A Critical Re-evaluation of "*Elucidating pathway-selective biased CCKBR agonism for Alzheimer's disease treatment*" by Wang *et al*., *Cell* 2025; doi:10.1016/j.cell.2025.10.034

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Abstract

Wang *et al*. (*Cell*, 2025) report that a newly synthesized small-molecule ligand, termed CCK-X, functions as a pathway-selective biased agonist of the cholecystokinin B receptor (CCKBR), leading to neuroprotection and cognitive improvement in Alzheimer's disease (AD) models. While the concept of exploiting protein–coupled receptor (GPCR) biased agonism for neurodegeneration is innovative and of substantial therapeutic interest, our in-depth analysis reveals that the evidence presented in the original study is insufficient to support several of its central conclusions. Quantitative pharmacological criteria required to establish true signaling bias are incompletely met, as operational model parameters are not reported, assay conditions are not matched, β-arrestin recruitment measurements lack kinetic resolution, and discrepancies occur between reported and plotted EC_{50} and Emax values. Cryo-EM structures are interpreted as indicative of a distinct biased agonist–bound CCKBR conformation, yet the reported map resolutions and validation metrics do not support side-chain interpretation or precise ligand positioning. Neuronal physiology studies and long-term potentiation experiments suffer from small sample sizes, limited biological replication, and absent blinding. Behavioral studies in AD mouse models show inconsistencies in experimental design, lack of locomotor controls, and possible smoothing or duplication of raw navigational traces. Biochemical assays, including ELISA-based quantification of amyloid and tau, exhibit nonlinear standard curves and insufficient validation. Transcriptomic and proteomic datasets display batch effects, limited depth, and inconsistent thresholds. Several Extended Data and Supplementary Figures raise internal consistency concerns that further weaken mechanistic and therapeutic claims. In summary, although the study proposes a promising strategy, its mechanistic interpretations and translational conclusions require substantial clarification and more rigorous experimental support.

1. Introduction and Background

1.1. Overview of CCKBR Biology and Its Relevance to Alzheimer's Disease

CCKBR, a class A GPCR, occupies a unique position in the neurochemical architecture of the mammalian brain[1,2]. Although traditionally associated with gastrointestinal function and satiety signaling, CCKBR is widely expressed in the central nervous system, particularly in the cortex, hippocampus, amygdala, basal ganglia, and select subcortical nuclei[1,2]. Its endogenous ligand, cholecystokinin, exists in multiple peptide isoforms that mediate a wide range of neuromodulatory effects, including anxiety regulation, synaptic plasticity modulation, neurotransmitter release, and memory processing. CCKBR couples to several intracellular pathways, including $G_{q/11}$-mediated phospholipase C activation, IP_3-dependent Ca^{2+} release, ERK/MAPK signaling cascades, and β-arrestin scaffolding functions[1,2]. Because of this signaling versatility, CCKBR has long been viewed as a complex receptor with pleiotropic outcomes depending on ligand identity, cellular context, and signaling kinetics.

In the context of AD, CCKBR has attracted renewed attention[3,4]. Evidence from transcriptomic and proteomic analyses suggests that cholinergic signaling pathways, glutamatergic neurotransmission, and calcium homeostasis all become progressively dysregulated during AD progression. As CCKBR participates in modulating excitatory neurotransmission and intracellular Ca^{2+} dynamics, it represents a potentially important node for neuromodulatory intervention[5]. Furthermore, alterations in CCK peptide levels have been observed in patients with cognitive impairment, and experimental modulation of CCKBR has been proposed to influence synaptic plasticity mechanisms such as long-term potentiation. Despite this promise, therapeutic exploitation of CCKBR has been limited by the broad and sometimes deleterious effects produced by non-selective agonists, which can trigger undesirable β-arrestin–dependent pathways linked to anxiety, dysphoria, or maladaptive plasticity. These concerns have fueled interest in designing ligands capable of selectively stabilizing CCKBR conformations that preferentially recruit beneficial signaling pathways while avoiding maladaptive ones.

1.2. Concepts and Challenges of GPCR Biased Agonism

Biased agonism, or functional selectivity, refers to the ability of different ligands acting at the same receptor to preferentially activate certain downstream signaling pathways over others[6-11]. In theory, a biased ligand can amplify beneficial effects while suppressing adverse outcomes by modulating the receptor's conformational ensemble. For GPCRs such as CCKBR, biased agonism often involves differential engagement of G-protein pathways versus β-arrestin pathways, each of which has

distinct spatiotemporal signaling patterns and physiological consequences. Demonstrating biased agonism in a rigorous pharmacological framework requires the use of standardized assays performed under matched experimental conditions, quantitative modeling of efficacy and potency, and formal estimation of bias factors using operational models such as the Black–Leff or Ehlert models. These models allow one to determine the degree to which a ligand shifts the relative preference of a receptor toward one signaling pathway over another.

However, the field of biased agonism is fraught with methodological challenges. Assay sensitivity, receptor expression levels, kinetic differences, and signal amplification can create apparent pathway selectivity that does not reflect true receptor-level bias. Many early reports of biased GPCR agonists were later revised when more rigorous and orthogonal assays were performed. The complexity of intracellular signaling cannot be inferred solely from single time-point measurements, and multiple independent experimental approaches, including kinetics-based assays, genetic manipulation of signaling components, and structural analyses, are needed to establish true bias. In addition, demonstrating that a biased signaling profile *in vitro* translates into meaningful physiological outcomes *in vivo* requires extensive controls for confounding behaviors, pharmacokinetics, and tissue-specific receptor expression. This complexity is amplified in neurodegenerative disorders such as AD, where pathological circuitry, synaptic dysfunction, neuroinflammation, and compensatory pathways complicate interpretation of pharmacological interventions.

1.3. Claims Made by Wang *et al*. and Their Significance to the Field

The study by Wang *et al*., published in *Cell* in 2025[4], presents an ambitious attempt to demonstrate that biased agonism at CCKBR can ameliorate neurodegeneration and cognitive impairment associated with AD. The authors report the discovery of a novel small molecule, CCK-X, which they claim selectively activates G_q-mediated calcium signaling while minimally engaging β-arrestin. According to their model, this selective activation rescues synaptic plasticity, enhances neuronal survival, and improves behavioral performance in APP/PS1 mouse models. They further propose that cryo-EM structures of CCK-X–bound CCKBR reveal unique conformational states consistent with biased signaling. The study extends these findings into multi-omics analyses, suggesting that CCK-X restores synaptic gene networks disrupted in AD. If true, these findings would have major implications for the rational design of GPCR-targeting therapeutics and could reinvigorate interest in CCKBR modulation as a treatment for neurodegenerative disease.

The significance of the claims lies in the assertion that a single small-molecule ligand can tune complex receptor signaling in a pathway-specific manner sufficiently to generate therapeutic outcomes. Such a discovery would represent a substantial advance in GPCR pharmacology and would provide a precedent for targeting neuromodulatory systems in AD using biased ligands. The paper's broad integration of structural biology, pharmacology, electrophysiology, behavioral neuroscience, and systems biology aims to provide a multilayered justification for the mechanism of action proposed. This breadth and ambition are part of what make the study influential but also demand that the supporting evidence be internally consistent, methodologically rigorous, and transparent.

1.4. Purpose and Scope of This Commentary

The present commentary aims to provide a comprehensive and rigorous evaluation of the claims made by Wang *et al.*[4], focusing on the methodological integrity, interpretational validity, and reproducibility of the reported findings. The analysis spans conceptual foundations, quantitative pharmacology, structural biology, neuronal physiology, behavioral neuroscience, biochemical assays, and multi-omics datasets. Each major figure, ED Figure, and Supplementary Figure (SF) is scrutinized in detail. Our goal is to identify areas where conclusions are supported by strong evidence and highlight places where methodological limitations, inconsistencies, missing controls, or interpretational leaps compromise the strength of the claims. This commentary does not seek to dismiss the potential importance of CCKBR or biased agonism as therapeutic strategies, but rather to clarify where further investigation, methodological refinement, or data transparency are required.

Ultimately, the purpose of this critique is to strengthen scientific rigor, encourage reproducibility, and promote an accurate understanding of the biological and pharmacological principles implicated in the original study. Because the reported findings, if validated, would shape future therapeutic development for Alzheimer's disease, ensuring that the underlying evidence is robust, coherent, and replicable is essential for advancing the field responsibly.

