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Effect of β-Arrestin 2 Downregulation on Morphine-Induced Antinociceptive Tolerance in Rodents: A Systematic Review and Meta-Analysis

Nirmay Rokad

pateln7@vcu.edu

Virginia Commonwealth University

Ohm Tripathi

University Of Conneticut

Systematic Review

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Abstract

Background

Morphine-induced antinociceptive tolerance severely limits the long-term clinical utility of opioid analgesics. β-arrestin 2 (βarr2), a G-protein-coupled receptor regulatory protein, has been proposed as a key mediator of μ-opioid receptor (MOR) desensitization and the consequent development of analgesic tolerance. Multiple preclinical studies employing diverse molecular strategies—including genetic knockout, antisense oligonucleotides, small interfering RNAs (siRNAs), antigene RNAs (agRNAs), and microRNA overexpression—have examined whether reducing βarr2 activity preserves morphine analgesia over time. However, no quantitative synthesis of this evidence has been performed.

Objectives

To quantitatively synthesize preclinical evidence on whether βarr2 downregulation attenuates morphine antinociceptive tolerance in rodent models, estimate the overall effect size, assess heterogeneity across studies, and contextualize these findings within the broader βarr2-biased agonism framework.

Methods

A systematic literature search identified rodent studies measuring antinociception (% maximum possible effect, %MPE) in both βarr2-deficient and wild-type/control groups following chronic morphine administration. Hedges' g was computed for each study as the primary effect size. A random-effects meta-analysis was conducted using the DerSimonian-Laird method. Cochran's Q and I^2 statistics quantified between-study heterogeneity.

Results

Six studies meeting pre-specified inclusion criteria (n = 6–12 animals/group) were included in the primary meta-analysis. βarr2 downregulation was associated with significantly preserved analgesia at Day 7 of chronic morphine treatment relative to controls. The pooled Hedges' g was 1.38 (95% CI: 0.94–1.83), indicating a large effect. Heterogeneity was negligible (I^2 = 0%, τ^2 = 0.000, Q = 1.72, df = 5, p = 0.89). A critical exception was identified: Bohn et al. (2002) demonstrated that βarr2-KO mice ultimately develop morphine tolerance in the spinal tail-flick paradigm, whereas tolerance was absent in the supraspinal hot-plate test, indicating CNS region-specific regulation. Sensitivity analyses including Kliewer et al. (2019), which employed a phosphorylation-deficient MOR knockin strategy, confirmed robustness of the primary estimate.

Conclusions

Across diverse molecular interventions and rodent species, βarr2 downregulation produces a large, consistent effect on preserving morphine antinociception during chronic administration. However, the effect is not universal: spinal analgesic pathways show residual tolerance independent of βarr2, implicating PKC-dependent mechanisms. These findings support βarr2 as a major, though not sole, determinant of morphine antinociceptive tolerance and inform the rational design of G-protein-biased MOR agonists.

1. Introduction

Morphine and related opioid analgesics remain the most effective pharmacological tools for managing moderate-to-severe pain. However, their sustained clinical utility is profoundly limited by the development of analgesic tolerance—a phenomenon in which repeated drug exposure leads to a progressive decline in pharmacological effect, necessitating dose escalation and heightening the risk of adverse outcomes including respiratory depression, constipation, and dependence.[1,2]

At the molecular level, morphine exerts its analgesic effects primarily through activation of the μ-opioid receptor (MOR), a G-protein-coupled receptor (GPCR) that signals predominantly via Gαi/o to inhibit adenylyl cyclase and downstream second messenger cascades.[3] Following agonist binding, MOR signaling is subject to rapid regulatory processes including receptor phosphorylation by G-protein-coupled receptor kinases (GRKs), subsequent recruitment of β-arrestin scaffolding proteins, and receptor internalization.[4,5] These events collectively dampen receptor responsiveness and constitute the cellular substrate of acute desensitization.

Two β-arrestin isoforms are expressed in the central nervous system: β-arrestin 1 and β-arrestin 2 (βarr2, also known as arrestin-3). Of these, βarr2 has received disproportionate attention in opioid pharmacology because of its preferential expression in neuronal tissues and its high efficiency in translocating to activated MOR complexes.[6,7] Seminal studies by Bohn et al. demonstrated that mice genetically lacking βarr2 (βarr2-KO mice) exhibited markedly enhanced and prolonged morphine antinociception in the hot-plate test and, crucially, failed to develop antinociceptive tolerance following repeated morphine administration—while still developing physical dependence.[8,9] These foundational observations established the conceptual framework that βarr2-mediated MOR desensitization is the proximal molecular event driving morphine tolerance, and that tolerance and dependence are dissociable phenomena.

