levels were significantly elevated in adipo- $\beta$ arr1-KO mice (Pydi et al., 2020b).

Mstn, a member of the transforming growth factor- $\beta$  superfamily, is named after its ability

to suppress skeletal muscle growth (Lee, 2023). Lineage tracing studies have shown that BAT cells are derived from Myf5-positive precursor cells that also give rise to skeletal muscle cells (Sanchez-Gurmaches and Guertin, 2014), explaining why genetic manipulation of BAT cell function can activate the transcription of genes that are normally expressed by skeletal muscle cells.

Several lines of evidence suggest that *Mstn* can also modulate skeletal muscle-independent physiological functions. For example, *Mstn* treatment of mice can induce peripheral insulin resistance (Pydi et al., 2020b). Strikingly, chronic treatment of HFD adipo- $\beta$ arr1-KO mice with an anti-*Mstn* antibody led to significant improvements in glucose homeostasis including improved glucose tolerance and reduced blood glucose levels (Pydi et al., 2020b).

Biochemical studies with cultured mouse BAT cells demonstrated that nuclear  $\beta$ arr1 can form a complex with PPAR $\gamma$  (Pydi et al., 2020b). Additional mechanistic data indicated that  $\beta$ arr1 interacts with the PPAR $\gamma$ /RXR $\alpha$  complex in the nucleus of brown adipocytes, thus interfering with the ability of PPAR $\gamma$  to activate the *Mstn* promoter.

In sum, the data presented by Pydi et al. (Pydi et al., 2020b) indicate that  $\beta$ arr1 represents an important negative regulator of *Mstn* expression in BAT. The ability of nuclear  $\beta$ arr1 to alter gene expression profiles in BAT provides a striking example for the ability of nuclear  $\beta$ arr1 to regulate whole body glucose homeostasis via a non-conventional mechanism of action (Fig. 4). As discussed above, the activity of nuclear  $\beta$ arr1 has also been implicated in carcinogenesis and other important physiological functions. Additional studies are needed to address the question whether the ability of  $\beta$ arr1 to inhibit the expression of myogenic genes in BAT is regulated by the activity of specific GPCRs. In any case, the development of novel strategies to modulate the expression or activity of nuclear  $\beta$ arr1 may prove beneficial in the treatment of various human

diseases including metabolic disorders such as T2D.

#### *D. Pancreatic $\beta$ -Cells*

The islets of Langerhans of the endocrine pancreas contain multiple cell types including the insulin-producing  $\beta$ -cells which release insulin after food intake to lower blood glucose levels. Besides peripheral insulin resistance, impaired  $\beta$ -cell function plays a key role in the pathogenesis of T2D (Eizirik et al., 2020; Vetere et al., 2014). Due to the deleterious effects of chronically elevated plasma glucose and lipid levels,  $\beta$ -cell function and number continue to decline, and insulin production eventually becomes inadequate to maintain euglycemia (Eizirik et al., 2020; Vetere et al., 2014). For this reason, pancreatic  $\beta$ -cells are considered a prime target for the development of novel antidiabetic drugs.

An early study (Kong et al., 2010) supported the concept that  $\beta$ -arrestins play a role in regulating the release of insulin from pancreatic  $\beta$ -cells. Specifically, a knock-in mouse strain expressing a phosphorylation-deficient mutant version of the  $M_3$  muscarinic receptor showed impaired insulin secretion (Kong et al., 2010). Mechanistic data indicated that this deficit was most likely due to the absence of phosphorylation/ $\beta$ -arrestin-dependent coupling of  $\beta$ -cell  $M_3$  receptors to protein kinase D1 (Kong et al., 2010).

*1.  $\beta$ arr2.* Mutant mice lacking  $\beta$ arr2 selectively in pancreatic  $\beta$ -cells ( $\beta$ - $\beta$ arr2-KO mice) displayed several major metabolic impairments in vitro and in vivo (Zhu et al., 2017a). For example, in vitro studies demonstrated that glucose-stimulated insulin secretion (GSIS) was greatly reduced in islets from  $\beta$ - $\beta$ arr2-KO islets, as compared to control islets (Zhu et al., 2017a). Similar results were obtained with EndoC- $\beta$ H1 cells, an immortalized human pancreatic  $\beta$ -cell

line (Scharfmann et al., 2014), following siRNA-mediated knockdown of  $\beta$ arr2 expression. Electrophysiological studies indicated that  $\beta$ arr2 deficiency resulted in impaired glucose-stimulated  $\text{Ca}^{2+}$  entry into  $\beta$ -cells due to reduced activity of voltage-dependent  $\text{Ca}^{2+}$  channels, associated with a resulting decrease action potential firing frequency (Zhu et al., 2017a). In agreement with the outcome of the in vitro studies,  $\beta$ - $\beta$ arr2-KO mice showed striking metabolic deficits in vivo, in particular when the mutant mice were maintained on a HFD (Zhu et al., 2017a). In HFD  $\beta$ - $\beta$ arr2-KO, GSIS was dramatically reduced, resulting in hyperglycemia and impaired glucose tolerance.