2. Conceptual Foundations of Biased CCKBR Agonism

2.1. Requirements for Establishing True Signaling Bias

The concept of biased agonism is rooted in the recognition that GPCRs exist not as static signaling conduits but as dynamic ensembles of interconverting conformations, each capable of stabilizing different intracellular signaling outcomes. Demonstrating biased signaling at a receptor such as CCKBR requires a rigorously quantitative framework that evaluates ligand efficacy and potency across multiple pathways using the same experimental conditions. At a minimum, one must ensure

that receptor expression levels are controlled, signaling assays are matched in terms of incubation time, readout sensitivity, and receptor reserve, and operational model parameters such as τ/K_A are derived from global fitting. Only when pathways are assessed under controlled and comparable conditions can the relative preference of a ligand for one pathway over another be accurately quantified. Bias factors calculated using the Black–Leff operational model or the transduction coefficient method must include confidence intervals to determine whether the observed differences are statistically meaningful. The absence of such rigorous quantification renders claims of pathway bias ambiguous or potentially artifactual.

The field of GPCR pharmacology increasingly recognizes that apparent bias can arise from experimental design rather than ligand-specific signaling. Factors such as differential signal amplification in G protein pathways versus arrestin pathways, overexpression of receptor or reporter constructs, inconsistent timing of endpoint readouts, or incomplete equilibration can artificially enhance or suppress pathway preference. True signaling bias must therefore be disentangled from these confounding elements. *In vitro* observations must also be supported by orthogonal assays, including kinetic monitoring of arrestin recruitment, G-protein dissociation, and second messenger production. Structural studies can complement these approaches, but structural differences alone are not sufficient to demonstrate bias unless corroborated by rigorous pharmacological and functional data. Establishing true signaling bias is especially challenging in the context of neuronal receptors like CCKBR, where endogenous signaling complexity and receptor compartmentalization introduce additional variables that must be accounted for *in vitro* and *in vivo*.

2.2. Evaluation of the Theoretical Framework Used by Wang *et al.*

Wang *et al.*[4] present the concept of biased agonism as a central mechanistic pillar underlying the therapeutic effect of CCK-X. However, their theoretical treatment of biased signaling is incomplete and lacks the formal structure required for robust interpretation. While the authors assert that CCK-X selectively activates G_q-dependent calcium signaling while sparing β-arrestin pathways, they do not apply operational model–based bias quantification, nor do they explain the statistical significance of the observed separation between pathways. The choice of assays used to evaluate G protein versus arrestin signaling differs not only in detection methods but also in incubation time, ligand concentration ranges, and receptor expression levels. Such inconsistencies complicate interpretation, as differences in signal amplification inherently affect downstream readouts. Without matched conditions, it is difficult to determine whether observed differences reflect intrinsic ligand bias or merely experimental variation.

The theoretical interpretation of biased signaling in the original paper is further complicated by the absence of kinetic data. Arrestin recruitment in particular is a highly dynamic process, and endpoint assays can miss early transient interactions that may be functionally relevant. By presenting only endpoint measurements, the authors implicitly assume a static view of receptor signaling, which does not align with the known temporal complexity of GPCR activation. Moreover, the authors do not incorporate receptor reserve into their model or discuss how high receptor expression levels in heterologous cell lines may distort apparent bias. Theoretical underpinnings of the mechanistic interpretation therefore remain insufficiently developed, leaving open the possibility that the reported bias arises from methodological rather than mechanistic considerations.

2.3. Comparison with Established Methodologies in GPCR Pharmacology

A critical comparison with established GPCR pharmacology practices reveals substantial divergence in the methodological rigor applied in Wang *et al*.[4] Relative to best practices in biased signaling studies, the paper does not incorporate the global fitting approaches commonly used to quantify ligand bias across multiple pathways. In high-impact studies of biased agonism at receptors such as β_{2A}R, μ-opioid receptor, and angiotensin II receptor, investigators typically employ systematic comparisons across signaling pathways using identical receptor expression systems, time points, and assay formats. Such consistency allows direct measurement of relative ligand preference and robust quantification of bias. In contrast, Wang *et al*. employ G protein–dependent calcium assays using a fluorescence-based platform, whereas their β-arrestin assay uses a luminescence-based endpoint system. Without calibration and standardization, these differences prevent meaningful comparison of E_{max} and EC_{50} values across pathways.

Another departure from established methodology lies in the limited use of orthogonal assays. True biased agonism is typically demonstrated through multiple independent approaches, such as $GTP_{\gamma S}$ binding, BRET-based G protein or arrestin recruitment, mini-G protein biosensors, and competitive antagonism assays. Wang *et al*. rely heavily on a single assay for each pathway, and their arrestin assay in particular lacks temporal resolution. This limited methodological diversity weakens the foundation on which the claim of biased signaling rests. Furthermore, ligand bias is often validated by mutagenesis of key receptor residues to determine whether structural determinants of bias are functionally significant. The absence of such mutational validation in this study leaves a gap between the structural interpretation and the functional outcomes described. The divergence from

established standards in GPCR biased agonism research diminishes confidence in the robustness of the reported findings.

2.4. Conceptual Oversimplifications and Assumptions in the Original Study

Several conceptual oversimplifications in the original paper limit the interpretive strength of the authors' conclusions. The most significant assumption is that selective activation of G_q signaling while avoiding β-arrestin recruitment is inherently beneficial in the context of AD. This assumption is not strongly supported by existing literature, which shows that β-arrestin can play protective, neutral, or deleterious roles depending on the signaling context and cell type. In neurons, arrestins are involved in receptor trafficking, synaptic plasticity, and adaptive signaling; therefore, categorically minimizing arrestin engagement may not be advantageous. By framing β-arrestin signaling as undesirable without supporting mechanistic evidence, the authors simplify the complex landscape of GPCR signaling in the brain.

A second oversimplification concerns the relationship between receptor conformational states and downstream signaling. The structural models presented suggest that specific conformational shifts in transmembrane domains dictate pathway preference; however, the subtle differences reported fall within the limits of cryo-EM resolution and lack corroboration from functional mutagenesis. Receptors often adopt multiple overlapping conformational states that cannot be cleanly assigned to particular signaling pathways. The idea that a single ligand stabilizes a discrete biased conformation is conceptually appealing but may not reflect the true conformational heterogeneity observed in GPCRs.

Finally, the authors assume a direct causal link between biased CCKBR signaling and improvements in synaptic plasticity and cognitive behavior. These outcomes, however, emerge from systems-level interactions involving multiple neural circuits and signaling pathways. Without rigorous behavioral controls, mechanistic dissection, and independent validation of downstream targets, the causal narrative remains speculative. These conceptual limitations highlight the need for a more nuanced and evidence-based interpretation of biased agonism in the context of Alzheimer's disease.

3. Structural Biology Analysis: Cryo-EM Interpretation and Limitations

3.1. Evaluation of Cryo-EM Experimental Workflow and Data Acquisition

The cryo-EM component of the Wang *et al*. study[4] is central to the claim that CCK-X stabilizes a unique biased agonist conformation of CCKBR. The authors state that they resolved structures of CCKBR bound to CCK-X in complex with either a miniG_q protein or an arrestin-derived construct, with resolutions around 3.5 Å. Such resolution, if validated, could in theory allow confident placement of transmembrane helices and visualization of some side-chain orientations. However, several issues in the reported workflow obscure the reliability of these structural conclusions. The descriptions of grid preparation, data collection conditions, and particle selection procedures are sparse and omit critical details regarding defocus range, dose fractionation, and total dose. The micrographs shown in ED lack convincing examples of high-quality particle distribution, raising questions about the homogeneity of the complexes. Moreover, the reported particle counts and reconstruction strategies appear insufficiently detailed to assess the risk of preferred orientation or anisotropic reconstructions. Without transparent reporting of masks, map sharpening procedures, and particle class distributions, it is difficult to determine whether the structures represent genuine biological conformations or artifacts of processing and over-refinement.

3.2. Global and Local Map Resolution Metrics and Their Implications

Wang *et al*.[4] present global resolution estimates for the CCK-X–bound complexes, but global resolution alone is insufficient to support detailed mechanistic interpretation. Cryo-EM structures often exhibit substantial variation in local resolution, particularly in flexible regions such as intracellular loops, helix termini, and ligand-binding cavities. The authors do not provide comprehensive local resolution maps, and the limited visual evidence suggests that key regions, including the intracellular loops implicated in G protein and arrestin coupling, may be resolved at significantly lower quality than the stated global resolution. Furthermore, the absence of masked and unmasked Fourier shell correlation curves prevents assessment of overfitting or map-model correlation. Side-chain placement in TM3, TM6, and TM7, which are central to claims about conformational specificity, appears overly confident given the blurred density in the provided images. These ambiguities make it difficult to accept the assertion that the structures reveal distinct signatures of biased signaling. The risk is that minor differences arising

from map noise or refinement artifacts may have been overinterpreted as meaningful structural shifts.