Subsequent work extended these findings using diverse molecular interventions designed to reduce βarr2 expression or activity without the developmental compensations associated with constitutive knockout. Antisense oligonucleotides (AS-ODNs),[10] small interfering RNAs (siRNAs) delivered to the spinal cord[11] or periaqueductal gray (PAG),[12] antigene RNAs (agRNAs),[13] and microRNA-365 overexpression[14] have all been reported to attenuate morphine tolerance in rodent models. Together,

these studies suggest a conserved role for βarr2 across multiple CNS regions, species, and methodological approaches.

The clinical relevance of these preclinical findings has gained considerable momentum with the emergence of the biased agonism hypothesis—the proposition that MOR agonists preferentially engaging G-protein signaling over βarr2 recruitment might achieve superior analgesia with attenuated tolerance and potentially reduced side effects.[15,16] The development of G-protein-biased MOR ligands has been partly predicated on the preclinical evidence reviewed here. However, more recent work has challenged key tenets of this hypothesis, particularly the role of βarr2 in mediating opioid side effects such as respiratory depression and constipation, and has highlighted important inconsistencies between studies using mixed genetic backgrounds.[17,18] Importantly, Kliewer et al. demonstrated using phosphorylation-deficient MOR knockin mice that preventing βarr2 recruitment greatly diminished tolerance but paradoxically worsened respiratory depression and constipation—findings that substantially complicate the biased agonism therapeutic narrative.[19]

Despite the accumulation of preclinical data across more than two decades, no formal quantitative synthesis of the βarr2-morphine tolerance literature has been conducted. Individual studies vary in species, brain region targeted, molecular intervention, and behavioral assay, creating challenges for interpreting the collective evidence. Furthermore, an important null result—Bohn et al. (2002) demonstrating that βarr2-KO mice do ultimately develop morphine tolerance in the spinal tail-flick test—has not been adequately integrated into the existing narrative.[20]

The present systematic review and meta-analysis addresses this gap. We quantitatively synthesize the preclinical evidence on whether βarr2 downregulation attenuates morphine antinociceptive tolerance in rodents, estimate the overall effect size using Hedges' g, assess between-study heterogeneity, identify moderating variables, and situate these findings within the contemporary βarr2-biased agonism literature. By doing so, we aim to provide a rigorous foundation for evaluating the translational potential of βarr2-targeted strategies in opioid pharmacotherapy.

2. Methods

2.1 Eligibility Criteria

Studies were eligible for inclusion if they: (1) used rodent models (mice or rats); (2) employed a genetic or molecular intervention specifically targeting βarr2, including global knockout (KO), regional knockdown via AS-ODNs, siRNA, agRNA, microRNA manipulation, or phosphorylation-deficient MOR knockin strategies that functionally prevent βarr2 recruitment; (3) administered morphine chronically via repeated injection, intrathecal infusion, or subcutaneous osmotic pump; (4) measured antinociception as %MPE using a validated behavioral assay (hot-plate, tail-flick/tail-immersion, or warm-water tail withdrawal); and (5) provided sufficient quantitative data at Day 7 of chronic treatment (mean and measure of variance) to compute effect sizes. Studies reporting only acute tolerance or acute morphine

response without a chronic tolerance paradigm were excluded. Review articles, in vitro studies, and studies examining opioids other than morphine as the primary agonist were excluded from the meta-analysis (but may be referenced in the systematic review portion).

2.2 Search Strategy

A systematic literature search was conducted in PubMed/MEDLINE using the following search terms: ("beta-arrestin 2" OR "β-arrestin 2" OR "arrestin-3" OR "ARRB2") AND ("morphine tolerance" OR "antinociceptive tolerance" OR "opioid tolerance") AND ("rodent" OR "mouse" OR "mice" OR "rat"). Additional searches combined ("mu-opioid receptor" OR "MOR") AND ("desensitization" OR "knockdown" OR "knockout" OR "siRNA" OR "antisense") AND ("tolerance" OR "antinociception"). Reference lists of identified articles were manually screened for additional eligible studies. No date restriction was applied. The search was conducted through April 2026.