Additional studies strongly suggested that the various deficits associated with the lack of  $\beta$ -cell  $\beta$ arr2 are caused by impaired activity of  $\beta$ -cell CAMKII, a multi-functional Ser/Thr protein kinase that promotes insulin secretion via phosphorylation of various signaling proteins (Dadi et al., 2014). Zhu et al. (Zhu et al., 2017a) observed that the biochemical, electrophysiological, and metabolic deficits caused by  $\beta$ -cell  $\beta$ arr2 deficiency are very similar to those displayed by a mouse strain expressing a dominant negative version of CAMKII selectively in  $\beta$ -cells (Dadi et al., 2014). Moreover, biochemical studies indicated that  $\beta$ arr2 is able to form a complex with CAMKII, thus facilitating CAMKII signaling in  $\beta$ -cells (Zhu et al., 2017a). Taken together, these observations strongly support the concept that  $\beta$ arr2 regulation of  $\beta$ -cell CAMKII activity is critical for proper  $\beta$ -cell function.

In agreement with the metabolic deficits observed with mice lacking  $\beta$ arr2 in  $\beta$ -cells, mutant mice that overexpressed  $\beta$ arr2 selectively in  $\beta$ -cells ( $\beta$ - $\beta$ arr2-OE mice) showed major metabolic improvements. For example, HFD  $\beta$ - $\beta$ arr2-OE mice displayed a striking increase in GSIS, leading to significantly improved glucose tolerance (Zhu et al., 2017a). Moreover, HFD  $\beta$ -

$\beta$ arr2-OE mice were resistant against most metabolic deficits associated with the consumption of a HFD (Fig. 4).

It remains to be explored whether the formation of  $\beta$ -cell  $\beta$ arr2/CAMKII complexes is regulated by signaling pathways activated by specific  $\beta$ -cell GPCRs. In any case, the striking phenotypes displayed by  $\beta$ -cell  $\beta$ arr2 mutant mice suggest the possibility that strategies capable of promoting  $\beta$ arr2/CAMKII interactions in  $\beta$ -cells may prove useful to restore euglycemia in T2D.

*2.  $\beta$ arr1 - Role in Sulphonylurea-Induced Insulin Secretion.* With one exception (see below), mutant mice lacking  $\beta$ arr1 selectively in pancreatic  $\beta$ -cells ( $\beta$ - $\beta$ arr1-KO mice) did not display any significant metabolic deficits when consuming regular chow (Barella et al., 2019). However, surprisingly, the ability of certain sulphonylurea drugs (SUs), including glibenclamide and tolbutamide, to promote insulin secretion was significantly reduced in  $\beta$ - $\beta$ arr1-KO mice (Barella et al., 2019). Previous studies have shown that glibenclamide, tolbutamide, and various other SUs can bind to and activate Epac2 in  $\beta$ -cells, thus contributing to SU-mediated insulin secretion (Zhang et al., 2009). Epac2 is a cAMP binding protein that promotes the activation of Rap1 by acting as a guanine nucleotide exchange factor, a process known to facilitate trafficking of insulin granules to the plasma membrane (Shibasaki et al., 2007). Insulin release studies carried out in the presence of a specific Epac2 inhibitor suggested that the presence of  $\beta$ arr1 is required for the proper function of Epac2 in  $\beta$ -cells (Barella et al., 2019). Moreover, the lack of  $\beta$ -cell  $\beta$ arr1 interfered with the ability of glibenclamide to stimulate Rap1 activation.

Co-immunoprecipitation assays revealed the existence of a  $\beta$ arr1/Epac2 complex in cultured mouse  $\beta$ -cells and demonstrated that glibenclamide is able to promote the formation of

this complex (Barella et al., 2019). Pull-down assays with purified proteins showed that  $\beta$ arr1 is capable of binding to Epac2 in a direct fashion. In sum, these observations strongly suggest that certain SUs, including glibenclamide and tolbutamide, promote the formation of a  $\beta$ arr1/Epac2 complex in  $\beta$ -cells which in turn promotes Rap1 activation and enhanced insulin exocytosis (Fig. 4). The development of strategies that can promote or stabilize  $\beta$ arr1/Epac2 interactions in  $\beta$ -cells may prove useful for stimulating insulin release for therapeutic purposes.