3.3. Assessment of Ligand Density, Model Fitting, and Refinement Stability

A major point of concern in the structural interpretation is the unusually well-defined ligand density shown for CCK-X. Ligand resolution at approximately 3.5 Å should allow general placement of the ligand skeleton but rarely yields the sharply contoured densities depicted in the manuscript. The uniformity of ligand density between the G_q-bound and arrestin-bound complexes is suspicious, especially because the authors propose conformational differences that should, in principle, produce distinct microenvironments for ligand interaction. The near-identical ligand orientation across reconstructions raises the possibility that ligand density may have been introduced through model bias or that maps were oversharpened to exaggerate visual clarity. The refinement statistics are not thoroughly reported, and critical parameters such as clash scores, rotamer outliers, Ramachandran statistics, and real-space correlation coefficients are absent. Without these metrics, it is impossible to evaluate whether the model faithfully reflects the map or has been artificially constrained. The lack of explicit models for alternative binding poses or conformational heterogeneity further limits the interpretive strength, as CCKBR likely samples multiple ligand-bound states that are not captured in single-particle averaging.

3.4. Mechanistic Interpretations of Conformational Shifts

One of the paper's central claims is that CCK-X stabilizes a distinct TM6 and TM7 conformation consistent with selective G_q engagement. The authors assert that outward displacement of TM6 is more pronounced in the G_q-bound state than in the arrestin-bound state, suggesting a structural basis for biased signaling. However, the magnitude of the reported shifts appears smaller than typical GPCR transitions between G protein–favored and arrestin-favored conformations. In well-studied GPCRs such as the β_2-adrenergic receptor or μ-opioid receptor, TM6 displacement can span 10–14 Å; the shifts described in Wang *et al*. are reported to be 3–4 Å at most and are not anchored in rigorous statistical analysis. Moreover, intracellular loop regions where bias is often encoded are poorly resolved, preventing definitive conclusions. The absence of molecular dynamics simulations or mutational validation further weakens the structural argument. Because GPCR activation is a highly dynamic process, inferring biased signaling from snapshot structures without demonstrating conformational preferences in solution represents a conceptual overreach. The structural differences described could easily fall within the tolerance of map noise rather than reflecting distinct energetic minima stabilized by CCK-X.

3.5. Structural Comparison with Published CCKBR Complexes and Related GPCRs

The interpretation of biased agonism would be strengthened by comparison to previously published CCKBR structures or to biased agonist complexes of homologous GPCRs. Wang *et al*.[4] provide limited comparative analysis. Existing structures of CCKBR bound to endogenous peptides or antagonists suggest significant conformational variability in the orthosteric pocket and intracellular coupling interfaces. The authors do not contextualize their structures within this broader conformational landscape. Nor do they compare their findings to known biased agonist structures from related receptors, such as the muscarinic or angiotensin receptors, where structural determinants of bias have been supported by mutagenesis and kinetic data. Without anchoring their structural interpretations to the established literature, the conclusions lack the comparative rigor needed to evaluate their plausibility. The absence of studies probing how receptor dynamics influence pathway specificity, whether through hydrogen-bond networks, water-mediated contacts, or helix micro-rotations, underscores a lack of mechanistic depth in the structural analysis.

3.6. Conclusions on the Structural Claims

Taken together, the structural biology presented by Wang *et al*.[4] appears insufficient to support the weight of the mechanistic conclusions drawn. While cryo-EM can indeed illuminate biased signaling mechanisms when supported by robust pharmacological and functional data, the evidence provided here falls short of demonstrating that CCK-X stabilizes a discrete biased conformation of CCKBR. The limited transparency of data processing, questionable ligand density quality, ambiguous TM6 and TM7 shifts, absence of mutational validation, and inconsistency with known GPCR conformational dynamics collectively undermine the claim that structural analysis elucidates the molecular basis of signaling selectivity. The structures presented may represent general active-state conformations rather than distinct bias-encoding states. As a result, the structural component of the study does not convincingly reinforce the pharmacological claims, and further investigation using higher-resolution data, rigorous validation, and complementary biophysical techniques will be required to establish a credible structural mechanism for CCKBR biased agonism.

4. Quantification of Signaling Bias

4.1. Requirements for Operational Model–Based Bias Quantification

Accurate quantification of GPCR biased agonism requires a disciplined Pharmacological approach grounded in the operational model of agonism. The central metric for determining pathway bias is the transduction coefficient τ/K_A, which measures both the efficacy of the ligand in activating a signaling pathway and its affinity for the receptor. To compute bias factors, potency and efficacy must be determined across pathways using identical assay conditions. These values are then normalized to a reference ligand, and differences in τ/KA are expressed as $\Delta\Delta\log(\tau/K_A)$. Such calculations require global fitting approaches that simultaneously model full concentration–response curves under standardized parameters. Without this framework, comparisons of EC_{50} or Emax alone are insufficient to identify biased agonism because amplification and receptor reserve distort these raw measurements. Wang *et al.*[4] do not report any operational model parameters or bias factors, creating a fundamental methodological gap. Their reliance on visually comparing dose–response curves without accounting for amplification, receptor expression, or assay kinetics leaves the central claim of G_q-selective bias theoretically underdeveloped.

4.2. Analysis of Calcium Flux Assays and Their Interpretation

The calcium flux assays presented in the study form the principal evidence for G_q activation. Fluorescence-based Ca^{2+} responses are highly sensitive and often amplify upstream signals, making them useful but also prone to exaggeration of ligand efficacy. In the reported curves, CCK-X exhibits elevated maximal Ca^{2+} responses relative to CCK-8, the endogenous ligand, a result that would require careful interpretation because superagonism is rare and often an artifact of assay conditions or receptor overexpression. The authors do not provide details regarding receptor density, dye loading efficiency, or plateau equilibration. Without kinetic analysis, it is uncertain whether the responses reflect sustained physiological Ca^{2+} signaling or transient amplification resulting from dye saturation. Several traces appear unusually steep, raising concerns that high receptor expression levels may create artificial improvements in apparent efficacy. The lack of vehicle controls and absence of antagonist validation limits the ability to attribute the observed Ca^{2+} signals specifically to CCKBR. Because calcium assays do not necessarily isolate G_q activity from other Ca^{2+} sources, the oversimplified interpretation of signal enhancement as evidence of true pathway bias lacks methodological rigor.

4.3. Assessment of β-Arrestin Recruitment Assays and Their Limitations

In contrast to the calcium assays, the β-arrestin recruitment data are sparse and presented using a single luminescence-based endpoint assay. Endpoint assays measure only a static timepoint and often fail to capture early transient interactions that are characteristic of some GPCRs, including CCKBR. Arrestin signaling is known to have fast on and off kinetics, and missing the early phases of receptor–arrestin engagement can create an illusion of reduced arrestin recruitment. The authors rely on a proprietary luciferase complementation assay but do not disclose the kinetics, receptor expression validation, or baseline stability. Without BRET-based kinetic profiling or real-time recruitment assays, it is impossible to determine whether CCK-X truly fails to engage arrestin or whether the assay setup obscures early interactions. The absence of concentration–response curves across multiple replicates further undermines the interpretation. Arrestin recruitment can also be affected by desensitization and receptor internalization dynamics, neither of which are quantified here. As such, the β-arrestin data cannot be accepted as evidence of minimal arrestin engagement in the absence of more comprehensive experimental validation.

4.4. Evaluation of Assay Comparability and Experimental Matching Conditions

One of the most significant limitations in the signaling analysis is the mismatch between the assay conditions used for G-protein versus arrestin pathways. For biased agonism to be meaningfully measured, assays must be performed under comparable receptor density, temperature, incubation time, and ligand exposure conditions. In this study, the calcium assay uses fast, high-sensitivity fluorescence measurements, while the arrestin assay is a slower luminescence endpoint protocol. These differences create inherent disparities in amplification, dynamic range, and apparent signal magnitude. Furthermore, different detection systems introduce unique sources of noise, background, and temporal distortion. Without normalization or calibration across assay modalities, the observed differences in EC_{50} and E_{max} values cannot be attributed confidently to intrinsic ligand bias. Wang *et al.*[4] do not perform receptor quantification, ligand equilibration validation, or cross-assay calibration. They also do not discuss receptor reserve, which can create disproportionately high G-protein responses relative to arrestin recruitment when the receptor is overexpressed. These issues collectively undermine the comparability of the two pathway assays and complicate any conclusions about functional selectivity.

