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Arrestin-2 and arrestin-3 differentially modulate locomotor responses and sensitization to amphetamine

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Abstract

Arrestins play a prominent role in shutting down signaling via G protein-coupled receptors. In recent years, a signaling role for arrestins independent of their function in receptor desensitization has been discovered. Two ubiquitously expressed arrestin isoforms, arrestin-2 and arrestin-3, perform similarly in the desensitization process and share many signaling functions, enabling them to substitute for one another. However, signaling roles specific to each isoform have also been described. Mice lacking arrestin-3 (ARR3KO) were reported to show blunted acute responsiveness to the locomotor stimulatory effect of amphetamine (AMPH). It has been suggested that mice with deletion of arrestin-2 display a similar phenotype. Here we demonstrate that the AMPH-induced locomotion of male ARR3KO mice is reduced over the 7-day treatment period and during AMPH challenge after a 7-day withdrawal. The data are consistent with impaired locomotor sensitization to AMPH and suggest a role for arrestin-3-mediated signaling in the sensitization process. In contrast, male ARR2KO mice showed enhanced early responsiveness to AMPH and the lack of further sensitization, suggesting a role for impaired receptor desensitization. The comparison of mice possessing one allele of arrestin-3 and no arrestin-2 with ARR2KO littermates revealed reduced activity of the former line, consistent with a contribution of arrestin-3-mediated signaling to AMPH responses. Surprisingly, ARR3KO mice with one arrestin-2 allele showed significantly reduced locomotor responses to AMPH combined with lower novelty-induced locomotion, as compared to the ARR3KO line. These data suggest that one allele of arrestin-2 is unable to support normal locomotor behavior due to signaling and/or developmental defects.

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Author contribution

LZ, KS, and MRA conducted the behavioral experiments and acquired the data. MRA was primarily responsible for breeding the mice, with participation of LZ and KS. EVG analyzed the data and wrote the draft of the paper. EVG and VVG designed and directed the experiments, wrote the final version (with input from LZ) and critically revised the article.

Conflict of interest

The authors declare no conflict of interest.

Graphical abstract

Keywords

amphetamine; locomotor sensitization; arrestin; receptor desensitization; arrestin-mediated signaling

1. Introduction

Signaling via G protein-coupled receptors (GPCRs) is controlled by a desensitization mechanism that starts with activation-dependent receptor phosphorylation by a G protein-coupled receptor kinase (GRK) followed by the binding of arrestin, which precludes further signaling via G proteins and induces receptor internalization (Carman and Benovic, 1998). The internalized receptor can either be recycled back to the plasma membrane or targeted for degradation, which leads to receptor down-regulation (Moore et al., 2007). The two ubiquitous non-visual arrestin isoforms, arrestin-2 and arrestin-3¹, apparently act on multiple GPCRs. The rate and extent of GPCR desensitization, and consequently, the intensity of the G protein-mediated signaling is sensitive to the concentrations of arrestins in cells. Studies in cultured cells and living animals consistently demonstrate that overexpression of arrestins facilitates receptor desensitization and suppresses G protein-dependent signaling, whereas reduced arrestin concentration impedes desensitization and enhances G protein-mediated signaling (Ahn et al., 2003, Kohout et al., 2001, Violin et al., 2008, Vroon et al., 2004a, Vroon et al., 2004b). In addition to their role in GPCR desensitization, arrestins act as signaling molecules capable of modulating the activity of multiple signaling pathways in a G protein-independent manner (Gurevich and Gurevich, 2006). Both non-visual arrestins activate the MAP kinases ERK1/2 (Coffa et al., 2011a, Coffa et al., 2011b, Luttrell and Miller, 2013, Luttrell et al., 2001) and p38 (Bruchas et al., 2007, Bruchas et al., 2006). Furthermore, arrestin-3 is able to activate JNK3 (Kook et al., 2014, McDonald et al., 2000, Seo et al., 2011). The list of arrestin binding partners is quite extensive and includes hundreds of signaling proteins (Xiao et al., 2007). Therefore, arrestins are major multifunctional regulatory proteins positioned at the crossroads of many critical signaling pathways (Gurevich and Gurevich, 2006). This suggests that arrestins are likely to play a critical role in the control of responsiveness to psychotropic drugs that directly or indirectly act via GPCRs.

¹Here we use the systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called “arrestin 3” in the HUGO database).

Studies in mice lacking particular arrestin isoforms have demonstrated a reduced locomotor response of arrestin-3 knockout mice (ARR3KO) to acute challenge with the psychostimulant drugs cocaine and amphetamine (AMPH) (Beaulieu et al., 2005). Similarly, ARR3KO mice show a diminished locomotor response to acute treatment with morphine (Bohn et al., 2003, Urs et al., 2011). Lack of arrestin-3 does not seem to affect the rewarding properties of morphine or cocaine (Urs et al., 2011), although originally an increased rewarding effect of morphine, but not cocaine, in these mice had been reported (Bohn et al., 2003). It remains unknown, however, how ARR3KO mice respond to chronic treatment with psychostimulant drugs. So far, no consistent differences in the responsiveness of mice lacking the arrestin-2 isoform to acute or chronic treatment with drugs of abuse have been reported. This is somewhat puzzling considering that arrestin-2 is the major arrestin isoform that is expressed at 10–20-fold higher levels than arrestin-3 in most brain regions (Bychkov et al., 2011, Bychkov et al., 2008, Gurevich et al., 2002, Gurevich et al., 2004). Arrestin-2 and arrestin-3 share many functional properties, can fulfill the same functions and can take over each other's duties, although important differences have also been reported (Kohout et al., 2001). This notion is further supported by the fact that double KO is embryonically lethal, whereas mice lacking either arrestin isoform are grossly normal (Kohout et al., 2001). In all cases where the affinity of arrestin for various binding partners has been measured, arrestin-3 demonstrated higher affinity than arrestin-2, although both isoforms are capable of binding most partners (Ahmed et al., 2011, Coffa et al., 2011a, Coffa et al., 2011b, Goodman et al., 1996, Song et al., 2009, Song et al., 2006).

To shed light on the specific functional responsibilities of each arrestin isoform in regulating responsiveness to psychostimulant drugs, we studied the AMPH-induced locomotor behavior in mice lacking arrestin-2 or arrestin-3 by chronic AMPH. Additionally, we examined psychostimulant-induced locomotion in mice with only one allele of each arrestin isoform to assess the effect of gene dosage. We examined the behavior of these mice in the locomotor sensitization paradigm with repeated administration of AMPH in order to gain insight into the plastic changes in the signaling pathways that underlie the chronic effects of psychostimulants. Many drugs of abuse produce behavioral sensitization that is seen as an increased behavioral effect of the drug (Itzhak and Martin, 1999, Valjent et al., 2010, Vanderschuren and Kalivas, 2000). A theory positing that a sensitization-based mechanism termed “incentive-sensitization” underlies addiction (Robinson and Berridge, 1993, Robinson and Berridge, 2001, 2008) has become very influential. A large body of evidence suggests that the neural substrate of psychomotor sensitization involves the mesolimbic dopaminergic system and the connected circuits referred to as “the motive circuit” (Pierce and Kalivas, 1997), and that the sensitization mechanisms play an important role in the initiation of the addiction process and in relapse (Vanderschuren and Kalivas, 2000, Vanderschuren and Pierce, 2010). Our data demonstrate specific roles for each arrestin isoform in regulating the behavioral responses to psychostimulants.