*3.  $\beta$ arr1 - Role in  $\beta$ -Cell Mass Expansion During Obesity.* It is well known that obesity promotes  $\beta$ -cell hypertrophy and proliferation, resulting in an increase in  $\beta$ -cell mass (Aguayo-Mazzucato and Bonner-Weir, 2018; Golson et al., 2010; Sachdeva and Stoffers, 2009). Before the development of overt T2D, this increase in  $\beta$ -cell mass can maintain euglycemia despite peripheral insulin resistance (Sachdeva and Stoffers, 2009). Strikingly, Barella et al. (Barella et al., 2021) recently demonstrated that  $\beta$ -cell  $\beta$ arr1 plays a critical role in obesity-induced  $\beta$ -cell mass expansion. Specifically, HFD  $\beta$ - $\beta$ arr1-KO mice ( $\beta$ - $\beta$ arr1-KO mice) showed a pronounced reduction in  $\beta$ -cell mass due to greatly reduced  $\beta$ -cell proliferation, as compared to HFD control mice. As a result, insulin content was reduced by ~50% in islets from HFD  $\beta$ - $\beta$ arr1-KO mice. In agreement with these findings, HFD  $\beta$ - $\beta$ arr1-KO mice showed pronounced metabolic deficits in vivo, including hyperglycemia, glucose intolerance, and greatly reduced GSIS.

Western blotting studies showed that the expression of Pdx1 was significantly decreased in the absence of  $\beta$ -cell  $\beta$ arr1 (HFD  $\beta$ - $\beta$ arr1-KO mice) (Barella et al., 2021). In the mature endocrine pancreas, Pdx1 acts as a key transcription factor required for the maintenance of proper  $\beta$ -cell function and for the increase in  $\beta$ -cell mass triggered by peripheral insulin resistance (Brissova et al., 2005; Kulkarni et al., 2004). In agreement with this finding,



overexpression of *Pdx1* in islets from HFD  $\beta$ -*barr1*-KO mice restored control-like GSIS (Barella et al., 2021). Interestingly, siRNA-mediated knockdown of *BARR1* expression in cultured human  $\beta$ -cells (EndoC- $\beta$ H1 cells) also resulted in a significant down-regulation of *PDX1* expression. This effect was accompanied by an almost complete loss in GSIS, suggesting that *barr1* regulates similar cellular function in mouse and human  $\beta$ -cells (Barella et al., 2021).

Expression of a *Pdx1* promoter construct in cultured mouse  $\beta$ -cells showed that the presence of *barr1* is required for efficient transcription from the *Pdx1* promoter (Barella et al., 2021). Moreover, chromatin immunoprecipitation experiments with cultured mouse  $\beta$ -cells demonstrated that nuclear *barr1* promotes *Pdx1* gene expression, most likely by facilitating the formation of a complex with p300, a histone acetyltransferase, and other nuclear factors. In agreement with this finding, Kang et al. (Kang et al., 2005) reported previously that nuclear *barr1* can stimulate the transcription of various other genes via binding to p300.

Barella et al. (Barella et al., 2021) also analyzed mice that selectively overexpressed *barr1* in  $\beta$ -cells ( $\beta$ -*barr1*-OE mice). When maintained on a HFD,  $\beta$ -*barr1*-OE mice displayed phenotypic changes that were opposite to those observed with HFD  $\beta$ -*barr1*-KO mice. Islets prepared from HFD  $\beta$ -*barr1*-OE mice showed a significant increase in insulin content and  $\beta$ -cell mass, as compared to islets from WT littermates. Consistent with these findings, HFD  $\beta$ -*barr1*-OE mice displayed a pronounced increase in GSIS, reduced blood glucose levels, and improved glucose tolerance (Barella et al., 2021).

In sum, metabolic studies with  $\beta$ -cell-specific *barr1* mutant mice strongly suggest that nuclear *barr1* plays an important role in regulating transcriptional processes required for  $\beta$ -cell mass expansion and replication under conditions of metabolic stress (Fig. 4). This observation

raises the possibility that agents that are able to promote the translocation of  $\beta$ arr1 into the nucleus may prove useful to stimulate  $\beta$ -cell replication in T2D and related disorders.

### *E. Skeletal Muscle*

Skeletal muscle (SKM) is the major tissue responsible for insulin-induced glucose disposal and utilization (Merz and Thurmond, 2020). Moreover, the inability of SKM to properly respond to insulin is considered the primary defect in the progression to T2D (DeFronzo and Tripathy, 2009). To explore the role of  $\beta$ -arrestins in regulating SKM function in the context of glucose homeostasis, Meister et al. (Meister et al., 2019) generated and analyzed mutant mice that lacked  $\beta$ arr1 and/or  $\beta$ arr2 selectively in SKM tissues. Somewhat surprisingly, SKM  $\beta$ arr1 and/or  $\beta$ arr2 deficiency had little or no effect on modulating whole body glucose homeostasis, SKM insulin sensitivity, and exercise performance (Meister et al., 2019). These findings do not rule out the possibility that  $\beta$ -arrestins may modulate other SKM functions under different experimental conditions (see the following paragraph).