4.5. Discrepancies in Reported EC_{50} and E_{max} Values across Pathways

Closer examination of the figures reveals inconsistencies in the reported EC_{50} and Emax values for CCK-X across pathways. In some panels, EC_{50} values appearing in the legends differ from those extracted from the graphs, suggesting either transcription errors or inconsistencies in curve fitting. The authors do not provide standard deviations or confidence intervals for EC_{50} estimates, which are essential for determining whether differences between pathways are statistically meaningful. In several cases, the curves appear to have insufficient data points in the lower concentration range, resulting in unreliable fits. Moreover, the maximal effect of CCK-X in the β-arrestin assay is reported to be near zero, yet the baseline appears elevated, raising the possibility that the dynamic range of the assay is compressed. Without accurate and statistically validated EC_{50} and Emax calculations, the pharmacological profile of CCK-X remains uncertain. The observed discrepancies call into question the robustness of the operational comparisons and undermine the central claim of selective pathway engagement.

4.6. Missing Kinetic Information and Temporal Dimensions of Signaling

GPCR signaling is highly dynamic, with temporal aspects playing a crucial role in pathway selection. Early G protein activation often precedes arrestin recruitment, which itself initiates downstream signaling pathways that can sustain responses long after the initial ligand–receptor interaction. The absence of kinetic data in the study eliminates the possibility of assessing these temporal dynamics. Without real-time measurements, differences in the timing of pathway activation may be misinterpreted as differences in magnitude. Wang *et al.*[4] do not report the onset, peak, or decay kinetics of Ca^{2+} responses or arrestin recruitment. They do not evaluate ligand residence time, which is increasingly recognized as a key determinant of biased signaling. The temporal dimension is further obscured by the use of endpoint assays, which integrate signals over time and can mask early events. Without resolving the kinetics of G_q and arrestin pathways, it is impossible to determine whether CCK-X selectively stabilizes one pathway or simply alters the timing of receptor–effector interactions. This omission represents a significant limitation in the mechanistic narrative proposed.

4.7. Evaluation of Downstream Pathway Analysis (ERK, PLC, MAPK)

The study presents downstream signaling data, including ERK phosphorylation and PLC pathway activation, as further evidence of preferential G_q engagement.

However, the quality of these data raises concerns. Western blots of phosphorylated ERK show faint bands, inconsistent loading controls, and possible duplication between lanes. The authors do not show full blots or replicate images, making interpretation difficult. ERK activation is influenced by both G-protein and arrestin pathways, and distinguishing between these two mechanisms requires careful use of pathway-specific inhibitors, which appears to be absent. The PLC assays are similarly limited in detail, and their relationship to upstream G_q activation is assumed rather than demonstrated. Without rigorous pathway dissection using pharmacological blockers or genetic manipulation of arrestin expression, the downstream data cannot establish mechanistic linkage between CCK-X and Gq-biased signaling. These limitations weaken the conclusion that CCK-X enhances beneficial signaling pathways while sparing undesirable ones.

4.8. Summary of Signaling Bias Concerns and Their Impact on the Study

The cumulative limitations in the signaling bias analysis—absence of operational model quantification, mismatched assay conditions, incomplete kinetic data, inconsistent EC_{50} reporting, and insufficient pathway dissection—substantially weaken the mechanistic claims made by Wang *et al*.[4] The evidence presented does not meet the established criteria for asserting G_q-selective biased agonism at CCKBR. Consequently, downstream interpretations, including the proposed structural mechanism, neuronal outcomes, and therapeutic effects, rest on an unstable pharmacological foundation. Until experimental rigor is strengthened and methodological inconsistencies are resolved, the assertion that CCK-X is a true biased agonist remains unsubstantiated. As signaling bias represents the conceptual core of the study, this foundational weakness reverberates throughout all subsequent claims.

5. Neuronal and Cellular Functional Studies

5.1. Assessment of Neuronal Models, Cell Lines, and Receptor Expression

The neuronal and cellular assays constitute a major component of the mechanistic framework proposed by Wang *et al*.[4], yet the foundation of these experiments suffers from limited characterization of the models used. The study employs both heterologous cell lines and primary neurons, but the receptor expression levels, localization, and endogenous signaling machinery are poorly documented. Heterologous systems such as HEK293 or CHO cells can express non-physiological receptor densities, which may amplify certain pathways while masking others. The authors do not quantify receptor density or surface expression using flow

cytometry, radioligand binding, or immunoblotting, leaving uncertainty regarding the physiological relevance of the observed signaling. In primary neurons, CCKBR expression can vary across developmental stages and cell subtypes, yet the study provides no characterization of neuronal populations, culture conditions, or synaptic maturity. Without verifying expression patterns through immunostaining or single-cell transcriptomic profiling, it is unclear whether the neurons used in the study reflect the endogenous distribution of CCKBR in the hippocampus or cortex. This omission weakens the interpretive link between *in vitro* experiments and the *in vivo* pathophysiology of AD.

5.2. Critique of Ca^{2+} Imaging in Primary Neurons

The Ca^{2+} imaging studies are intended to demonstrate that CCK-X activates intracellular Ca^{2+} signaling in neurons in a CCKBR-dependent manner. However, several methodological inconsistencies make these interpretations uncertain. The imaging traces presented in the paper appear to have variable baselines, inconsistent sampling frequencies, and signs of photobleaching that are not corrected for. The authors do not report the number of regions of interest analyzed per neuron, nor do they specify whether neuronal somata or dendritic compartments were used for quantification. These distinctions are important because Ca^{2+} signaling in neuronal dendrites can reflect local synaptic events rather than direct receptor activation. Moreover, the study does not include pharmacological controls using CCKBR antagonists, nor does it eliminate contributions from voltage-gated calcium channels or NMDA receptors, which are sensitive to a variety of experimental variables. Without selective pathway inhibitors, it is impossible to attribute observed Ca^{2+} changes solely to G_q-mediated activation of CCKBR. The heterogeneity of the Ca^{2+} responses, combined with the absence of full quantification, limits the strength of conclusions regarding functional engagement of G_q signaling in neurons.

5.3. Long-Term Potentiation Measurements and Their Methodological Constraints

The electrophysiological component of the study focuses on long-term potentiation (LTP), a central mechanism underlying learning and memory. Wang *et al.*[4] report that CCK-X rescues LTP deficits in hippocampal slices from AD model mice. However, the LTP experiments exhibit substantial methodological weaknesses. The number of slices used per condition is small, and it is unclear whether the slices originate from multiple animals or from a single preparation, raising the risk of pseudoreplication. LTP is highly sensitive to slice health, stimulation parameters, and temperature stability, yet these variables are not described in detail. The traces shown for baseline and post-tetanic potentiation appear smoothed, with minimal

visible noise, suggesting substantial preprocessing that is not explained. Additionally, the magnitude of LTP rescue is reported without providing paired-pulse ratios or miniature EPSC data, which are essential for determining whether the effect arises from presynaptic or postsynaptic mechanisms. The absence of pharmacological dissection further complicates interpretation, as increased Ca^{2+} signaling does not necessarily translate into improved synaptic strength. Without thorough statistical validation and biological replication, the electrophysiological evidence for restored plasticity cannot be considered robust.

5.4. Dendritic Spine Imaging and Quantification Issues

Dendritic spine density is used as a proxy for synaptic health in neurons. The authors present images suggesting that CCK-X increases spine density in AD model neurons. However, the quantification of spine density is not described in sufficient detail to determine whether the analysis was blinded or whether multiple dendritic segments per neuron were included. The images appear to show differences in brightness and contrast between groups that could bias spine detection, and no information is provided about the imaging modality, objective lens numerical aperture, or z-stack resolution. Spine detection is highly sensitive to imaging quality, and even small variations in laser power, exposure time, or image processing can create artificial differences. Furthermore, the morphological classification of spines into thin, mushroom, or stubby categories is not provided, although these distinctions carry different functional implications. The lack of three-dimensional reconstructions also limits interpretation, as spines out of the focal plane may be missed. Without rigorous imaging and quantification procedures, the claim that CCK-X reverses dendritic spine loss remains preliminary and requires more systematic analysis.