2.3 Data Extraction

Two reviewers independently extracted the following variables: first author and year, animal species and strain, βarr2 intervention type and target region, morphine dose and route of administration, behavioral assay, assessment timepoint, group sample sizes (n), mean %MPE at Day 7 for control (WT/scramble/vehicle) and βarr2-deficient (experimental) groups, and measure of variance (SEM or SD). Where multiple behavioral assays were reported in the same study at Day 7, data from the assay most consistent with the primary outcome measure (tail-flick or hot-plate) were extracted. Standard deviations were calculated from reported SEM values using the formula SD = SEM × √n.

2.4 Effect Size Calculation

The primary effect size metric was Hedges' g, a bias-corrected standardized mean difference appropriate for small samples. For each study, Cohen's d was first calculated as:

$$d = (M_{exp} - M_{ctrl}) / SD_{pooled}$$

where SD_{pooled} is the pooled standard deviation computed from both groups. Hedges' g was then obtained by applying the correction factor $J = 1 - [3 / (4df - 1)]$, where $df = n_{ctrl} + n_{exp} - 2$. The variance of g was estimated as:

$$Var(g) = (n_{ctrl} + n_{exp}) / (n_{ctrl} \times n_{exp}) + g^2 / [2(n_{ctrl} + n_{exp})]$$

2.5 Meta-Analytic Model

Given the diversity of molecular interventions, species, and CNS target regions anticipated across eligible studies, a random-effects model was pre-specified as the primary analytic approach. The DerSimonian-Laird (DL) method was used to estimate the between-study variance component τ^2. Random-effects pooled Hedges' g was computed as the inverse-variance-weighted mean of individual g values, where each weight incorporates both within-study sampling variance and τ^2.

Between-study heterogeneity was assessed using Cochran's Q statistic and the I^2 index, where $I^2 \leq 25\%$ indicates low, 25–50% moderate, and > 50% high heterogeneity.[21] Ninety-five percent confidence intervals (CIs) for pooled estimates were calculated as: pooled $g \pm 1.96 \times SE$, where $SE = \sqrt{(1/\Sigma w_i)}$.

2.6 Sensitivity and Subgroup Analyses

Pre-specified sensitivity analyses included: (1) restriction to studies using %MPE as the outcome measure (primary analysis), excluding Bu et al. (2015) which used von Frey paw withdrawal threshold; (2) inclusion of Bohn et al. (2002) to examine the impact of the null tail-flick finding; (3) separate subgroup analyses by intervention type (genetic KO vs. molecular knockdown), species (mouse vs. rat), and behavioral assay (hot-plate vs. tail-flick). Subgroup analyses were exploratory given the limited number of studies.

2.7 Systematic Review Scope

The systematic review component additionally examined studies reporting on opioid side effects (respiratory depression, constipation, physical dependence) in the context of βarr2 manipulation, studies using non-morphine MOR agonists (fentanyl, methadone, oxycodone, kurkinorin, kurkinol), and the phosphorylation-deficient MOR knockin studies of Kliewer et al. (2019). These studies are discussed qualitatively where they inform interpretation of the meta-analytic findings or the biased agonism framework, but are not included in the primary quantitative pooling due to outcome measure or agonist heterogeneity.

3. Results

3.1 Study Selection

The literature search identified 11 primary research articles examining βarr2 manipulation and morphine tolerance in rodents. Following screening against eligibility criteria, 8 studies provided Day 7%MPE or equivalent behavioral data. Of these, 6 were included in the primary meta-analysis (reporting %MPE outcomes), and 2 were retained for sensitivity or subgroup analyses: Bohn et al. (2002), whose outcome demonstrated a null result uniquely relevant to CNS-region specificity, and Bu et al. (2015), which used von Frey mechanical threshold (grams) rather than %MPE. Kliewer et al. (2019) was included as a pre-specified sensitivity analysis given its use of a phosphorylation-deficient MOR knockin model that functionally prevents βarr2 recruitment. The Raehal and Bohn (2011) study was included in the systematic review for its examination of βarr2 effects across multiple opioid agonists but excluded from the primary meta-analysis as it did not report %MPE at Day 7 for morphine in a manner directly comparable to the other studies. Van de Wetering et al. (2025) was included in the systematic review for its examination of novel MOR agonists kurkinorin and kurkinol in βarr2-KO mice.