Clenbuterol is a  $\beta$ 2-AR agonist that is used by some bodybuilders to enhance SKM mass due to its anabolic effects (Spiller et al., 2013). Interestingly,  $\beta$ arr1 deficiency reduced the increase in SKM mass and strength observed after chronic clenbuterol treatment of WT mice, suggesting that  $\beta$ arr1 plays a key role in mediating  $\beta$ 2AR-dependent SKM growth and strength (Kim et al., 2018). In agreement with this finding, chronic treatment of mice with carvedilol, a  $\beta$ -arrestin-biased  $\beta$ 2-AR agonist, was able to enhance the contractile force of SKM in a  $\beta$ arr1-dependent fashion (Kim et al., 2020). Clearly, these observations are translationally relevant for the development of novel classes of drugs useful for the treatment of various diseases characterized by SKM wasting.

### *F. AgRP Neurons*

Neurons contained within the arcuate nucleus of the hypothalamus (ARC) play key roles in regulating appetite as well as glucose and energy homeostats (Deem et al., 2022). Agouti-related peptide (AgRP) neurons of the ARC are best known for their role in stimulating food intake due to their ability to synthesize and release several appetite-inducing agents, including AgRP, NPY, and GABA (Deem et al., 2022). Modulation of the activity of AgRP neurons can also lead to altered peripheral glucose metabolism and carbohydrate utilization independent of the orexigenic activity of these neurons (Cavalcanti-de-Albuquerque et al., 2019; Steculorum et al., 2016). Like other cell types, AgRP neurons express numerous GPCRs which, following their activation by agonist ligands, are likely to recruit  $\beta$ -arrestins (Cowley et al., 2003; Nakajima et al., 2016; Ren et al., 2012).

To investigate the potential physiological relevance of the two  $\beta$ -arrestins expressed by AgRP neurons, Pydi et al. generated mutant mice that lacked  $\beta$ arr1 or  $\beta$ arr2 selectively in AgRP neurons (Pydi et al., 2020a). Elimination of  $\beta$ arr2 in AgRP neurons had no detectable effect on whole body glucose homeostasis, insulin sensitivity, and several other metabolic parameters, independent of the diet that the mice consumed (regular chow or HFD) (Pydi et al., 2020a). In contrast, mutant mice lacking  $\beta$ arr1 selectively in AgRP neurons (AgRP- $\beta$ arr1-KO mice) displayed significant deficits in glucose tolerance, enhanced hepatic glucose production (HGP), and impaired insulin sensitivity when maintained on a HFD. Interestingly, these metabolic phenotypes were not observed after surgical dissection of the hepatic branch of the vagus nerve, suggesting that the lack of  $\beta$ arr1 in AgRP neurons enhances vagal outflow to the liver, resulting in increased HGP (Pydi et al., 2020a). Metabolic studies also suggested that  $\beta$ arr1 expressed by

AgRP neurons suppresses the activity of a neuronal pathway that causes the sympathetic activation of adipose tissue, explaining why plasma FFA levels were significantly increased in HFD AgRP- $\beta$ arr1-KO mice.

It is well known that insulin hyperpolarizes AgRP neurons, resulting in various metabolic changes including the suppression of vagus-mediated HGP (Huang et al., 2018c; Konner et al., 2007; Steculorum et al., 2016). Strikingly, insulin was unable to hyperpolarize AgRP neurons lacking  $\beta$ arr1 (Pydi et al., 2020a). Additional electrophysiological studies showed that  $\beta$ arr1 is required for proper insulin signaling in AgRP neurons upstream of PI3 kinase, most likely at the level of IRS-1 (Pydi et al., 2020a) (Fig. 4). However, the precise molecular mechanisms underlying this  $\beta$ arr1 activity remain to be explored.

In contrast to HFD AgRP- $\beta$ arr1-KO mice, HFD mice that overexpressed  $\beta$ arr1 in AgRP neurons (AgRP- $\beta$ arr1-OE mice) showed beneficial metabolic outcomes, including improved glucose tolerance and insulin sensitivity, as compared to HFD control mice (Pydi et al., 2020a). This observation suggests that strategies aimed at enhancing  $\beta$ arr1 expression levels in AgRP neurons could prove clinically useful to treat impairments in glucose homeostasis.

In sum, these findings demonstrate that the lack of a single  $\beta$ -arrestin isoform in a single neuronal subpopulation can have striking effects on whole body glucose homeostasis. It should be of considerable interest to explore the potential roles of  $\beta$ arr1 and  $\beta$ arr2 in modulating the activity of other neuronal subpopulations known to regulate key metabolic functions.

## VIII. Targeting $\beta$ -Arrestins for Therapeutic Purposes

As shown in Fig. 1,  $\beta$ -arrestins consist of two cup-like domains. GPCRs engage residues on the concave side of both domains, while most non-receptor signaling partners are predicted to bind to the opposite side of the molecule. The C-terminal portion of  $\beta$ -arrestins contains binding sites for trafficking proteins such as clathrin and its adaptor AP2 (Fig. 1). This distinct pattern of functionally relevant arrestin regions should make it possible to independently manipulate the receptor-binding surface, the effector-binding side, or the elements that engage trafficking proteins.