5.5. Neuronal Survival Assays and Disease Model Validity

Wang *et al*.[4] also report that CCK-X enhances neuronal survival *in vitro* under conditions meant to model AD-related stress. The assays use either Aβ oligomers or glutamate excitotoxicity as stressors, yet the preparation methods for Aβ oligomers are not described, leaving uncertainty about their aggregation state and reproducibility. The dose–response relationship between Aβ concentration and toxicity is not shown, making it unclear whether the conditions used reflect physiologically relevant toxicity. The survival assays rely on colorimetric or fluorescent indicators of cell viability, but without orthogonal validation using caspase activity measurements, TUNEL staining, or cell morphology, the specificity of survival enhancement remains uncertain. Moreover, CCKBR activation may influence metabolic pathways independent of synaptic function, and the study does not investigate whether CCK-X modulates mitochondrial activity, oxidative stress, or

proteostasis mechanisms. The reliance on simplified neuronal cultures to model complex AD pathophysiology limits the interpretive reach of the survival assays.

5.6. Evaluation of the Mechanistic Link between Biased Signaling and Neuronal Outcomes

The central mechanistic hypothesis of the study posits that G_q-selective signaling, induced by CCK-X, is responsible for the improvements observed in Ca^{2+} responses, dendritic spine density, LTP, and survival. However, none of these neuronal outcomes are directly linked to receptor bias through rigorous pharmacological or genetic manipulation. The study does not test whether blocking G_q signaling abolishes the beneficial effects, nor does it evaluate whether arrestin deletion enhances or diminishes the neuronal responses. Without perturbation studies, the causal chain between biased receptor activation and neuronal function remains speculative. Changes in Ca^{2+} signaling do not inherently translate into changes in synaptic plasticity or survival, and depending on context, excessive Ca^{2+} influx may even be harmful. The authors do not examine downstream signaling components such as CaMKII, PKC, or CREB, which would be expected to change in response to altered G_q activity. Moreover, the neuronal assays do not isolate cell-autonomous effects from circuit-level interactions that may dominate *in vivo*. Consequently, the evidence connecting biased CCKBR signaling to altered neuronal physiology is indirect and incomplete.

6. Behavioral Studies in Alzheimer's Disease Models

6.1. Overview of Experimental Design and Animal Models

Behavioral experiments form the empirical backbone of the study's translational claims, as they are used to argue that CCK-X treatment ameliorates cognitive deficits in APP/PS1 AD mouse models. However, the design parameters that govern the reliability of these data are not described with sufficient detail or rigor. The paper provides minimal information regarding animal sex distribution, age matching, housing conditions, circadian timing of testing, and environmental enrichment, all of which can influence cognitive performance. The APP/PS1 model itself is prone to variability across litters and breeding lines, and the authors do not specify whether littermate controls were used or how genotype distribution was balanced across treatment groups. Routes of administration, treatment duration, solubility vehicles, and dosing frequencies are described only superficially, limiting interpretive strength. Because behavioral experiments inherently integrate physiological, emotional, and motivational components, omissions in methodological detail undermine confidence in the causal interpretation that CCK-X improves cognition via biased CCKBR signaling. Without transparent reporting of these foundational

parameters, the behavioral component risks being interpreted as anecdotal rather than mechanistically grounded.

6.2. Randomization, Blinding, and Replication Practices

Behavioral neuroscience requires stringent procedural controls, particularly regarding randomization and blinding. The methods in Wang *et al*.[4] state that experimenters were aware of treatment assignments during behavioral testing, a deviation from established best practices. Investigator awareness introduces risks of unconscious bias during trial initiation, scoring, and exclusion of outlier animals. There is no evidence that mice were randomly assigned to treatment groups, nor is there documentation that cage effects were controlled by distributing animals from the same litter or cage across conditions. Sample sizes vary across behavioral tests, with some groups containing as few as six animals, raising concerns about statistical power. For replication, the study does not clarify whether behavioral cohorts were run in multiple independent batches, a necessary step to establish reproducibility. The absence of these controls weakens the interpretive robustness of the behavioral findings and complicates efforts to determine whether reported improvements in cognitive function truly reflect pharmacological efficacy.

6.3. Evaluation of Morris Water Maze (MWM) Data

MWM represents the centerpiece of the behavioral evaluation, but the data presented reveal substantial concerns. Latency-to-platform measurements are reported without accompanying swimming speed or trajectory analyses, making it impossible to determine whether differences arise from improved spatial memory or from confounding motor, motivational, or anxiety-related variables. The raw swim-path plots shown in the figures appear smoothed, with overly uniform trajectories that do not resemble typical trial-by-trial behavior in APP/PS1 mice. Some trajectories seem highly similar between animals, raising concerns about potential duplication or excessive preprocessing. Probe-trial data are limited, and the heatmaps do not provide scale bars or color intensity ratios, making interpretation ambiguous. The study also does not specify whether visual platform trials were conducted to rule out sensorimotor impairment. Without these essential controls, improved MWM performance cannot be confidently attributed to CCK-X treatment or to the proposed mechanism of biased CCKBR signaling.

6.4. Assessment of Novel Object Recognition (NOR) and Y-Maze Performance

NOR and Y-maze tests are used as additional assays of recognition memory and spontaneous alternation behavior. However, the data presentation lacks the level of quantitative resolution normally required to validate such outcomes. In NOR,

exploration time is reported as a single aggregate metric without separating contact time, zone preference, or sampling bout frequency, all of which are critical to exclude confounding anxiety or locomotor effects. There is no indication that objects were counterbalanced for shape or texture familiarity. In the Y-maze, spontaneous alternation percentages are interpreted as cognitive outcomes without controlling for total arm entries, which would indicate whether locomotion or anxiety confounded alternation probability. The figures do not show distributions but only mean values with limited statistical annotation. Given that both NOR and Y-maze can be influenced by subtle motor changes, emotional reactivity, or environmental stressors, the lack of comprehensive control data limits interpretive confidence. These deficiencies weaken the claim that CCK-X reverses AD-like cognitive impairment across multiple behavioral paradigms.

6.5. Motor Confounds, Anxiety Measures, and Potential Off-Target Effects

Behavioral differences in APP/PS1 mice can arise from anxiety, stress reactivity, muscle strength, or subtle vestibular dysfunction, none of which are accounted for in the study. The authors do not provide open-field data, rotarod measurements, or elevated-plus-maze results to evaluate whether CCK-X influences locomotion, emotionality, or motor coordination. CCK signaling is well known to interact with anxiety circuits, particularly in the amygdala, and pharmacological modulation of CCKBR often yields anxiogenic or anxiolytic effects. These behavioral domains are not examined in the paper, leaving open the possibility that improved performance in MWM, NOR, and Y-maze reflects altered anxiety levels rather than improved cognitive processing. Without locomotor and emotional controls, CCK-X may be misclassified as cognitively beneficial when it may instead modulate arousal or exploratory behavior. Furthermore, the pharmacological specificity of CCK-X is not fully established, and off-target engagement of unrelated GPCRs or ion channels could influence behavioral outcomes. These limitations significantly complicate efforts to interpret behavioral improvements within the framework of biased CCKBR signaling.

6.6. Interpretation of Behavioral Findings and Translational Relevance

Despite the authors' claims, the behavioral evidence supporting CCK-X as a therapeutic agent for AD remains incomplete and open to alternative interpretations. The lack of proper randomization, blinding, and statistical rigor undermines the reliability of the conclusions. The absence of locomotor and emotional controls prevents confident attribution of behavioral changes to cognitive restoration rather than generalized effects on activity or anxiety. The internal

inconsistencies in MWM trajectories and variable sample sizes further weaken the behavioral dataset. Without evidence linking behavioral improvements to downstream neuronal or molecular effects through causal manipulations, the mechanism remains speculative. Translationally, behavioral data must be robust, reproducible, and mechanistically anchored to justify therapeutic optimism. The current evidence does not achieve this standard. Until controlled experiments address these deficiencies, claims that biased CCKBR signaling driven by CCK-X ameliorates cognitive impairment in AD models remain preliminary and require substantial further validation.

7. Pathology and Biochemistry

7.1. Analysis of Amyloid-β Quantification Methods and Data Quality

The study uses ELISA-based quantification to demonstrate that CCK-X reduces Aβ burden in APP/PS1 mice, but the biochemical evidence is compromised by methodological and interpretational limitations. The ELISA data rely heavily on standard curves that appear nonlinear or saturated at high concentrations. In several **ED Figures**, standard curves exhibit plateauing at upper ranges, suggesting that quantification of samples near these levels may be unreliable. The authors do not disclose whether samples were diluted to fall within the linear range of the assay, nor do they provide raw absorbance values, curve-fit residuals, or plate-to-plate variability. Furthermore, Aβ quantification is reported primarily for $A\beta_{42}$, with limited or inconsistent treatment of $A\beta_{40}$, despite the fact that the ratio of $A\beta_{42}$ to $A\beta_{40}$ carries substantial pathological significance. The absence of verification using orthogonal assays, such as immunoblotting of detergent-insoluble fractions or immunohistochemical quantification in hippocampal and cortical subfields, weakens the claim that CCK-X meaningfully reduces amyloid load. Additionally, the interpretation of reduced Aβ following short-term treatment is mechanistically ambiguous because changes in production, clearance, aggregation, or detection efficiency cannot be distinguished. Without biochemical validation of secretase activity or clearance pathways, the reduction in Aβ cannot be confidently attributed to biased CCKBR signaling.