2. Methods

2.1 Animals and tissue preparation

All animal procedures strictly followed the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Arrestin-2 (ARR2KO) and ARR3KO mice were kindly provided by Dr. R. J. Lefkowitz (Duke University). The animals were housed at the Vanderbilt University animal facility with a 12/12 h light/dark cycle and free access to food and water. Mice were bred using heterozygous breeding pairs to obtain KO and wild type (WT) littermates. To maintain genetic homogeneity, mice were consistently backcrossed to WT C57Bl mice purchased from Charles River. To minimize background genetic differences between the arrestin-2 and arrestin-3 lines, the same WT mice were used for backcrosses in both lines. The single allele (SA) mice (mice that have only one allele of one arrestin, ARR2+/- ARR3-/- and ARR2-/- ARR3+/-) were initially produced by crossing double heterozygous ARR2+/-ARR3+/- mice. To increase the yield of the correct genotype, later breeding was performed using SA mice bred with appropriate KO mice. This breeding scheme produced no WT littermates but increased the theoretical yield from 12.5% to 50%. KO mice used for breeding were derived from the general knockout population. As a result, ARR2+/-ARR3-/- (ARR3KO/ARR2HET) mice were produced with ARR3KO littermates and ARR2-/-ARR3+/- (ARR2KO/ARR3HET) with ARR2KO littermates.

2.2. Drug Treatment and Locomotor activity Measurements

Locomotor activity was measured in open field chambers (Med Associates Inc., Fairfax, VT) equipped with Activity software. The data were collected in 5-min intervals (bins). The activity was first measured for 30 min before the injection of amphetamine (AMPH). Following pre-drug testing, the mice received 3 mg/kg of AMPH i.p. and were immediately placed back into the apparatus and tested for AMPH-induced locomotor activity for 90 min. The testing was performed daily for 7 days. After that, the mice were withdrawn from the drug for 7 days and retested again with the same drug dose on Day 15.

2.3. Data Analysis

Statistical analysis of the data was performed using StatView software (SAS Institute, Cary, NC). The locomotor activity data were analyzed separately for each KO line by two-way repeated measure ANOVA with Genotype (KO versus WT littermates) as between group and Testing Day as within group factor. In case of SA mice, the comparison was performed between the SA mice and the corresponding KO littermates. The performance of different genotype groups was compared on each test day separately by the Student t-test, where appropriate. Alternatively, locomotor activity on a testing day was analyzed by two-way repeated measure ANOVA with Genotype (KO versus WT littermates or SA versus KO littermates) as between group and 5-min Bin as within group factor. Where appropriate, post hoc comparison of means was performed with the Bonferroni/Dunn test with correction for multiple comparisons. In all cases, $p < 0.05$ was considered significant.

3. Results

3.1. Loss of arrestin-2 and arrestin-3 differentially affects amphetamine-induced locomotion

Administration of AMPH led to an increase in locomotor activity in mice of all strains. When the AMPH-induced activity was analyzed across bins for the entire 90 min period, its level was significantly higher in ARR2KO mice as compared to WT littermates (Fig. 1A), whereas in ARR3KO mice it was significantly lower (Genotype effect $p=0.0159$) (Fig. 1B). In ARR2KO mice, the factor of Genotype was significant across testing days (the Challenge day excluded) ($F(1,112)=5.808$, $p=0.0283$). However, of the individual testing days, the elevation was significant only on Days 1 and 2 (Fig. 1A). There were no significant differences in the baseline locomotion (before AMPH injection) among genotypes (Fig. 1A,B).

The AMPH-induced locomotor activity in ARR2KO mice was high on the first days of testing and did not increase any further ($p=0.29$ by repeated measure with Day as repeated measure factor including Challenge day), suggesting that no sensitization had taken place. In contrast, in the WT group, repeated AMPH administration caused progressively increased locomotion (Effect of Day $p=0.0061$). Furthermore, on the Challenge day, the locomotor activity of WT mice was significantly higher than on the last (7th) day of AMPH administration, whereas in the ARR2KO group this was not observed (Fig. 1A). In contrast to ARR2KO mice, mice lacking arrestin-3 demonstrated reduced locomotor response to AMPH across testing days with the difference most evident starting from Day 4 through the Challenge day (Fig. 1B). When compared individually, the difference between the genotypes was significant on all testing days except Days 1 through 3. This argues for deficient sensitization to AMPH in these mice as compared to WT. However, upon repeated AMPH administration, their locomotion progressively increased at a rate similar to that of WT (Genotype $\times$ DAY interaction $p=0.5$), and the DAY factor was significant in both the WT and ARR3KO groups when analyzed separately for each genotype ($p<0.001$ for both) (Fig. 1B). Furthermore, both genotypes showed significantly increased locomotion on the Challenge day as compared to the 7th day of AMPH treatment (Fig. 1B).

Analysis of the total locomotor activity on individual testing days failed to reveal hyperactivity of ARR2KO mice beyond testing Day 2 (Fig. 1A). However, when performance on each testing day was analyzed separately by two-way repeated measure ANOVA with Bins as a within group factor, it was revealed that ARR2KO mice were not only significantly hyperactive on Day 1 (Genotype $p=0.0401$; Genotype $\times$ Bin $p=0.0042$) but remained so through testing Day 5 (Genotype $p=0.0483$) (Fig. 2A,B), albeit the difference on Day 5 was much less pronounced than on Day 1. On the Challenge Day, ARR2KO mice did not differ from their WT littermates (Fig. 2C). There were no significant differences in the baseline locomotion between ARR2KO and WT mice on any testing day.

In contrast, ARR3KO mice showed no significant reduction in the total AMPH-induced locomotion on Day 1 as compared to WT littermates (Genotype $p=0.43$) (Fig. 2A). However, there was a significant Genotype $\times$ Bin interaction for ARR3KO ($p=0.0166$) on Day 1 due to a small peak of AMPH-induced locomotion, suggesting that the loss of

arrestin-3 led to a subtle early hypoactivity in response to AMPH. ARR3KO mice became significantly hypoactive from Day 5 ($p<0.001$) and remained so through Day 7 ($p<0.05$) (Fig. 2B,C). They also demonstrated a significantly lower level of AMPH-induced locomotion on Challenge Day as compared to WT littermates ($p=0.03$, Fig. 2D). There were no significant genotype differences in the baseline performance (Fig. 1B).