#### *A. Biased GPCR Ligands*

The therapeutic potential of G protein- or arrestin-biased GPCR ligands has been discussed in several excellent review articles (Eiger et al., 2022; Peterson and Luttrell, 2017; Rajagopal et al., 2010b; Shonberg et al., 2014). Likewise, the mechanistic underpinnings of GPCR signaling bias and the potential caveats associated with the development of biased GPCR ligands as novel therapeutic agents have been reviewed in detail recently (Gurevich and Gurevich, 2020; Seyedabadi et al., 2022). Examples for the potential therapeutic use of G protein- or arrestin-biased GPCR ligands are given throughout this review.

#### *B. Other Small Molecules*

$\beta$ -Arrestins are multi-functional cytoplasmic proteins that are expressed by virtually all cell types. As a result, modulating  $\beta$ -arrestin function for therapeutic purposes appears to be a very challenging task. However, accumulating evidence suggests that different  $\beta$ -arrestin interfaces are involved in facilitating interactions with different intracellular signaling proteins (Chaturvedi et al., 2018; Shukla and Dwivedi-Agnihotri, 2020). This observation suggests that it might be

possible to develop small molecules that target distinct  $\beta$ -arrestin subdomains to modulate specific signaling cascades for therapeutic purposes. The potential feasibility of this approach is exemplified by the recent discovery of barbadin, a small molecule that inhibits the interaction of  $\beta$ -arrestins with the  $\beta$ 2-adaptin subunit of the clathrin adaptor protein AP2 via binding to  $\beta$ 2-adaptin (Beautrait et al., 2017). Since  $\beta$ 2-adaptin is a key component of clathrin-coated pits, barbadin interferes with the internalization of various GPCRs without affecting receptor-mediated  $\beta$ -arrestin recruitment (Beautrait et al., 2017). Studies with selected GPCRs demonstrated that barbadin treatment of cultured cells can affect the magnitude of activation and kinetics of distinct intracellular signaling cascades (Beautrait et al., 2017). Moreover, additional signaling studies involving the use of barbadin confirmed the concept that GPCR-mediated cAMP signaling persists after activation of  $G_s$ -coupled receptors (Beautrait et al., 2017), suggesting that GPCR endocytosis can promote GPCR signaling in certain cases.

### *C. Aptamers*

Recent studies suggest that the use of RNA aptamers (short sequences of artificial DNA or RNA that bind a specific target molecule) may also prove useful to target  $\beta$ -arrestins in a direct fashion (Chaturvedi et al., 2018). For example, a sophisticated screening strategy led to the identification of RNA aptamers that can bind  $\beta$ -arrestins with high affinity (Kotula et al., 2014). Several of these aptamers displayed pronounced selectivity for  $\beta$ arr2, as compared with  $\beta$ arr1, and were able to inhibit distinct intracellular signaling pathways in cultured cells (Kotula et al., 2014). Although this hypothesis has not been tested experimentally, it may be feasible to develop aptamers that target specific  $\beta$ -arrestin surfaces, thus modifying  $\beta$ -arrestin-mediated functions in a more targeted fashion. Clearly, studies in this area are of considerable therapeutic interest.

#### *D. Synthetic Intrabodies*

A recent study identified several antigen-binding fragments (Fabs) that were able to distinguish between  $\beta$ arr1 and  $\beta$ arr2 (Ghosh et al., 2017). Several of these Fabs selectively modulated the interaction of  $\beta$ -arrestins with clathrin and the ERK signaling cascade. One of the newly identified Fabs selectively interfered with  $\beta$ arr2-clathrin binding (Ghosh et al., 2017). An intrabody (an antibody that works within the cell) derived from this Fab strongly inhibited the agonist-dependent endocytosis of several GPCRs but did not interfere with GPCR-induced ERK signaling (Ghosh et al., 2017). These findings suggest the possibility that intrabodies that can selectively interfere with distinct  $\beta$ -arrestin functions may prove useful for the treatment of various diseases characterized by abnormal  $\beta$ -arrestin expression and/or function.

### **IX. Conclusion**

As discussed in this review article,  $\beta$ arr1 and  $\beta$ arr2 are involved in a very large number of physiological functions, and altered  $\beta$ arr1/2 expression levels or activity are predicted to play key roles in the pathogenesis of many important pathophysiological conditions. On the basis of these findings, it should be possible to design novel therapeutic strategies that target  $\beta$ -arrestins or their associated signaling molecules and networks for therapeutic purposes. The identification of  $\beta$ arr1/2-regulated signaling cascades that show increased or decreased activity in specific cell types or tissues should greatly aid this endeavor.