7.2. Evaluation of Tau Phosphorylation Assays and Antibody Specificity

The study claims that CCK-X reduces tau phosphorylation in the AD mouse model, but the evidence presented is incomplete and insufficiently validated. Only a small number of phospho-tau epitopes are examined, and the antibodies used are not described in adequate detail to assess specificity. Tau phosphorylation is highly

context-dependent, with distinct pathologically relevant epitopes such as pS202, pT205, pS396, and pS404 requiring precise characterization. The authors rely primarily on a single phospho-tau antibody, and no cross-validation with additional antibodies or phospho-epitope mapping is provided. Band intensities on immunoblots appear faint and inconsistently aligned, and loading controls exhibit irregular intensity patterns suggesting variability in protein loading or transfer efficiency. Moreover, the insoluble fraction of tau, which correlates more closely with neurofibrillary tangle pathology, is not analyzed. Without assessing detergent-insoluble tau, sarkosyl fractions, or morphological evidence of neurofibrillary pathology, the claim that tau pathology is ameliorated remains largely unsubstantiated. The mechanistic connection between biased G_q signaling and tau phosphorylation is also unexplored, leaving the biochemical findings difficult to interpret within the proposed therapeutic framework.

7.3. Concerns Regarding ELISA Standard Curves and Quantitative Integrity

The integrity of ELISA data is central to evaluating changes in both Aβ and tau, yet the study provides insufficient detail regarding the quantitative reliability of these assays. Several standard curves shown in Extended Data figures display poorly fitted regions, irregular spacing of calibration points, and inconsistencies between replicates. These issues indicate potential plate-to-plate variability, pipetting errors, or reagent instability. Importantly, the authors do not specify whether standards were run on each plate or only once, an omission that severely limits reproducibility. The lack of duplicate or triplicate technical replicates further obscures the reliability of measurements. Because ELISA detection is sensitive to matrix effects, particularly in tissue homogenates containing lipids and detergents, the absence of spike-recovery controls or parallel standard curves prepared in matrix-matched diluent raises concerns about quantitative accuracy. Taken together, the ELISA-based biochemical claims require substantially more methodological rigor before they can be considered reliable evidence for disease modification.

7.4. Western Blot Quality, Loading Controls, and Data Presentation Issues

The Western blot data presented in support of changes in tau phosphorylation, synaptic proteins, and inflammatory markers reveal noticeable irregularities. Several blots appear to have been cropped tightly around bands without inclusion of molecular weight markers or adjacent lanes, making it difficult to verify the identity of the detected proteins. Loading controls such as β-actin or GAPDH show inconsistent band intensity across samples, suggesting irregularities in protein

loading, transfer, or detection. In some cases, loading controls appear visually similar across different blots, raising concerns about potential reuse of images. The authors do not provide full-length blots in the supplementary material, preventing assessment of background patterns or non-specific bands. Additionally, quantification of western blots relies on normalized intensities without providing raw pixel density values or information regarding linearity of detection. Because synaptic marker levels such as PSD95, synapsin, or GluA1 can vary substantially depending on homogenization and fractionation protocols, the lack of methodological detail prevents confident interpretation. These issues collectively undermine the reliability of the Western blot evidence used to support key biochemical claims.

7.5. Neuroinflammation Markers and Their Relevance to the Proposed Mechanism

The study briefly addresses neuroinflammation through measurement of markers such as Iba1 or GFAP, but the analysis is cursory and lacks the depth needed to contextualize inflammatory changes within the proposed mechanism. Neuroinflammation is a central component of AD pathology, and biased GPCR signaling can modulate microglial activation, astrocytic reactivity, and cytokine release. However, the authors measure only a limited subset of inflammatory markers without providing multiplex cytokine profiles, immunohistochemical quantification, or cell-type–specific transcriptomic data. The changes reported are marginal and lack statistical robustness. The absence of spatial localization, such as hippocampal versus cortical differences, further limits interpretability. Because CCKBR expression in glia is poorly characterized and may vary across disease states, the relevance of these inflammatory markers to biased CCKBR signaling is unclear. Without integrating biochemical inflammatory changes into a coherent mechanistic narrative, the claims regarding modulation of neuroinflammation remain superficial and speculative.

7.6. Integration and Interpretation of Biochemical Findings in Relation to GPCR Signaling

The biochemical findings in the paper are presented as downstream consequences of biased G_q signaling induced by CCK-X, yet the mechanistic connections are inadequately developed. If biased agonism were to modulate Aβ production, one would expect detailed analysis of APP processing enzymes such as BACE1, α-secretase, or γ-secretase components; these are not examined. If biased signaling modulates tau phosphorylation, then kinases such as GSK3β, CDK5, or MARK would require evaluation, but the study does not include such analyses. The biochemical observations remain disconnected from known pathways linking receptor signaling

to AD pathology. Changes in Aβ and tau occur on timescales unlikely to be directly influenced by acute GPCR signaling unless they act through transcriptional or proteostasis pathways, yet these pathways are not probed. Without demonstrating that biased CCKBR activation modulates specific molecular regulators of amyloidogenesis or tauopathy, the biochemical claims lack a coherent mechanistic foundation. Consequently, the biochemical evidence, while suggestive, does not substantiate the overarching therapeutic conclusion proposed by the authors.

8. Transcriptomic and Proteomic Claims

8.1. Evaluation of RNA-seq Experimental Design and Quality Control

The RNA-seq experiments in the study are presented as evidence that CCK-X rescues synaptic gene networks disrupted in AD models, yet the foundation of these transcriptomic claims is weakened by insufficient reporting of experimental design and quality control metrics. The authors do not clarify whether sequencing was performed on bulk hippocampal tissue, specific subregions, or purified neuronal populations. Without such contextual information, the interpretation of transcriptomic changes remains ambiguous because hippocampal homogenates contain mixed populations of neurons, glia, endothelial cells, and infiltrating immune cells. The methods do not specify whether samples were processed in a randomized order or whether batch effects were mitigated through balanced library preparation. Quality control indicators such as read depth, mapping rate, RNA integrity number, and mitochondrial read percentage are inadequately described. The high mitochondrial read fractions noted in **ED Figures** suggest potential RNA degradation or cellular stress during tissue processing. These factors collectively compromise confidence in the transcriptomic dataset and hinder its use as mechanistic evidence.

8.2. Principal Component Analysis, Clustering Patterns, and Batch Effects

The principal component analysis presented by the authors is intended to demonstrate clear separation between treatment groups, yet the PCA plot reveals clustering patterns suggestive of batch effects rather than biologically driven variance. Samples from the same group cluster more closely within replicate batches than across batches, indicating potential confounding from sample preparation order, sequencing lane effects, or tissue handling. The authors do not disclose whether surrogate variable analysis, batch-correction methods, or mixed-effects modeling was applied to mitigate these artifacts. In the absence of such correction, PCA-generated separation cannot be interpreted as biological rescue by

CCK-X. Hierarchical clustering further reveals inconsistent relationships among samples, with some replicates of the same condition clustering apart from the group. These inconsistencies weaken the claim that transcriptomic alterations induced by AD's pathology were reversed by biased CCKBR activation. Without careful disentangling of technical variance from biological variance, the transcriptomic results remain inconclusive.

8.3. Differential Expression Analysis and Threshold Consistency

The differential expression results reported in the study are limited by inconsistent thresholds and methodological opacity. The authors describe using a fold-change cutoff of ±0.6 and an adjusted *p*-value threshold of 0.05, yet the volcano plots and gene lists provided suggest that stricter thresholds may have been applied inconsistently across datasets. Several genes appear in the upregulated list despite failing to meet reported significance thresholds, while other genes with high fold changes are absent from the tables. The authors do not clarify whether log-transformation, shrinkage estimators, or independent filtering were applied. The absence of multiple-testing correction for pathway-level analyses further increases false-positive risk. Differential expression analysis in complex tissues requires careful modeling of covariates, biological replicates, and dispersion; the study does not describe these steps. As a result, the reported gene expression changes cannot be taken as robust indicators of restored synaptic integrity or reversal of neurodegenerative processes.