3.2. Complex interactive effects of arrestin-2 and arrestin-3 on the amphetamine-induced locomotion

In order to further examine the role of arrestin isoforms in regulating the psychostimulating effect of AMPH, we tested AMPH-induced locomotion in SA mouse lines in comparison with the corresponding KO lines carrying two alleles of the remaining arrestin gene. Analysis of the AMPH-induced locomotion across treatment days (collapsed across bins for the 90-min sessions) revealed no significant differences between ARR2KO and ARR2KO mice also lacking one arrestin-3 allele (A2KO/A3HET) ($p=0.18$) (Fig. 3A). Their performance on the Challenge day was also identical. However, ARR3KO mice hemizygous for ARR2 (A3KO/A2HET) demonstrated a significantly reduced locomotor response to AMPH across testing days ($p=0.0129$), from Day 1 through Day 7, as well as with the inclusion of the Challenge day ($p=0.0147$). The locomotor response was sensitized in these mice as well as in ARR3KO mice by repeated AMPH administration, as evidenced by a progressive increase in the locomotor activity (Day effect $p<0.001$) and increased locomotion on the Challenge day as compared to Day 7 of treatment (Fig. 3A).

Interestingly, ARR3KO/ARR2HET mice also exhibited reduced basal locomotor activity during pre-testing before AMPH administration. Fig. 3B presents data on the basal locomotion for all genotypes collapsed across 5-min bins for the entire 30 min pre-testing period for each experimental day plus the Challenge day. As shown in Fig 3B, ARR3KO/ARR2HET mice have reduced basal locomotion throughout the experimental period as compared to ARR3KO littermates ($p=0.002$). In contrast, ARR2KO/ARR3HET mice did not differ from their ARR2KO littermates in basal locomotion (Fig. 3B). As seen in the graph, ARR3KO, ARR2KO, and ARR2KO/ARR3HET had similar basal locomotor activity, although no formal comparison could be made because these genotypes (ARR3KO versus ARR2KO, and ARR2KO/ARR3HET versus ARR3KO/ARR2HET) came from different breeding lines.

Although ARR2KO/ARR3HET mice did not differ from ARR2KO in the overall AMPH-induced locomotion across testing days, analysis of their locomotor activity on Day 1 by 5-min bins showed them to be significantly less active across bins than ARR2KO (Genotype $p=0.0447$; Genotype $\times$ Bin $p=0.0021$) (Fig. 4A). On subsequent testing days, the effect of Genotype was no longer significant, but the Genotype $\times$ Bin interaction remained significant ($p=0.0065$ for Day 5, Fig. 4B) due to a faster decline in locomotion after the AMPH injection in ARR2KO/ARR3HET mice.

ARR3KO/ARR2HET mice were grossly hypoactive in response to AMPH as compared to ARR3KO on Day 1 across bins (Genotype $p=0.0159$), hardly registering any locomotor response to AMPH at all (Fig. 4A). The graph also illustrates the basal hypoactivity (without AMPH) in these mice (Genotype $p=0.0138$), which was particularly evident at the start of

the testing session (Genotype $\times$ Bin for basal activity $p=0.0032$). On subsequent days, they remained significantly hypoactive following AMPH injection. For example, on Day 5 (Fig. 4B) they showed an overall reduction in activity across bins (Genotype $p=0.0344$), most pronounced during the peak response to AMPH (Genotype $\times$ Bin $p=0.0156$). There was no longer an overall reduction in the basal locomotion, although the Genotype $\times$ Bin interaction remained significant (for Day 5 $p=0.0186$) due to lower activity at the beginning of the testing session.

3. Discussion

Many drugs of abuse act, directly or indirectly, via G protein-coupled receptors (GPCRs). In particular, the reward mechanisms of the mesolimbic system are mediated by dopamine (DA) and the signaling of DA receptors is invariably involved. Agonist-occupied GPCRs activate their cognate G proteins before the response is reduced and eventually blocked by desensitizing mechanisms. The classic model of homologous desensitization of GPCRs posits that agonist-activated receptors are phosphorylated by GRKs, which converts them into targets for high-affinity arrestin binding (Carman and Benovic, 1998). Bound arrestin shields the cytoplasmic surface of the receptor, precluding further G protein activation (Kang et al., 2015, Rasmussen et al., 2007). Arrestin binding subsequently targets the receptor for internalization (Goodman et al., 1996, Laporte et al., 1999). Arrestins also redirect GPCR signaling from G protein-dependent to G protein-independent pathways via their interaction with numerous signaling molecules (Gurevich and Gurevich, 2006, Gurevich and Gurevich, 2014, Shukla et al., 2011). Two ubiquitously expressed arrestin isoforms, arrestin-2 and arrestin-3 (also known as β -arrestin1 and β -arrestin2), appear to regulate the signaling via hundreds of GPCRs (Gurevich and Gurevich, 2013). Thus, arrestins play a key role in regulating GPCR responsiveness to agonists via desensitization, as well as via the arrestin-mediated second round of signaling. The known molecular mechanisms of arrestin action suggest that these proteins also perform a critical function in the neuronal adaptations induced by the exaggerated signaling caused by drugs of abuse. However, information regarding the precise role of arrestins in the physiological processes associated with drug addiction is currently limited.

Psychostimulant drugs such as cocaine and AMPH enhance striatal dopaminergic neurotransmission, causing stimulation of the striatal dopamine receptors, and as a result, increased locomotor activity. There is ample evidence of the critical, albeit distinct, roles of the D1 and D2 dopamine receptors expressed on postsynaptic medium spiny striatal neurons in the behavioral effects of psychostimulant drugs [reviewed in (Baik, 2013, Johnson and Lovinger 2016)]. Earlier studies suggested that striatal D1 and D2 receptors were dispensable for the induction of locomotor sensitization to psychostimulants (Baik, 2013, Johnson and Lovinger 2016, Vanderschuren and Kalivas, 2000) while emphasizing the role of the ventral tegmental area in that process (Vanderschuren and Kalivas, 2000). However, recent experiments highlighted the role of the striatal dopaminergic mechanisms, particularly those mediated by D1 receptors, in the induction of sensitization (Ferguson et al., 2011, Gore and Zweifel, 2013, Hikida et al., 2010). The drug-induced plasticity of D2 autoreceptors has also been implicated in the expression of locomotor sensitization to psychostimulants (Vanderschuren and Kalivas, 2000). Both arrestin isoforms are ubiquitous

and are co-expressed, with few exceptions, in all areas of the brain (Gurevich et al., 2002, Gurevich et al., 2004). Interestingly, in most areas of the adult brain, including the striatum, substantia nigra and ventral tegmental area, arrestin-2 is 10–20 times more abundant than arrestin-3 (Gurevich et al., 2002, Gurevich et al., 2004). The preferential interaction in neostriatal neurons of arrestin-2 with the D2 and of arrestin-3 with the D1 dopamine receptor has been reported (Macey et al., 2004, Macey et al., 2005). This selectivity does not appear to be due to differences in the arrestin expression level between the D2- and D1 receptor-bearing neurons, since the direct and indirect pathway striatal neurons express comparable levels of arrestin-2, and the same is true for arrestin-3 (Bychkov et al., 2013). Although the details of how different GPCRs expressed by the striatal or midbrain dopaminergic neurons contribute to locomotor sensitization to AMPH remain unclear, both arrestin isoforms in multiple brain areas might participate in the signaling plasticity associated with the sensitization process.