### **Authorship Contributions**

Wrote or contributed to the writing of the manuscript: Wess, Oteng, Rivera-Gonzalez, VV

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## Figure legends

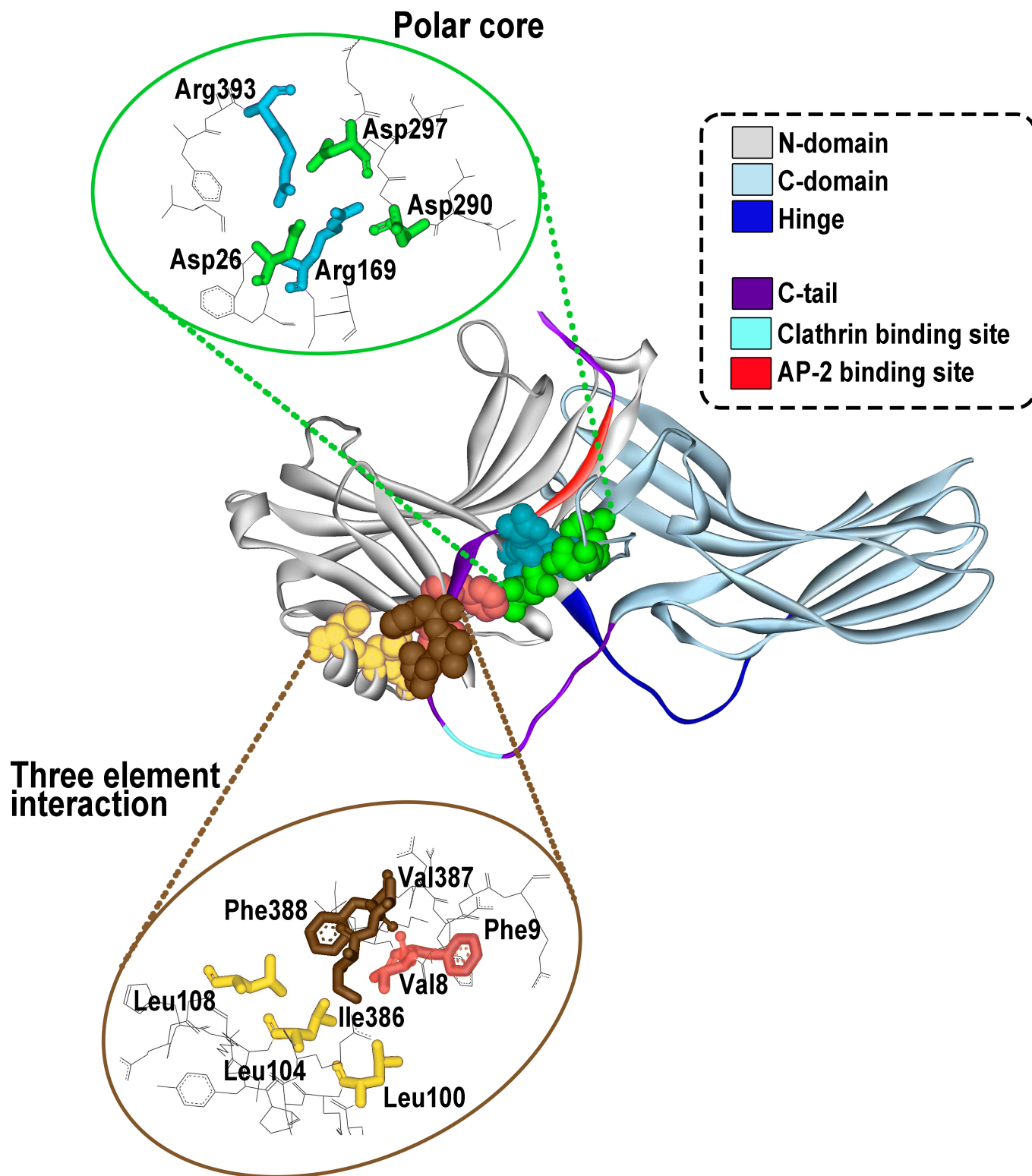
**Fig. 1.** Arrestin structure and important functional elements. All arrestins consist of the N-domain (grey), the C-domain (blue-grey), and the C-terminus (C-tail, magenta) that emerges from the C-domain and is anchored to the N-domain via the so-called three-element interaction and the polar core (side chains participating in these two interactions are shown as CPK models on the structure; their special arrangement is shown in the insets). The N- and C-domains are connected by a 12-residues hinge region (dark blue). The C-terminus contains binding sites for clathrin (light blue) and clathrin adaptor AP2 (red). The basal arrestin conformation is stabilized by two intramolecular interactions. One is the polar core, an arrangement of five charged side chains, two of which are supplied by the N-domain, two by the C-domain, and one by the C-terminus (upper inset). The other one is three-element interaction mediated by hydrophobic side chains of  $\beta$ -strand I and  $\alpha$ -helix I of the N-domain and  $\beta$ -strand XX of the C-terminus (lower inset). Both of these interactions are destabilized by GPCR binding, which results in the release of the C-terminus and twisting of the two domains relative to each other by  $\sim 20^\circ$ .