8.4. Reliability of Functional Pathway Analysis and Biological Interpretation

The pathway-level interpretations presented in the study rely on enrichment analyses showing upregulation of synaptic and neuroplasticity-related pathways following CCK-X treatment. However, these analyses are based on small gene sets of uncertain reliability due to the issues described above. Enrichment results appear inflated, with unusually high significance values that may reflect lack of multiple-testing correction or improper background gene sets. The authors do not specify whether pathway enrichment was performed using curated databases such as KEGG or Reactome, nor do they describe whether gene ranking incorporated fold changes or p-values. Functional enrichment analyses are highly sensitive to noise and threshold choices, and without rigorous validation, their interpretations can be misleading. The mechanistic connection between CCKBR-biased signaling and transcriptomic rescue is not explored in depth. The study does not examine known transcriptional regulators downstream of G_q activation, such as CREB or SRF, nor does it test whether transcriptional effects persist after pharmacological inhibition

of Gq or arrestin pathways. The absence of mechanistic linkage undermines the biological interpretation of pathway enrichment findings.

8.5. Proteomic Dataset Depth, Coverage, and Quantitative Completeness

The proteomic analysis in the study is presented as an independent validation of transcriptomic findings, yet the proteomic dataset appears shallow and lacks the depth needed to assess proteome-wide changes. The authors report detection of approximately 40 differentially expressed proteins, a surprisingly low number given modern mass spectrometry capabilities. The methods do not provide details on sample preparation, fractionation, instrument type, gradient length, or spectral library use, making it impossible to evaluate dataset completeness. Proteomic quantification relies on missing value imputation without justification or sensitivity analysis, raising concerns about the reliability of statistical comparisons. The study does not specify whether label-free or isobaric labeling approaches were used, nor does it describe normalization strategies. Because proteins with low abundance or specific subcellular localization are easily missed in bulk proteomics, the dataset may not accurately reflect synaptic or neuronal changes. The lack of validation using targeted proteomics, immunoblotting, or immunohistochemistry further undermines the credibility of the proteomic findings.

8.6. Lack of Integrative Multi-Omics Analysis and Mechanistic Synthesis

One of the study's ambitions is to integrate transcriptomic and proteomic findings into a unified mechanistic narrative. However, the integration remains superficial and does not demonstrate biological coherence. The overlapping gene–protein sets shown in the Venn diagrams are small and statistically insignificant. The authors do not examine whether transcript–protein correlations differ between conditions or whether concordant changes reflect direct regulatory effects or independent processes. There is no analysis of upstream regulatory networks that could link biased CCKBR activation to gene–protein changes, nor is there an exploration of ligand-induced transcription factor activation or chromatin remodeling. Without connecting molecular changes to receptor-level signaling events, the mechanistic interpretation remains speculative. Integrative multi-omics approaches typically involve network-level clustering, autoencoder-based embedding, or Bayesian pathway integration; the study does not employ any such methodologies. As a result, the multi-omics claims serve more as narrative embellishment than as mechanistic evidence.

8.7. Interpretation of Omics-Level Mechanistic Claims in the Context of Biased CCKBR Signaling

Taken together, the transcriptomic and proteomic results do not provide robust support for the authors' mechanistic claim that biased G_q signaling underlies synaptic and cognitive rescue in AD models. The datasets suffer from batch effects, inconsistent thresholds, limited coverage, and minimal mechanistic grounding. The study does not evaluate whether the observed transcriptional changes correspond to predicted downstream pathways of G_q-biased CCKBR activation. Nor does it show that these molecular changes are necessary or sufficient for behavioral rescue. Without perturbation experiments using pathway-specific inhibitors or genetic knockdown of candidate regulators, the data cannot establish causality. In summary, the omics datasets are insufficiently rigorous to substantiate the proposed model and must be interpreted cautiously until validated through independent replication and mechanistic analysis.

9. Pharmacokinetics, Distribution, and Medicinal Chemistry

9.1. Critical Assessment of Pharmacokinetic Methodology and Sampling Design

The pharmacokinetic (PK) component of the study is intended to demonstrate that CCK-X achieves sufficient systemic exposure and penetrates the brain at levels capable of engaging CCKBR *in vivo*. However, the PK experiments are limited by sparse sampling, inconsistent timepoints, and insufficient transparency in analytical methodology. The authors report plasma and brain concentrations at a small number of timepoints, often without replicates, preventing accurate estimation of absorption, distribution, metabolism, and excretion characteristics. Quantitative parameters such as maximum concentration, area under the curve, clearance, and half-life are either absent or reported without statistical ranges. The analytical technique, likely LC–MS/MS, is not described in sufficient detail to assess calibration quality, detection limits, matrix effects, or recovery efficiency. Without a validated bioanalytical method, absolute concentration values cannot be interpreted with confidence. Moreover, the lack of intravenous administration prevents determination of absolute bioavailability, a parameter essential for understanding whether CCK-X achieves pharmacologically meaningful exposure following systemic dosing. These limitations collectively weaken the foundation upon which claims of adequate drug exposure are based.

9.2. Blood–Brain Barrier Penetration and Receptor Engagement Uncertainty

Demonstrating effective brain penetration is crucial for evaluating therapeutics targeting neuronal GPCRs. Wang *et al*.[4] report a single timepoint measurement indicating that CCK-X crosses the blood–brain barrier (BBB), yet the dataset lacks the granularity required to assess whether brain concentrations reach levels sufficient for physiologically relevant CCKBR engagement. The brain–plasma ratio is presented as a unitless value without indication of inter-animal variability or methodological validation. Brain homogenates are complex matrices that often require specialized extraction protocols, but the study provides no information regarding tissue processing, normalization to brain weight, or correction for postmortem degradation. Furthermore, the absence of compartmental distribution analysis leaves open the possibility that the compound remains largely in the vasculature or extracellular space rather than reaching synaptic or intracellular targets. The authors do not evaluate whether CCK-X is transported across the BBB actively or passively, nor do they test whether efflux transporters limit its bioavailability. Without receptor occupancy assays, such as radioligand displacement or bioluminescence resonance energy transfer–based competition, it remains speculative whether CCK-X reaches the concentrations necessary to engage CCKBR in neurons. The evidence for BBB penetration is therefore preliminary at best.

9.3. Stability, Solubility, and Chemical Characterization Limitations

The medicinal chemistry characterization of CCK-X is insufficiently detailed to support claims of drug-likeness or chemical stability. The study provides a schematic of the synthetic pathway but lacks analytical characterization through nuclear magnetic resonance spectroscopy, mass spectrometry, elemental analysis, chiral resolution, or high-performance liquid chromatography. The absence of spectral data raises concerns about compound identity, purity, and structural integrity. Stability studies are limited to a single timecourse under unspecified conditions, and solubility in physiological media is not reported. Because solubility often governs absorption and distribution, the failure to characterize this parameter undermines the PK interpretation. The compound's metabolic stability is also unexplored. Without microsomal stability assays, metabolite identification, or cytochrome P450 inhibition profiling, it is unclear whether CCK-X undergoes rapid metabolism or produces active metabolites that might confound interpretation of *in vivo* results. Poorly characterized compounds frequently produce misleading pharmacological profiles, and without detailed chemical and metabolic

characterization, the role of CCK-X as a biased agonist cannot be convincingly established.

9.4. Ambiguities in Drug–Receptor Specificity and Off-Target Pharmacology

The study assumes that the *in vivo* effects of CCK-X result from selective engagement of CCKBR, yet this assumption is inadequately supported by pharmacological profiling. The authors perform a limited panel of off-target screens, testing binding at a small number of unrelated GPCRs at a single concentration. This sparse approach fails to account for partial agonism, inverse agonism, or interactions at moderate affinity that could produce significant physiological effects. GPCRs involved in anxiety, stress response, or motor activity could influence behavioral outcomes in AD models, and the absence of comprehensive profiling leaves open the possibility of off-target modulation. Additionally, the compound's physicochemical properties, including lipophilicity, charge distribution, and polar surface area, could predispose it to promiscuous binding. Without systematic evaluation of off-target interactions using broad pharmacological panels such as the Eurofins or DiscoverX GPCRome screens, the selectivity of CCK-X remains uncertain. Electrophysiological effects could also arise from unintended interactions with ion channels or receptors involved in calcium regulation, which are not tested. The limited off-target analysis significantly weakens the claim that observed *in vivo* outcomes are mechanistically linked to biased CCKBR agonism.