3.2 Study Characteristics

The 6 studies included in the primary meta-analysis (Table 1) were published between 2000 and 2019 and collectively enrolled 83–120 rodents across experimental and control groups. Three studies employed mouse models and three employed rat models. Molecular interventions represented four distinct mechanistic strategies: (1) global constitutive knockout of the Arrb2 gene (Bohn et al., 2000); (2) antisense oligonucleotide (AS-ODN) knockdown of spinal βarr2 (Przewlocka et al., 2002); (3) regional siRNA-mediated knockdown via lentiviral delivery to PAG (Li et al., 2009) or intrathecal administration (Yang et al., 2011); (4) post-transcriptional regulation via miR-365 overexpression (Wang et al., 2016); and (5) phosphorylation-deficient MOR knockin preventing βarr2 recruitment (Kliewer et al., 2019). Morphine dosing paradigms ranged from 10 mg/kg subcutaneous daily injection (Bohn, Li, Yang) to intrathecal infusion (2 nmol/h; Yang) and osmotic pump delivery (17 mg/kg/day; Kliewer). Behavioral assays included the tail-immersion/tail-flick test (Bohn 2000, Przewlocka, Yang, Wang) and the hot-plate test (Li, Kliewer). All studies assessed antinociception at Day 7 as the primary tolerance endpoint.

Table 1
Characteristics of Studies Included in the Primary Meta-Analysis

Study	Model / Species	Intervention	Assay	n ctrl	n exp	Mean ctrl (%MPE)	Mean exp (%MPE)
Bohn et al., 2000 [9]	Mouse (C57BL/6 × 129SvJ)	Global βarr2-KO	Tail-immersion 54°C	7	8	21	79
Przewlocka et al., 2002 [10]	Rat (Wistar)	AS-ODN i.th.	Tail-flick	6	6	13	30
Li et al., 2009 [12]	Mouse (C57BL/6)	siRNA lentivirus (PAG)	Hot-plate 52°C	10	9	12	45
Yang et al., 2011 [11]	Rat (Sprague-Dawley)	siRNA i.th.	Tail-flick 54°C	10	10	15	38
Wang et al., 2016 [14]	Rat (Sprague-Dawley)	miR-365 LV i.th.	Tail-flick	6	6	0	10
Kliewer et al., 2019 [19]*	Mouse (C57BL/6J)	Phospho-deficient MOR KI (10S/T-A)	Hot-plate 56°C	9	9	15	50

**Kliewer et al. (2019) used a phosphorylation-deficient MOR knockin model (10S/T-A) rather than a direct βarr2 knockout; this model prevents βarr2 recruitment. AS-ODN = antisense oligonucleotide; i.th. = intrathecal; LV = lentivirus; PAG = periaqueductal gray; KI = knockin.*

3.3 Effect Sizes and Meta-Analytic Pooling

Individual study effect sizes (Hedges' g) ranged from 0.91 to 1.78, with all primary studies demonstrating a positive effect favoring βarr2-deficient animals (Table 2). The pooled random-effects estimate yielded a Hedges' g of 1.38 (95% CI: 0.94–1.83, SE = 0.23), indicating a large and statistically significant protective effect of βarr2 downregulation on morphine antinociception at Day 7 of chronic treatment. By conventional benchmarks (Cohen, 1988), g values of 0.2, 0.5, and 0.8 correspond to small, medium, and large effects, respectively; the present estimate of 1.38 substantially exceeds the threshold for a large effect.[22]

Between-study heterogeneity was remarkably low: Cochran's Q = 1.72 (df = 5, p = 0.89), I^2 = 0%, and τ^2 = 0.000. This absence of meaningful heterogeneity is notable given the diversity of interventions, species, and assays represented, and suggests a highly consistent biological effect across the conditions examined. No evidence of a systematic moderating influence of species, intervention type, or behavioral assay was identified in exploratory subgroup analyses, though power for detecting true moderation is limited by the small number of studies.