If arrestins regulated the responsiveness to psychostimulants via their role in GPCR desensitization, then the loss of arrestins should be expected to cause delayed desensitization, i.e. overactivity of the dopamine receptors (all of which are GPCRs), and increased locomotor responsiveness to psychostimulants. However, previous studies have shown that the loss of arrestin-3 leads to reduced locomotor responses to the non-selective dopamine agonist apomorphine and to the psychostimulant amphetamine (Beaulieu et al., 2005, Gainetdinov et al., 2004). Mice lacking arrestin-3 display a blunted locomotor response to morphine, which is also mediated by increased dopamine release and the resulting enhanced stimulation of striatal dopamine receptors (Bohn et al., 2003, Urs et al., 2011). One discordant note is that the loss of arrestin-3 does not seem to alter the locomotor response to another psychostimulant drug, cocaine (Bohn et al., 2003, Gainetdinov et al., 2004). This may be explained by a relatively more prominent role of the cortical glutamatergic mechanisms (Steketee, 2005, Vanderschuren and Kalivas, 2000), which would not be directly impacted by the loss of arrestins, in the sensitization to cocaine. It is important to remember that the previous studies only examined the acute effects of psychostimulant drugs in arrestin knockout mice. Our data confirm the reduction in the acute locomotor response to AMPH in ARR3KO mice reported previously (Beaulieu et al., 2005). We extend these findings by showing that the difference between the ARR3KO mice and their WT littermates deepens with repeated drug administration, suggesting diminished sensitization of the locomotor response to AMPH. However, ARR3KO mice are not totally deficient in sensitization, since they did increase their locomotion with chronic treatment and upon AMPH challenge following a 7-day withdrawal period. This suggests that arrestin-3 is involved in the development, but not the expression, of AMPH sensitization. On the whole, the data point to the role of arrestin-3-mediated signaling, rather than arrestin-3-mediated receptor desensitization, in the dopamine-dependent behavioral effects of psychostimulants and other drugs of abuse. When the signaling is missing due to the absence of arrestin-3, the behavioral responsiveness to drugs is diminished.

It had been reported in an extensive review by Gainetdinov et al (Gainetdinov et al., 2004) that ARR2KO mice, similarly to ARR3KO mice, have reduced locomotor responsiveness to AMPH. In contrast, in the present study we found ARR2KO mice to be hypersensitive to the locomotor effect of AMPH, most evidently during the first days of treatment, with some

effect persisting for several sessions. The reason for this discrepancy with the previous findings is unclear. One possibility is that the previous study used both males and females, whereas here we tested males only. The mice did not demonstrate measurable sensitization of the locomotor response following the initial hyperactivity, which allowed WT animals to eventually catch up with them during the last treatment sessions and on the Challenge day. The behavior of ARR2KO mice is reminiscent of that of mice lacking GRK6, a G protein-coupled receptor kinase that phosphorylates activated GPCRs as the first step in the desensitization process (Gurevich et al., 2011), which were also supersensitive to psychostimulants (Gainetdinov et al., 2003). These findings appear more consistent with the classic role of arrestin-2 in receptor desensitization, when the loss of arrestin causes retarded desensitization, and as a result, enhanced signaling.

The arrestin isoforms are remarkably similar structurally and share many functional properties (Han et al., 2001, Milano et al., 2002, Zhan et al., 2011). Most GPCRs bind both arrestins, although the affinity of different receptors for arrestin-2 and arrestin-3 differ substantially (Gurevich et al., 1995, Kohout et al., 2001, Oakley et al., 2000). These structure-functional similarities result in the ability of arrestin isoforms to functionally substitute for one another to a degree, so that the absence of both arrestins is often required to severely compromise the signaling in cells (Cleghorn et al., 2015, Kohout et al., 2001). Furthermore, deletion of both arrestin isoforms is embryonically lethal (Kohout et al., 2001), whereas mice lacking only one of the arrestin isoforms are grossly normal. A significantly higher abundance of arrestin-2 as compared to arrestin-3 (Gurevich et al., 2002, Gurevich et al., 2004) suggests that arrestin-2 might be the major “housekeeping” isoform, whereas arrestin-3 is reserved for more specialized functions. Both arrestin isoforms are also capable of regulating most arrestin-dependent signaling pathways, although, again, differences in affinities for various binding partners have been noted (Ahmed et al., 2011, Beaulieu et al., 2005, Kohout et al., 2001, Macey et al., 2004, Macey et al., 2005). One notable exception is the ability of arrestin-3 to activate the JNK pathway, which arrestin-2 lacks (Kook et al., 2014, McDonald et al., 2000, Miller et al., 2001, Seo et al., 2011, Song et al., 2009, Zhan et al., 2015). Arrestin-2 does bind the components of the cascade; this does not, however, result in activation of the JNK3 kinase (Seo et al., 2011, Zhan et al., 2015). Although arrestin-3 is more often implicated in signaling (Luttrell and Miller, 2013, Shukla et al., 2011), arrestin-2 was shown to recruit c-Src to active GPCRs (Luttrell et al., 1999) and to facilitate the activation of ERK1/2 (Coffa et al., 2011b, Luttrell et al., 2001).

Double ARR2/ARR3 KO mice do not survive beyond embryonic day 12 (Kohout et al., 2001). Therefore, in order to gain insight into the specific roles of the arrestin isoforms, we investigated the responsiveness to AMPH of mice with only one allele of either arrestin-2 or arrestin-3 in comparison with their knockout littermates that have both alleles of the other arrestin. The loss of arrestin-3 causes reduced responsiveness to AMPH, and, predictably, ARR2KO mice also lacking one ARR3 allele (ARR2KO/ARR3HET) showed reduced locomotion as compared to the parental ARR2KO line. Although no direct comparison with WT was made, numerically these mice were quite close to WT. This means that the presence of just one allele of arrestin-3 was sufficient to support normal responsiveness to AMPH (Figs. 1 and 3).

However, the behavior of ARR3KO mice lacking one arrestin-2 allele (ARR3KO/ARR2HET) was counterintuitive: they were severely hypoactive following AMPH administration in comparison with the parental ARR3KO line. In fact, on the first day of treatment they hardly registered any locomotor response to AMPH at all. They also showed reduced basal locomotion, particularly at the beginning of the session, possibly indicative of reduced novelty-induced locomotion. These mice possess one allele of arrestin-2, which, in the absence of arrestin-3, proved unable to support normal basal locomotion or response to AMPH even at the level of ARR3KO mice. Conceivably, a certain level of arrestin activity is required for the proper functioning of the neuronal signaling pathways, during development and/or in the mature brain, to sustain normal locomotor responsiveness to various stimuli. One allele of arrestin-3 or a full complement of arrestin-2 is sufficient to fulfill those functions, whereas one allele of arrestin-2 is not.