**Fig. 2.** Opioid receptor subtypes: signal transduction, agonist effects, and localization. (A) The three opioid receptor subtypes ( $\mu$ ,  $\kappa$ , and  $\delta$ ) are G protein-coupled receptors that primarily couple to the  $G_{i/o}$  subfamily of G proteins. The ligand-activated receptors are phosphorylated by G protein-coupled receptor kinases, resulting in the recruitment of  $\beta$ -arrestins (ARR), followed by receptor desensitization and/or internalization, and, most likely,  $\beta$ -arrestin-dependent modulation of intracellular signaling. (B) The analgesic effects of  $\mu$ ,  $\kappa$ , and  $\delta$  opioid receptor agonists are usually accompanied by a series of side effects. The potential involvement of  $\beta$ -arrestin-dependent signaling in mediating these side effects is currently a hotly debated issue (Gurevich and Gurevich, 2020). (C) A simplified diagram of the ascending pain pathway [for details see (De Ridder et al., 2021; Wang et al., 2022a)] showing the brain regions with the highest expression levels of the three opioid receptor subtypes (red circles,  $\mu$ ; blue,  $\kappa$ ; green,  $\delta$ ). Although the striatum (CPu and NAc) is technically not part of the pain pathway, this brain region expresses high levels of  $\mu$  and  $\kappa$  receptors. Hipp, hippocampus; VP, ventral posterior nucleus of the thalamus; Amg, amygdala; CPu, caudate-putamen; NAc, nucleus accumbens;

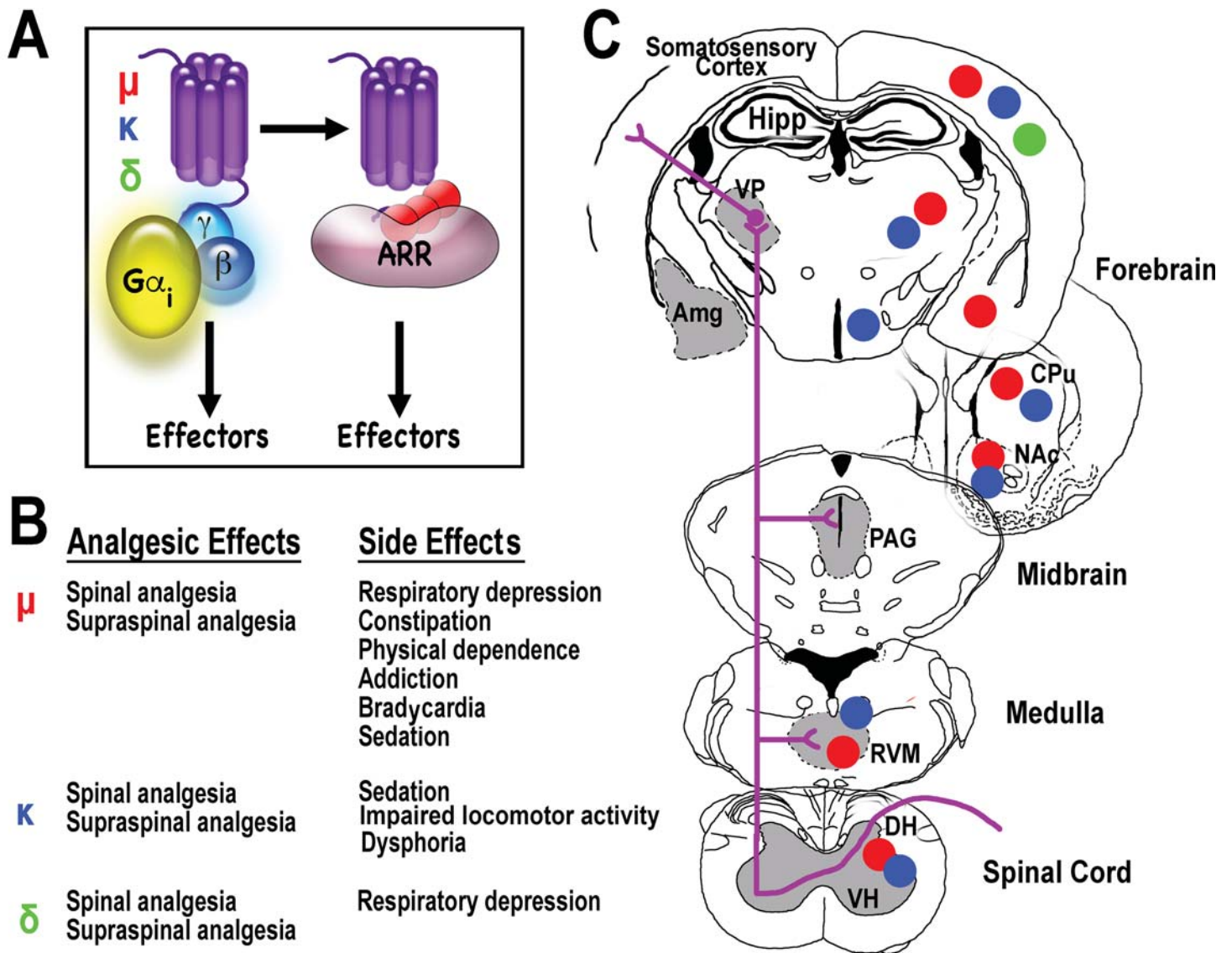
PAG, periaqueductal grey; RVM, rostroventral medulla; DH, dorsal horn of the spinal cord; VH, ventral horn of the spinal cord.