Table 2
Individual and Pooled Effect Size Estimates (Hedges' g)

Study	n ctrl	n exp	SD ctrl	SD exp	Pooled SD	Hedges' g (95% CI)
Bohn et al., 2000	7	8	26.46	33.94	30.71	1.78 [0.58, 2.97]
Przewlocka et al., 2002	6	6	12.25	19.60	16.34	0.96 [− 0.24, 2.16]
Li et al., 2009	10	9	15.81	24.00	20.09	1.57 [0.54, 2.60]
Yang et al., 2011	10	10	12.65	15.81	14.32	1.54 [0.54, 2.54]
Wang et al., 2016	6	6	7.35	12.25	10.10	0.91 [− 0.28, 2.10]
Kliewer et al., 2019*	9	9	15.00	30.00	23.72	1.41 [0.37, 2.44]
Pooled (RE model)						**1.38 [0.94, 1.83]**

*RE = random-effects; SD values derived from reported SEM (SD = SEM × √n); 95% CI = 95% confidence interval. *Sensitivity analysis inclusion; phosphorylation-deficient MOR knockin.*

Heterogeneity statistics: Q = 1.72, df = 5, p = 0.89; I^2 = 0.0%; τ^2 = 0.000.

3.4 The Bohn et al. (2002) Null Result: CNS Region-Specific Tolerance

A critical finding that informs interpretation of the primary meta-analysis is the partial null result reported by Bohn et al. (2002) in the Journal of Neuroscience.[20] Using the warm water tail-immersion (tail-flick) assay—which primarily measures spinal reflex responses—this study demonstrated that βarr2-KO mice, despite showing substantially enhanced acute morphine antinociception and no tolerance on Days 1–4, exhibited a precipitous loss of morphine responsiveness by Days 4–5 and were fully tolerant by Day 7,

reaching levels indistinguishable from wild-type controls. At the same time, a parallel hot-plate test on Day 7 confirmed that the identical βarr2-KO mice retained full morphine responsiveness supraspinally, demonstrating that the dissociation was anatomically specific rather than reflecting residual systemic tolerance.

The effect size for this study (g ≈ 0.00 at Day 7, 95% CI: −1.05 to 1.05) stands in stark contrast to all other studies in this review. Inclusion of this study in the meta-analysis (sensitivity analysis) reduces the pooled estimate but does not substantially alter the conclusion, given that the null effect at Day 7 is accompanied by a strong positive effect at Days 1–4. Importantly, Bohn et al. (2002) identified PKC-dependent signaling as the mechanism responsible for the residual βarr2-independent tolerance in spinal cord: the PKC inhibitor chelerythrine completely reversed Day 7 morphine tolerance in βarr2-KO mice in the tail-flick test but had minimal effect on tolerant wild-type animals, where βarr2-mediated desensitization remains the dominant regulatory pathway.[20]

This finding has important implications: βarr2 is the dominant but not exclusive mechanism of morphine antinociceptive tolerance, and its contribution is pathway-specific. Supraspinal (PAG, brainstem) MOR circuits are more dependent on βarr2 for tolerance development, while spinal cord MOR circuits have sufficient redundancy via PKC-dependent pathways to develop tolerance even in the absence of βarr2.

3.5 Sensitivity Analyses

Several sensitivity analyses were conducted to test the robustness of the primary estimate. First, inclusion of Kliewer et al. (2019) as a sixth primary study (g = 1.41) yielded a pooled estimate of g = 1.38 (95% CI: 0.94–1.83), virtually unchanged from the five-study estimate. Second, inclusion of Bohn et al. (2002) with its null Day 7 tail-flick finding (g = 0.00) reduced the pooled estimate to g = 1.14 (95% CI: 0.58–1.70), still large and statistically significant. Third, analysis of the Bu et al. (2015) mechanical threshold data (von Frey, grams), which employed agRNA lentiviral delivery with intracerebroventricular administration, yielded a Hedges' g of 1.39 (95% CI: 0.13–2.65), consistent with the primary %MPE-based estimate despite measuring a different sensory modality and using a different outcome metric.

Subgroup analysis by intervention type showed that genetic KO (Bohn 2000) produced the largest point estimate (g = 1.78), while molecular knockdown studies ranged from g = 0.91 (miRNA; Wang 2016) to g = 1.57 (siRNA; Li 2009). Subgroup analysis by species did not reveal systematic differences between mouse and rat studies, consistent with a conserved biological mechanism. Subgroup analysis by assay type suggested numerically larger effects in hot-plate (supraspinal) versus tail-flick (spinal) studies, which is mechanistically coherent with the region-specificity observed by Bohn et al. (2002), though the difference did not reach statistical significance given limited study numbers.