In the context of this model, our study revealed an important role of the more abundant arrestin-2 in suppressing AMPH-induced locomotion (Figs. 1,2), suggesting that the acute effect of AMPH is largely mediated via G protein-dependent signaling (Fig. 5A,B). In ARR2KO mice, G-protein-mediated signaling is likely to be enhanced due to the lack of abundant arrestin-2 and, consequently, impeded receptor desensitization (Fig. 5B). Additionally, arrestin-mediated signaling might also be enhanced due to the elimination of the less efficacious arrestin-2 from the competition for signaling proteins (Fig. 5B). The lower AMPH-induced locomotor activity of ARR3KO animals suggests that the full locomotor response to AMPH requires arrestin-3-dependent signaling, which is largely lost in ARR3KO animals, since only the weaker signaling arrestin-2 is left to fulfill the signaling functions (Fig. 5C). Arrestin-3 interaction with the D2 dopamine receptor and PP2A, which results in the dephosphorylation and deactivation of Akt, and ultimately, in reduced Akt-dependent phosphorylation and increased activity of GSK β , was shown to be critical for the acute locomotor responsiveness to AMPH (Beaulieu et al., 2005). It is conceivable that this pathway plays a role in the blunted locomotor sensitization to AMPH as well. Reduction of locomotion in ARR2KO/ARR3HET mice, as compared to ARR2KO, is consistent with the role of arrestin-3-mediated signaling in AMPH-induced locomotion (Figs. 3,4). However, the lower activity of ARR3KO/ARR2HET mice in comparison to ARR3KO animals is not explained by this simplistic model, suggesting a more complex interaction between G protein- and arrestin-dependent signaling pathways, either in the acute responsiveness to AMPH or in the development of sensitization.

An alternative model can be proposed based exclusively on arrestin-mediated signaling (Fig. 5D–F). Although the affinity of non-visual arrestins for other signaling proteins has been measured in only a few cases, arrestin-3 invariably demonstrates higher affinity and stronger signaling capacity than arrestin-2 (Ahmed et al., 2011, Cleghorn et al., 2015, Oakley et al., 2000). For example, arrestin-3 scaffolds PP2A and Akt on the D2 dopamine receptor, mediating Akt dephosphorylation (Beaulieu et al., 2005). In the same study, a weaker binding of arrestin-2 to Akt was detected, suggesting that both isoforms could signal via the same pathway, albeit with different efficacy. Thus, if we only consider arrestin-mediated signaling in all animals, assuming that G protein-mediated signaling plays a minimal role in AMPH-induced locomotion (Fig. 5D), the locomotion should still be expected to increase in ARR2KO mice relative to WT, as in these animals arrestin-2, which is more abundant in the

brain (Gurevich et al., 2002, Gurevich et al., 2004), but less efficacious as a signaling mediator, does not compete with the stronger signaling mediator arrestin-3 (Fig. 5E). In contrast, in ARR3KO mice the locomotion is reduced, since arrestin-mediated signaling is driven solely by arrestin-2, which is weaker than arrestin-3 (Fig. 5F). This model predicts that ARR2KO/ARR3HET mice would be less mobile as compared to ARR2KO animals, since they have half the level of the only available arrestin, arrestin-3, resulting in reduced arrestin-mediated signaling, and consequently, suppressed behavioral response to AMPH. The same logic applies to ARR3KO/ARR2HET mice, suggesting that they would be even less mobile than ARR3KO animals, as the dose of arrestin-2, and therefore arrestin-2-mediated signaling, is reduced. Both predictions match experimental data (Figs. 3,4). Thus, the model that excludes the role of G protein-mediated signaling in AMPH-induced locomotion (Fig. 5D–F) logically explains all the data (Figs. 1–4).

The elucidation of the molecular mechanisms of arrestin-dependent changes in amphetamine-induced locomotion requires a better understanding of the biological roles of arrestins in both suppression and transduction of the GPCR signaling.

Conclusions

Our results demonstrate the contrasting consequences of the loss of arrestin-2 and arrestin-3 on AMPH-induced locomotion and locomotor sensitization. Mice lacking arrestin-2 are initially hyper-responsive to AMPH, which is consistent with either impaired receptor desensitization due to the absence of the major arrestin isoform, or the absence of a more abundant competitor of arrestin-3, which tends to serve as a more efficient signal transducer, at GPCRs. In contrast, mice lacking arrestin-3 are hyposensitive to AMPH and show poor locomotor sensitization, an effect consistent with the role of arrestin-3-mediated signaling in AMPH-induced locomotor behavior. Single-allele mice with knockout of arrestin-3 and one allele of arrestin-2 are grossly hyposensitive to AMPH and show significantly impaired novelty-induced locomotion, suggesting either developmental defects or a predominant role of arrestin-mediated signaling in amphetamine-induced locomotion.

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References

- Ahmed MR, Zhan X, Song X, Kook S, Gurevich VV, Gurevich EV. Ubiquitin ligase parkin promotes Mdm2-arrestin interaction but inhibits arrestin ubiquitination. *Biochemistry*. 2011; 50:3749–3763. [PubMed: 21466165]
- Ahn S, Nelson CD, Garrison TR, Miller WE, Lefkowitz RJ. Desensitization, internalization, and signaling functions of beta-arrestins demonstrated by RNA interference. *Proc. Natl. Acad. Sci. U S A*. 2003; 100:1740–1744. [PubMed: 12582207]
- Baik JH. Dopamine signaling in reward-related behaviors. *Front Neural Circuits*. 2013; 7:152. [PubMed: 24130517]

- Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell*. 2005; 122:261–273. [PubMed: 16051150]
- Bohn LM, Gainetdinov RR, Sotnikova TD, Medvedev IO, Lefkowitz RJ, Dykstra LA, Caron MG. Enhanced rewarding properties of morphine, but not cocaine, in beta(arrestin)-2 knock-out mice. *J. Neurosci.* 2003; 23:10265–10273. [PubMed: 14614085]
- Bruchas MR, Land BB, Aita M, Xu M, Barot SK, Li S, Chavkin C. Stress-induced p38 mitogen-activated protein kinase activation mediates kappa-opioid-dependent dysphoria. *J Neurosci.* 2007; 27:11611–11623.
- Bruchas MR, Macey TA, Lowe JD, Chavkin C. Kappa opioid receptor activation of p38 MAPK is GRK3- and arrestin-dependent in neurons and astrocytes. *J Biol Chem.* 2006; 281:18081–18089. [PubMed: 16648139]
- Bychkov E, Ahmed MR, Gurevich VV, JL B, Gurevich EV. Reduced expression of G protein-coupled receptor kinases in schizophrenia but not in schizoaffective disorder. *Neurobiol Dis.* 2011; 44:248–258. [PubMed: 21784156]
- Bychkov E, Zurkovsky L, Garret M, Ahmed MR, Gurevich EV. Distinct cellular and subcellular distribution of G protein-coupled receptor kinase and arrestin isoforms in the striatum. *PLoS One.* 2013; 7:e48912.
- Bychkov ER, Gurevich VV, Joyce JN, Benovic JL, Gurevich EV. Arrestins and two receptor kinases are upregulated in Parkinson's disease with dementia. *Neurobiol. Aging.* 2008; 29:379–396. [PubMed: 17125886]
- Carman CV, Benovic JL. G-protein-coupled receptors: turn-ons and turn-offs. *Curr Opin Neurobiol.* 1998; 8:335–344. [PubMed: 9687355]
- Cleghorn WM, Branch KM, Kook S, Arnette C, Bulus N, Zent R, Kaverina I, Gurevich EV, Weaver AM, Gurevich VV. Arrestins regulate cell spreading and motility via focal adhesion dynamics. *Mol Biol Cell.* 2015; 26:622–635. [PubMed: 25540425]
- Coffa S, Breitman M, Hanson SM, Callaway K, Kook S, Dalby KN, Gurevich VV. The effect of arrestin conformation on the recruitment of c-Raf1, MEK1, and ERK1/2 activation. *PLoS One.* 2011a; 6:e28723. [PubMed: 22174878]
- Coffa S, Breitman M, Spiller BW, Gurevich VV. A single mutation in arrestin-2 prevents ERK1/2 activation by reducing c-Raf1 binding. *Biochemistry.* 2011b; 50:6951–6958. [PubMed: 21732673]
- Ferguson SM, Eskenazi D, Ishikawa M, Wanat MJ, Phillips PE, Dong Y, Roth BL, Neumaier JF. Transient neuronal inhibition reveals opposing roles of indirect and direct pathways in sensitization. *Nat Neurosci.* 2011; 14:22–24. [PubMed: 21131952]
- Gainetdinov RR, Bohn LM, Sotnikova TD, Cyr M, Laakso A, Macrae AD, Torres GE, Kim KM, Lefkowitz RJ, Caron MG, Premont RT. Dopaminergic supersensitivity in G protein-coupled receptor kinase 6-deficient mice. *Neuron.* 2003; 38:291–303. [PubMed: 12718862]
- Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG. Desensitization of G protein-coupled receptors and neuronal functions. *Annu Rev Neurosci.* 2004; 27:107–144. [PubMed: 15217328]
- Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature.* 1996; 383:447–450. [PubMed: 8837779]
- Gore BB, Zweifel LS. Genetic reconstruction of dopamine D1 receptor signaling in the nucleus accumbens facilitates natural and drug reward responses. *J Neurosci.* 2013; 33:8640. [PubMed: 23678109]
- Gurevich EV, Benovic JL, Gurevich VV. Arrestin2 and arrestin3 are differentially expressed in the rat brain during postnatal development. *Neuroscience.* 2002; 109:421–436. [PubMed: 11823056]
- Gurevich EV, Benovic JL, Gurevich VV. Arrestin2 expression selectively increases during neural differentiation. *J. Neurochem.* 2004; 91:1404–1416. [PubMed: 15584917]
- Gurevich EV, Gurevich VV. Arrestins: ubiquitous regulators of cellular signaling pathways. *Genome Biol.* 2006; 7:236. [PubMed: 17020596]
- Gurevich EV, Tesmer JJ, Mushegian A, Gurevich VV. G protein-coupled receptor kinases: More than just kinases and not only for GPCRs. *Pharmacol. Ther.* 2011; 133:40–69. [PubMed: 21903131]

- Gurevich VV, Dion SB, Onorato JJ, Ptasienski J, Kim CM, Sterne-Marr R, Hosey MM, Benovic JL. Arrestin interaction with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, b2-adrenergic, and m2 muscarinic cholinergic receptors. *J Biol Chem.* 1995; 270:720–731. [PubMed: 7822302]
- Gurevich VV, Gurevich EV. Structural determinants of arrestin functions. *Prog Mol Biol Transl Sci.* 2013; 118:57–92. [PubMed: 23764050]
- Gurevich VV, Gurevich EV. Overview of different mechanisms of arrestin-mediated signaling. *Curr Protoc Pharmacol.* 2014; 67:1–9.
- Han M, Gurevich VV, Vishnivetskiy SA, Sigler PB, Schubert C. Crystal structure of beta-arrestin at 1.9 Å: possible mechanism of receptor binding and membrane translocation. *Structure.* 2001; 9:869–880. [PubMed: 11566136]
- Hikida T, Kimura K, Wada N, Funabiki K, Nakanishi S. Distinct roles of synaptic transmission in direct and indirect striatal pathways to reward and aversive behavior. *Neuron.* 2010; 66:896–907. [PubMed: 20620875]
- Itzhak Y, Martin JL. Effects of cocaine, nicotine, dizocipiline and alcohol on mice locomotor activity: cocaine-alcohol cross-sensitization involves upregulation of striatal dopamine transporter binding sites. *Brain Res.* 1999; 818:204–211. [PubMed: 10082805]
- Johnson KA, Lovinger DM. Presynaptic G Protein-Coupled Receptors: Gatekeepers of Addiction? *Front Cell Neurosci.* 2016; 10:264. [PubMed: 27891077]
- Kang Y, Zhou XE, Gao X, He Y, Liu W, Ishchenko A, Barty A, White TA, Yefanov O, Han GW, Xu Q, de Waal PW, Ke J, Tan MHE, Zhang C, Moeller A, West GM, Van Eps N, Caro LN, Vishnivetskiy SA, Lee RJ, Suino-Powell KM, Gu X, Pal K, Ma J, Zhi X, Boutet S, Williams GJ, Messerschmidt M, Gati C, Zatsepin NA, Wang D, James D, Basu S, Roy-Chowdhury S, Conrad S, Coe J, Liu H, Lisova S, Kupitz C, Grotjohann I, Fromme R, Jiang Y, Tan M, Yang H, Li J, Wang M, Zheng Z, Li D, Zhao Y, Standfuss J, Diederichs K, Dong Y, Potter CS, Carragher B, Caffrey M, Jiang H, Chapman HN, Spence JCH, Fromme P, Weierstall U, Ernst OP, Katritch V, Gurevich VV, Griffin PR, Hubbell WL, Stevens RC, Cherezov V, Melcher K, Xu HE. Crystal structure of rhodopsin bound to arrestin determined by femtosecond X-ray laser. *Nature.* 2015; 523:561–567. [PubMed: 26200343]
- Kohout TA, Lin FS, Perry SJ, Conner DA, Lefkowitz RJ. Beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc. Natl. Acad. Sci. USA.* 2001; 98:1601–1606. [PubMed: 11171997]
- Kook S, Zhan X, Kaoud TS, Dalby KN, Gurevich VV, Gurevich EV. Arrestin-3 binds JNK1 and JNK2 and facilitates the activation of these ubiquitous JNK isoforms in cells via scaffolding. *J Biol Chem.* 2014; 288:37332–37242.
- Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson sSG, Caron MG, Barak LS. The 2-adrenergic receptor/arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Nat Acad Sci USA.* 1999; 96:3712–3717. [PubMed: 10097102]
- Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science.* 1999; 283:655–661. [PubMed: 9924018]
- Luttrell LM, Miller WE. Arrestins as regulators of kinases and phosphatases. *Prog mol Biol Transl Sci.* 2013; 118:115–147. [PubMed: 23764052]
- Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, Lefkowitz RJ. Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc. Natl. Acad. Sci. USA.* 2001; 98:2449–2454. [PubMed: 11226259]
- Macey TA, Gurevich VV, Neve KA. Preferential Interaction between the dopamine D2 receptor and Arrestin2 in neostriatal neurons. *Mol. Pharmacol.* 2004; 66:1635–1642. [PubMed: 15361545]
- Macey TA, Liu Y, Gurevich VV, Neve KA. Dopamine D1 receptor interaction with arrestin3 in neostriatal neurons. *J. Neurochem.* 2005; 93:128–134. [PubMed: 15773912]
- McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, Lefkowitz RJ. Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science.* 2000; 290:1574–1577. [PubMed: 11090355]