**Fig. 3.**  $\beta$ -arrestins play important roles in regulating carcinogenic processes. The cancer types shown in this figure are briefly discussed in the text. In most but not all cases,  $\beta$ -arrestins contribute to tumor formation by stimulating processes that promote cell proliferation. See text for details.

**Fig. 4.** Metabolic roles of  $\beta$ arr1 and  $\beta$ arr2 in metabolically important cell types. The overview given in this figure is based on data obtained with mutant mice selective lacking or overexpressing either of the two  $\beta$ -arrestins in hepatocytes, adipocytes, pancreatic  $\beta$ -cells, or AgRP neurons of the arcuate nucleus of the hypothalamus (Pydi et al., 2022).



**Fig. 1**



**Fig. 2**

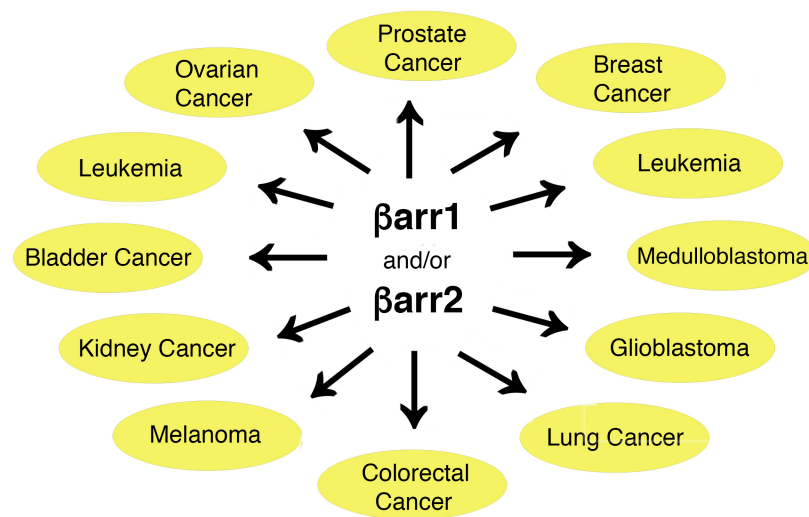


Fig. 3

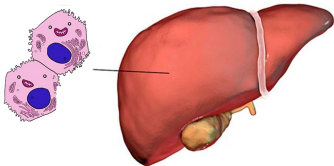
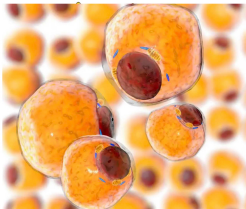
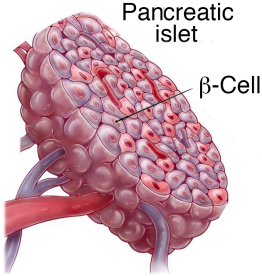
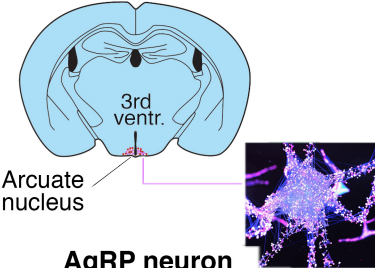
Cell type	Metabolic role of $\beta arr1$	Metabolic role of $\beta arr2$
 <b>Hepatocytes</b>	Unknown <i>Zhu et al., 2017</i>	Suppression of GCGR signaling  Maintenance of euglycemia  <i>Zhu et al., 2017</i>
 <b>Adipocytes</b>	Suppression of the expression of myogenic genes in BAT  Maintenance of proper glucose tolerance and insulin sensitivity  <i>Pydi et al., 2020b</i>	Inhibition of signaling via $\beta$ -ARs ( $\beta 3$ in the mouse)  Regulation of energy and glucose homeostasis  <i>Pydi et al., 2019</i>
 <b><math>\beta</math>-Cells of the endocrine pancreas</b>	Enhancement of insulin release by promoting the function of CAMKII  Protection against HFD-induced metabolic deficits  <i>Zhu et al., 2017a</i>	Increase in the activity of SU drugs via formation of $\beta arr1$ /Epac2 complex  <i>Barella et al., 2019</i>  Stimulation of $\beta$ -cell proliferation during peripheral insulin resistance by promoting the expression of Pdx-1  <i>Barella et al., 2021</i>
 <b>AgRP neuron</b>	Essential for insulin-mediated hyperpolarization of AgRP neurons  Maintenance of glucose homeostasis  <i>Pydi et al., 2020a</i>	Unknown  <i>Pydi et al., 2020a</i>

Fig. 4