4. Systematic Review: Broader Pharmacological Context

4.1 Opioid Agonist Selectivity for βarr2-Dependent Tolerance

A critical insight from the broader literature is that the role of βarr2 in analgesic tolerance is not uniform across opioid agonists. Raehal and Bohn (2011) conducted a systematic comparison of βarr2-KO and wild-type mice under chronic infusion with morphine, methadone, fentanyl, and oxycodone via osmotic pump.[23] While βarr2-KO mice showed markedly attenuated morphine tolerance (approximately 10-fold lower rightward shift in ED_{50} at Day 7 compared to wild-type), tolerance developed equivalently in both genotypes following chronic treatment with methadone, fentanyl, and oxycodone. These findings are consistent with the cell biology literature demonstrating that morphine, unlike fentanyl or DAMGO, produces only minimal GRK-mediated phosphorylation and βarr2 recruitment in vitro—leaving the receptor free to desensitize via alternative mechanisms when a strong agonist drives robust phosphorylation.[24,25]

This agonist selectivity has profound implications for preclinical-to-clinical translation: G-protein-biased agonists may selectively reduce βarr2-mediated desensitization while leaving βarr2-independent tolerance mechanisms intact. Furthermore, for highly efficacious opioids that produce robust receptor phosphorylation independently of morphine-specific mechanisms, βarr2 targeting may offer limited benefit.

4.2 βarr2 and Opioid Side Effects: A Contested Relationship

Early studies in βarr2-KO mice suggested that several limiting side effects of morphine—including respiratory depression, constipation, and urinary retention—are attenuated when βarr2 signaling is absent, supporting the hypothesis that G-protein-biased agonists might have an improved therapeutic window.[26] This view was influential in driving the development of G-protein-biased MOR ligands including TRV-130 (oliceridine), PZM21, and related compounds.

However, the genetic background of the original βarr2-KO mice (mixed C57BL/6 × 129SvJ) has been identified as a significant confound, and numerous subsequent studies using congenic strains have failed to replicate the attenuated side-effect profiles.[17,27] Most definitively, Kliewer et al. (2019) generated phosphorylation-deficient MOR knockin mice (10S/T-A and 11S/T-A) in which all 11 carboxyl-terminal serine and threonine residues were mutated to alanine, completely preventing GRK-mediated phosphorylation and βarr2 recruitment.[19] These mice showed dramatically reduced analgesic tolerance to both morphine and fentanyl—confirming the role of phosphorylation/βarr2 recruitment in tolerance—but exhibited unchanged or significantly worsened respiratory depression and constipation at equianalgesic doses. The correlation between ED_{50} values for analgesia and side effects across genotypes was remarkably high ($R^2 = 0.92–0.97$), indicating that both beneficial and adverse effects scale proportionally with G-protein signaling intensity, not βarr2 engagement.

Van de Wetering et al. (2025) extended this analysis to novel MOR agonists kurkinorin and kurkinol using congenic C57BL/6J βarr2-KO mice.[28] Knockout of βarr2 increased antinociceptive potency for morphine and kurkinorin but had no impact on respiratory depression, constipation, conditioned reward, or

antinociceptive tolerance across all drugs tested. These results, in a well-controlled genetic background, provide strong evidence that βarr2 signaling does not drive MOR-mediated side effects and that the biased agonism hypothesis—at least as applied to separating analgesia from adverse effects—requires fundamental revision.

4.3 Molecular Mechanisms Linking βarr2 to MOR Desensitization and Tolerance

The mechanistic basis for βarr2-mediated morphine tolerance has been elucidated through a series of complementary studies. Following morphine activation of MOR, GRK5 preferentially phosphorylates the receptor at serine-375 in the carboxyl terminus.[19,24] This monophosphorylation event, while insufficient to drive robust βarr2 recruitment or receptor internalization in vitro, appears sufficient to initiate a βarr2-dependent conformational change that promotes receptor desensitization in vivo. Upon chronic morphine exposure, this process is amplified and sustained, resulting in functional uncoupling of MOR from Gαi/o and loss of downstream analgesic signaling.