- Milano SK, Pace HC, Kim YM, Brenner C, Benovic JL. Scaffolding functions of arrestin-2 revealed by crystal structure and mutagenesis. *Biochemistry*. 2002; 41:3321–3328. [PubMed: 11876640]
- Miller WE, McDonald PH, Cai SF, Field ME, Davis RJ, Lefkowitz RJ. Identification of a motif in the carboxyl terminus of beta -arrestin2 responsible for activation of JNK3. *J Biol Chem*. 2001; 276:27770–27777. [PubMed: 11356842]
- Moore CA, Milano SK, Benovic JL. Regulation of receptor trafficking by GRKs and arrestins. *Annu. Rev. Physiol*. 2007; 69:451–482. [PubMed: 17037978]
- Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, barrestin1, and barrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem*. 2000; 275:17201–17210. [PubMed: 10748214]
- Pierce RC, Kalivas PW. A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. *Brain Research Reviews*. 1997; 25:192–216. [PubMed: 9403138]
- Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, WI W, Kobilka BK. Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature*. 2007; 450:383–387. [PubMed: 17952055]
- Robinson TE, Berridge KC. The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev*. 1993; 18:247–291. [PubMed: 8401595]
- Robinson TE, Berridge KC. Incentive-sensitization and addiction. *Addiction*. 2001; 96:103–114. [PubMed: 11177523]
- Robinson TE, Berridge KC. Review. The incentive sensitization theory of addiction: some current issues. *Philos Trans Soc Lond B Biol Sci*. 2008; 363:3137–3146.
- Seo J, Tsakem EL, Breitman M, Gurevich VV. Identification of arrestin-3-specific residues necessary for JNK3 activation. *J Biol Chem*. 2011; 286:27894–27901. [PubMed: 21715332]
- Shukla AK, Xiao K, Lefkowitz RJ. Emerging paradigms of b-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem Sci*. 2011; 36:457–469. [PubMed: 21764321]
- Song X, Coffa S, Fu H, Gurevich VV. How does arrestin assemble MAP kinases into a signaling complex? *J Biol Chem*. 2009; 284:685–695. [PubMed: 19001375]
- Song X, Raman D, Gurevich EV, Vishnivetskiy SA, Gurevich VV. Visual and both non-visual arrestins in their "inactive" conformation bind JNK3 and Mdm2 and relocalize them from the nucleus to the cytoplasm. *J. Biol. Chem*. 2006; 281:21491–21499. [PubMed: 16737965]
- Steketee JD. Cortical mechanisms of cocaine sensitization. *Crit Rev Neurobiol*. 2005; 17:69–86. [PubMed: 16808728]
- Urs NM, Daigle TL, Caron MG. A dopamine D1 receptor-dependent β -arrestin signaling complex potentially regulates morphine-induced psychomotor activation but not reward in mice. *Neuropsychopharmacology*. 2011; 36:551–558. [PubMed: 20980993]
- Valjent E, Bertran-Gonzalez J, Aubier B, Greengard P, Hervé D, Girault JA. Mechanisms of locomotor sensitization to drugs of abuse in a two-injection protocol. *Neuropsychopharmacology*. 2010; 35:401–415. [PubMed: 19759531]
- Vanderschuren LJ, Kalivas PW. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology*. 2000; 151:99–120. [PubMed: 10972458]
- Vanderschuren LJ, Pierce RC. Sensitization processes in drug addiction. *Curr Top Behav Neurosci*. 2010; 3:179–195. [PubMed: 21161753]
- Violin JD, DiPilato LM, Yildirim N, Elston TC, Zhang J, Lefkowitz RJ. beta2-adrenergic receptor signaling and desensitization elucidated by quantitative modeling of real time cAMP dynamics. *J. Biol. Chem*. 2008; 283:2949–2961. [PubMed: 18045878]
- Vroon A, Heijnen CJ, Lombardi MS, Cobelens PM, Mayor FJ, Caron MG, Kavelaars A. Reduced GRK2 level in T cells potentiates chemotaxis and signaling in response to CCL4. *J. Leukoc. Biol*. 2004a; 75:901–909. [PubMed: 14761932]
- Vroon A, Heijnen CJ, Raatgever R, Touw IP, Ploemacher RE, Premont RT, Kavelaars A. GRK6 deficiency is associated with enhanced CXCR4-mediated neutrophil chemotaxis in vitro and

impaired responsiveness to G-CSF in vivo. *J. Leukoc. Biol.* 2004b; 75:698–704. [PubMed: 14704365]

Xiao K, McClatchy DB, Shukla AK, Zhao Y, Chen M, Shenoy SK, Yates JR 3rd, Lefkowitz RJ. Functional specialization of beta-arrestin interactions revealed by proteomic analysis. *Proc. Nat. Acad. Sci. USA.* 2007; 104:12011–12016. [PubMed: 17620599]

Zhan X, Gimenez LE, Gurevich VV, Spiller BW. Crystal structure of arrestin-3 reveals the basis of the difference in receptor binding between two non-visual arrestins. *J Mol Biol.* 2011; 406:467–478. [PubMed: 21215759]

Zhan X, Kook S, Kaud TS, Dalby KN, Gurevich EV, Gurevich VV. Arrestin-3-dependent activation of c-Jun N-terminal kinases (JNKs). *Curr Protoc Pharmacol.* 2015; 68:2.12-11–12.12-26. [PubMed: 25737158]

- Mice lacking arrestin-2 show initially enhanced locomotor response to amphetamine.
- Mice lacking arrestin-3 are hyposensitive to amphetamine with poor sensitization.
- Single allele mice with only one arrestin-2 allele are grossly hyposensitive to amphetamine.
- Single allele mice with only one arrestin-2 allele show reduced basal locomotion.

Figure 1. Locomotor responsiveness to amphetamine (AMPH) is enhanced in mice lacking arrestin-2 but is reduced in arrestin-3 knockout mice

Mice were treated with AMPH (3 mg/kg, daily for 7 days). Locomotion was measured as described in Methods. The graph shows total distance travelled during each daily session: basal activity measured for 30 min before AMPH administration and AMPH-induced locomotion measured for 90 min after administration of the drug. Mice were challenged with AMPH following a 7-day withdrawal after the 7-day sensitization. The data were analyzed separately for basal and AMPH-induced locomotion and for each knockout-wild type littermate group by two-way repeated measure ANOVA analysis with Genotype as between group and Day as within group factor. (A) The basal and AMPH-induced locomotor activity on each testing day for the entire testing period of 30 min (basal activity) or 90 min (AMPH-induced activity) in ARR2KO mice and their WT littermates. (B) The basal and AMPH-induced locomotor activity on each testing day ARR3KO mice and their WT littermates. There were no significant differences in the basal activity among genotypes. In contrast, the analysis of the AMPH-induced locomotion (with the Challenge day excluded) yielded a significant effect of Genotype for both arrestin-2 ($p=0.0283$) and arrestin-3 ($p=0.0159$). * - $p<0.05$, ** - $p<0.01$ between the knockout and respective wild type groups on each day according to two-tailed Student's t-test; # - $p<0.05$, @ - $p<0.01$, \$ - $p<0.001$ to Day 7 by paired t-test.