In the spinal cord, where Bohn et al. (2002) demonstrated residual βarr2-independent tolerance, protein kinase C γ (PKCγ) plays an important compensatory role.[20,29] PKCγ-dependent phosphorylation of MOR—likely at residues distinct from the GRK sites—can promote receptor desensitization through a pathway that persists in the absence of βarr2. This is consistent with studies showing that PKC inhibitors attenuate spinal morphine tolerance in wild-type animals and completely reverse the delayed tolerance that develops in βarr2-KO mice. The interplay between GRK/βarr2 and PKC pathways thus represents a redundant, region-specific regulatory system governing MOR responsiveness.

microRNA-mediated regulation of βarr2 expression adds an additional layer of endogenous control. Wang et al. (2016) demonstrated that chronic morphine administration downregulates miR-365 in the spinal dorsal horn, leading to de-repression of βarr2 mRNA and upregulation of βarr2 protein.[14] This positive feedback mechanism—where morphine increases βarr2 expression, which in turn promotes MOR desensitization and tolerance—provides a molecular explanation for the progressive nature of tolerance development. Restoring miR-365 levels via intrathecal lentiviral delivery reversed established tolerance, demonstrating that this pathway is not merely correlative but causally involved.

5. Discussion

This systematic review and meta-analysis represents, to our knowledge, the first formal quantitative synthesis of the preclinical evidence linking βarr2 to morphine antinociceptive tolerance. The primary finding is robust: across six studies employing diverse molecular interventions, rodent species, CNS target regions, and behavioral assays, βarr2 downregulation is associated with a large, consistent preservation of morphine analgesia at Day 7 of chronic treatment (pooled Hedges' g = 1.38, 95% CI: 0.94–1.83). The near-zero heterogeneity (I^2 = 0%) is remarkable given the methodological diversity of the

included studies and suggests that the biological phenomenon is highly reproducible across experimental contexts.

The magnitude of the pooled effect size merits attention. A Hedges' g of 1.38 corresponds to approximately 1.4 standard deviations of separation between the analgesia distributions of βarr2-deficient and control groups—a difference that, in the context of preclinical pharmacology, represents a substantial and clinically meaningful preservation of drug efficacy. Translated to the laboratory setting, this means that animals lacking functional βarr2 maintain roughly 30–50 percentage points greater %MPE than controls at the time point (Day 7) when tolerance is typically well-established in standard rodent models.

However, several important qualifications temper this finding. First, the Bohn et al. (2002) study provides unambiguous evidence that βarr2 is necessary but not sufficient to explain all forms of morphine antinociceptive tolerance. The complete tolerance that developed in βarr2-KO mice in the spinal tail-flick test by Day 7—while the same animals remained fully responsive in the hot-plate test—reveals a fundamental anatomical dissociation in the regulatory mechanisms governing supraspinal versus spinal opioid analgesia. This dissociation was mechanistically explained by the reversal of spinal tolerance with PKC inhibition, implicating PKCγ as a βarr2-independent desensitization pathway that is particularly prominent in dorsal horn neurons. Any therapeutic strategy targeting βarr2 would therefore be expected to preserve supraspinal analgesia more effectively than spinal analgesia.

Second, the translational trajectory of the βarr2-biased agonism hypothesis—which was substantially motivated by the very preclinical evidence synthesized here—has encountered significant challenges. While the development of G-protein-biased MOR ligands was predicated on the expectation that reduced βarr2 engagement would simultaneously improve analgesia, reduce tolerance, and attenuate side effects, the empirical evidence for the last of these benefits has not held up under rigorous scrutiny.[17,18,19,27,28] The Kliewer et al. (2019) study, which most definitively dissected the consequences of βarr2 elimination using a genetics-based approach that avoids pharmacological confounds, found that completely preventing βarr2 recruitment enhanced analgesia and reduced tolerance (consistent with our meta-analysis) but actually exacerbated respiratory depression and constipation at equianalgesic doses. This paradox—that sustained G-protein signaling simultaneously improves the desired effect and worsens side effects—suggests that both analgesic and adverse MOR effects are mediated primarily through G-protein pathways, with βarr2 actually serving a protective role in limiting the magnitude of pharmacological responses.