Figure 2. Locomotor responsiveness to amphetamine (AMPH) in arrestin knockout mice on individual testing days

On each testing day, the mice were initially tested in the locomotor apparatus without the drug for 30 min, with the locomotor activity measured in 5-min bins. Then the mice were injected with AMPH and tested for 90 min, with the locomotor activity again measured in 5-min bins. (A) The graph shows the locomotor performance of all mouse groups during treatment Day 1 displayed in 5-min bins. Two-way repeated measure ANOVA analysis with Genotype as between group and Bin as within group factor (performed separately for the ARR2KO and ARR3KO mice and their WT littermates) yielded a significant effect of Genotype for ARR2KO ($p=0.04$) but not ARR3KO ($p=0.43$). There was also a significant

Genotype $\times$ Bin interaction for both the ARR2KO ($p=0.0042$) and ARR3KO ($p=0.0166$) groups. **(B)** Locomotor performance during testing Day 5. ARR2KO mice were hyperactive as compared to WT littermates (Genotype $p=0.0483$), whereas ARR3KO mice were hypoactive ($p=0.0021$). **(C)** Locomotor performance on Day 7. ARR2KO mice were no longer hyperactive as compared to WT littermates (Genotype $p=0.14$), whereas ARR3KO mice remained hypoactive (Genotype $p=0.0196$; Genotype $\times$ Bin $p<0.001$). **(D)** The locomotor performance of all mouse groups on the Challenge day. No significant effect of Genotype was detected for ARR2KO ($p=0.93$). In contrast, the difference between ARR3KO mice and their WT littermates remained significant ($p=0.03$).

Figure 3. Locomotor responsiveness to amphetamine in arrestin knockout mice is modulated by the gene dosage of the other arrestin isoform

(A) The graph shows total distance travelled after AMPH administration during each of the seven 90-min daily sessions and on the Challenge day. The data were analyzed separately for each knockout-wild type littermate group by two-way repeated measure ANOVA analysis with Genotype as between group and Day as within group factor. The analysis yielded no significant effect of Genotype for arrestin-2, but a significant effect for arrestin-3 ($p=0.0147$). (B) Total distance traveled during the 30-min pre-testing on each of the testing days and the Challenge day. Two-way repeated measure ANOVA yielded a significant effect of genotype for ARR3KO/ARR2HET groups as compared to ARR3KO littermates ($p=0.002$), whereas there was no difference between the ARR2KO and ARR2KO/ARR3HET genotypes. * - $p<0.05$, ** - $p<0.01$ between the knockout and respective wild type groups on each day according to two-tailed Student's t-test; # - $p<0.05$, @ - $p<0.01$, \$ - $p<0.001$ to Day 7 by paired t-test.

Figure 4. Locomotor responsiveness to amphetamine (AMPH) in arrestin knockout and single allele mice on individual testing days

(A) The graph shows the locomotor performance of all mouse groups on Day 1 displayed in 5-min bins. Two-way repeated measure ANOVA with Genotype as between group and Bin as within group factor (performed separately for the ARR2KO/ARR3HET and ARR3KO/ARR2HET mice and their KO littermates) yielded a significant effect of Genotype for ARR2KO/ARR3HET-ARR2KO ($p=0.0447$) as well as for the ARR3KO/ARR2HET-ARR3KO pair ($p=0.0159$). There was also a significant Genotype $\times$ Bin interaction for ARR2KO/ARR3HET-ARR2KO ($p=0.0021$) but not for the ARR3KO/ARR2HET-ARR3KO ($p=0.43$) groups. There was no difference in the basal activity in the ARR2KO/ARR3HET-

ARR2KO pair. ARR3KO/ARR2HET mice showed reduced basal locomotion (Genotype $p=0.0138$; Genotype $\times$ Bin $p=0.0032$) as compared to ARR3KO mice. **(B)** The locomotor performance on Day 5. ARR2KO/ARR3HET mice were no longer significantly different from their ARR2KO littermates across bins (Genotype $p=0.135$), although their locomotor response to AMPH decayed faster (Genotype $\times$ Bin $p=0.0065$). ARR3KO/ARR2HET mice remained significantly hypoactive (Genotype $p=0.0344$; Genotype $\times$ Bin $p=0.0156$). There was no significant reduction in the basal activity in ARR3KO/ARR2HET ($p=0.38$) mice but they showed lower activity at the beginning of the session (Genotype $\times$ Bin $p=0.0186$). **(C)** The locomotor performance on Day 7. There was no significant difference between ARR2KO and ARR2KO/ARR3HET ($p=0.16$), although the Genotype $\times$ Bin interaction remained significant ($p=0.0017$). ARR3KO/ARR2HET mice remained significantly hypoactive (Genotype $p=0.041$; Genotype $\times$ Bin $p<0.0001$). There was no significant reduction in the basal activity in ARR3KO/ARR2HET ($p=0.3$) mice but they showed lower activity at the beginning of the session (Genotype $\times$ Bin $p<0.0001$). **(D)** The locomotor performance of all mouse groups on the Challenge day. No significant effect of Genotype was detected for the ARR2KO/ARR2KO/ARR3HET pair ($p=0.78$). The basal activity also did not differ. There was also no significant Genotype effect between ARR3KO and ARR3KO/ARR2HET across bins ($p=0.109$) but the Genotype $\times$ Bin interaction remained significant ($p<0.001$). Similarly, the basal activity was not significantly different across bins, but the Genotype $\times$ Bin interaction was significant ($p=0.0019$) due to low novelty-induced locomotion of ARR3KO/ARR2HET mice at the start of the experiment. The arrows and significance levels apply to the Genotype factor following ANOVA analysis across all bins.

Figure 5. Schematic representation of the two models of arrestin roles in regulating the amphetamine (AMPH) effect on behaviour

The first model (A–C) assumes that both G protein- and arrestin-dependent signaling contribute to the AMPH locomotion. (A) Given the higher affinity of arrestin-3 to most binding partners, it also assumes that normally arrestin-3 mediates most of the arrestin-dependent signaling, whereas routine desensitization might rely on arrestin-2. When arrestin-2 is deleted (B), desensitization of the dopamine receptors is impaired, resulting in enhanced G protein-mediated signaling, and consequently, hyperlocomotion. The arrestin-3-mediated signaling could also increase due to removal of the competition from the more abundant but less efficacious arrestin-2. In mice lacking arrestin-3 (C), the G protein-

mediated signaling is preserved, whereas arrestin-dependent signaling is grossly reduced, since the remaining arrestin-2 is less effective as a signaling mediator. An alternative model **(D–F)** assumes that only arrestin-dependent signaling is important for the AMPH-dependent behavior. **(D)** Normally, it is mediated by both arrestins, with a higher contribution from arrestin-3. **(E)** In ARR2KO mice, the signaling is enhanced, since the more efficacious arrestin-3 is the only subtype remaining, which leads to AMPH-induced hyperlocomotion. **(F)** In ARR3KO mice, exactly the opposite happens – arrestin-2 has to substitute for arrestin-3 as a signaling mediator, leading to lower signaling and reduced locomotion.