Third, the agonist-selectivity of βarr2 involvement in tolerance is an important consideration. The robust effect seen in morphine studies does not extend to fentanyl, methadone, or oxycodone, where tolerance develops equivalently in βarr2-KO and wild-type mice.[23] This likely reflects the distinct phosphorylation profiles elicited by different opioid agonists at MOR: morphine drives predominantly GRK5-mediated monophosphorylation at S375 without triggering multisite hierarchical phosphorylation and robust βarr2 engagement, whereas high-efficacy opioids drive multisite phosphorylation that promotes strong βarr2

interactions through a GRK2/3-dependent mechanism.[19,24] The clinical opioids most relevant to the current pain crisis (fentanyl, oxycodone) may therefore fall outside the domain of βarr2-dependent tolerance.

The diversity of molecular interventions represented in this meta-analysis—spanning constitutive genetic knockout, antisense knockdown, siRNA, agRNA, and microRNA strategies—provides important mechanistic triangulation. Each approach has distinct limitations: constitutive knockouts carry developmental compensations and genetic background effects; viral siRNA delivery is region-restricted; agRNAs and antisense approaches achieve partial and temporally variable knockdown. Yet the consistency of large positive effects across all these methodological approaches, in both mice and rats, and in both supraspinal and spinal regions (with the important exception of Day 7 in the tail-flick), argues strongly against a spurious or method-specific result.

Several limitations of this meta-analysis should be acknowledged. The number of eligible studies is small (n = 6 primary studies), limiting statistical power for subgroup analyses and heterogeneity assessment. Effect size estimates relied on figure-derived values where exact numerical data were not reported in text, introducing potential measurement error. The included studies span more than two decades and employ variable morphine dosing regimens, raising questions about dose-response relationships that cannot be addressed with the current data. Publication bias—the tendency for positive results to be published more readily—cannot be excluded, though the inclusion of a prominent null result (Bohn 2002) and studies with non-significant individual CIs (Przewlocka 2002, Wang 2016) partially mitigates this concern. Finally, all included studies used male rodents, and the potential for sex-specific differences in βarr2-mediated tolerance—a relevant concern given known sex differences in opioid pharmacology—cannot be addressed from the existing literature.

6. Conclusions

This systematic review and meta-analysis establishes, with quantitative precision, that βarr2 downregulation produces a large, consistent, and highly reproducible attenuation of morphine antinociceptive tolerance across preclinical rodent models (pooled Hedges' g = 1.38, 95% CI: 0.94–1.83; I^2 = 0%). The effect is robust across species, molecular intervention types, and CNS target regions, and is qualitatively replicated in phosphorylation-deficient MOR knockin models that functionally prevent βarr2 recruitment. These findings place βarr2 firmly as the dominant, proximal molecular determinant of morphine-specific antinociceptive tolerance.

However, the complete picture is substantially more complex than a simple βarr2-tolerance equation. Spinal cord circuits retain a βarr2-independent, PKC-dependent mechanism of MOR desensitization that produces full tolerance even in the absence of βarr2, specifically in assays measuring spinal reflexes. βarr2 involvement is agonist-selective, applying robustly to morphine but not to fentanyl, methadone, or oxycodone. Most importantly, the emerging consensus from phosphorylation-deficient knockin studies and congenic βarr2-KO experiments indicates that βarr2 does not mediate the major adverse effects of

opioids (respiratory depression, constipation) and may in fact limit their severity by constraining the duration of G-protein signaling. The original promise of G-protein-biased MOR agonists—separating analgesia from side effects by eliminating βarr2 engagement—is therefore not supported by the most rigorous preclinical evidence.

Collectively, these findings reframe βarr2 as a precision target for reducing morphine-specific antinociceptive tolerance without the expectation of broadly improved side-effect profiles. Future work should address sex as a biological variable, examine chronic pain models (as opposed to naive rodent assays), and investigate the βarr2-independent tolerance mechanisms in spinal circuits as additional therapeutic targets.

Declarations

7.1 Ethics approval and consent to participate

Not Applicable.

7.2 Consent for publication

Not Applicable.

7.3 Availability of data and materials

The dataset(s) supporting the conclusions of this article is(are) included within the article

7.4 Competing interests

The authors declare that they have no competing interests

7.5 Funding

Not Applicable.

7.6 Authors' contributions

NR provided the conceptual and literary framework for systemic review and meta-analysis. OT analysed and interpreted results, and was a major contributor to writing the manuscript. All authors read and approved the final manuscript.

7.7 Acknowledgements

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