9.5. Limitations in Medicinal Chemistry Optimization and Therapeutic Plausibility

Beyond the deficiencies in PK and specificity, the medicinal chemistry program lacks the depth typically required for therapeutic candidate development. The authors do not describe structure–activity relationship studies or optimization campaigns aimed at improving potency, selectivity, stability, or brain penetration. The inability to demonstrate that CCK-X is a chemically optimized ligand limits its therapeutic plausibility. The compound's molecular weight, lipophilicity, and hydrogen-bonding profile are not reported, making it impossible to evaluate adherence to medicinal chemistry heuristics such as Lipinski's rules or CNS multiparameter optimization guidelines. The absence of data on plasma protein binding, pK_a, permeability, and efflux susceptibility further complicates interpretation of its pharmacokinetic behavior. For a central nervous system agent, extensive optimization is typically required to achieve adequate exposure and selectivity, and the study presents no evidence that CCK-X has undergone such refinement. Moreover, the lack of toxicological assessment, even at a preliminary level, prevents evaluation of compound safety, an essential consideration for therapeutic relevance. The

pharmacological and biochemical claims therefore rest on a compound whose chemical properties, metabolic fate, and safety profile remain insufficiently defined.

10. Figure-by-Figure Critique of Main Figures

10.1. Figure 1: Identification and Initial Characterization of CCK-X

Figure 1 establishes the foundational evidence for the discovery of CCK-X as a biased agonist of CCKBR, yet multiple methodological and interpretational deficiencies undermine the credibility of the data. The dose–response curves for calcium signaling show unusually steep slopes and high maximal responses relative to the endogenous ligand, suggesting possible assay saturation or non-physiological receptor expression. No surface expression analysis or receptor density quantification is provided to ensure comparability across experiments. The β-arrestin recruitment data are represented by a single luminescence endpoint, lacking kinetic resolution and therefore incapable of detecting transient arrestin engagement. The absence of error bars in some subpanels raises concerns about the number of replicates performed. Structural modeling within Figure 1 depicts a predicted binding pose for CCK-X but lacks validation through mutagenesis or alternative docking approaches. Brain–plasma ratios shown in Figure 1g rely on a small number of animals without proper statistical presentation, rendering the pharmacokinetic inference incomplete. As the entry point to the study's mechanistic claims, **Figure 1** fails to provide the rigorous quantitative grounding necessary for establishing a biased agonist.

10.2. Figure 2: Cryo-EM Structures and Conformational Comparisons

Figure 2 presents the cryo-EM structures of CCKBR bound to CCK-X in complex with either miniG_q or an arrestin-derived construct. Although the maps are described as having resolutions around 3.5 Å, the density images appear inconsistent with this level of detail. The ligand density is particularly suspicious due to its excessive sharpness and uniformity, which is incongruent with the surrounding blurred densities in transmembrane regions. The purported structural differences between G_q- and arrestin-bound complexes are small and fall within the expected noise margin of cryo-EM reconstructions at the reported resolution. The authors highlight subtle shifts in TM6 and TM7 but do not present quantitative distance or angular measurements, leaving the interpretation largely speculative. Without displaying masked and unmasked Fourier shell correlation curves, the structural analysis lacks the validation required to distinguish true conformational features from refinement

artifacts. Thus, the structural interpretation provided in **Figure 2** is insufficient to support claims of a unique biased conformation induced by CCK-X.

10.3. Figure 3: Dissection of Signaling Pathways

Figure 3 is intended to demonstrate that CCK-X preferentially activates G_q-PLC–Ca^{2+} signaling and minimally engages MAPK and arrestin-associated pathways. However, the data presentation is fragmented and lacks methodological consistency. The ERK phosphorylation blots appear faint and irregular, and loading controls do not convincingly demonstrate equal sample loading. Some panels appear cropped in ways that obscure non-specific bands or background artifacts. The PLC activity assays rely on indirect measurements and do not include timecourses, inhibitor controls, or calibration with known activators. The relationship between calcium flux and downstream PLC activation is assumed rather than shown. β-arrestin signaling is again assessed through a single endpoint assay, reiterating the limitations identified in earlier figures. Because **Figure 3** attempts to establish mechanistic selectivity across divergent pathways, its deficiencies in assay design and execution significantly weaken the study's central claim that CCK-X exhibits functional selectivity at CCKBR.

10.4. Figure 4: Neuronal Physiology, LTP Recording, and Dendritic Structure

Figure 4 attempts to establish that CCK-X improves neuronal function in AD model neurons by restoring LTP, enhancing dendritic spine density, and modulating Ca^{2+} dynamics. The electrophysiological data lack sufficient replicates and fail to disclose whether slices were derived from multiple animals. The LTP traces appear overly smoothed, suggesting substantial preprocessing, and the magnitude of potentiation does not align with the visual representation of the traces. Quantification of LTP does not include baseline variability, input–output curves, or paired-pulse ratios. The images of dendritic spines show differences in brightness and contrast that may bias manual counting. No information regarding blinding, z-stack depth, or analysis software is provided. The Ca^{2+} imaging traces are displayed without clear temporal alignment or amplitude calibration. Because Figure 4 links neuronal outcomes directly to the proposed mechanism of biased CCKBR signaling, the absence of methodological rigor critically undermines the interpretation. The inconsistencies in imaging, limited electrophysiological replicates, and lack of causal manipulation present significant challenges to accepting the neuronal benefits attributed to CCK-X.

10.5. Figure 5: Behavioral and Pathological Outcomes in APP/PS1 Mice

Figure 5 is central to the translational claim that CCK-X improves cognitive performance and reduces pathological hallmarks in AD mice. However, multiple issues compromise the reliability of these findings. The Morris water maze trajectories appear unnaturally smooth and sometimes strikingly similar across trials, raising concerns about preprocessing or even potential duplication. Latency differences are interpreted as cognitive improvements despite the absence of swimming speed data, visual platform trials, or emotionality controls. The pathological measurements rely on ELISA quantification of Aβ and tau, but the underlying standard curves shown in Extended Data figures are nonlinear and sometimes saturated. Immunohistochemical analysis is either absent or minimally presented, depriving readers of spatial context for pathological changes. The sample sizes differ across panels, and statistical annotations do not provide exact p-values or confidence intervals. Because **Figure 5** attempts to show both behavioral rescue and pathological improvement, its inconsistencies and methodological omissions cast doubt on the study's therapeutic conclusions.

10.6. Figure 6: Transcriptomic and Proteomic Changes Attributed to CCK-X

Figure 6 presents transcriptomic and proteomic analyses as evidence that CCK-X restores synaptic gene expression profiles in AD models. The PCA plots reveal clustering patterns better explained by batch effects than biological differences. Volcano plots use inconsistent thresholds and appear to highlight genes that do not meet the stated significance criteria. The proteomic data are shallow and detect an unusually low number of proteins, suggesting limited analytical depth or improper sample preparation. Venn diagrams illustrating overlap between transcriptomic and proteomic changes show minimal intersection, contradicting the narrative of coordinated synaptic rescue. Without gene regulatory network analysis or evaluation of upstream transcriptional regulators, the mechanistic relevance of these changes remains unclear. Because **Figure 6** is used to argue that biased CCKBR signaling produces coherent molecular benefits, its methodological weaknesses significantly undermine the study's multi-omics claims.

10.7. Figure 7: Proposed Mechanistic Model and Integrative Interpretation

Figure 7 synthesizes the study's findings into a proposed mechanism whereby CCK-X stabilizes a biased conformation of CCKBR, leading to selective G_q activation, restored neuronal physiology, improved cognition, and reduced pathology.

However, each step of this proposed signaling cascade relies on experimental data that are incomplete, inconsistent, or insufficiently validated. The structural component lacks the resolution needed to assert a unique configuration. The signaling data suffer from methodological mismatches. The neuronal and behavioral analyses lack rigorous controls. The transcriptomic and proteomic findings do not coherently unify with the proposed mechanism. As a result, the schematic in **Figure 7** represents a speculative narrative rather than a conclusion supported by convergent evidence. While graphical models can be useful for conceptual development, they must remain grounded in experimentally validated mechanisms, which is not achieved here.

11. Detailed Critique of ED Figures

11.1. ED Figure 1: Compound Screening and Hit Identification

ED Figure 1 presents the initial screening pipeline that purportedly led to the identification of CCK-X as a biased CCKBR agonist. However, the screening data lack essential methodological transparency. The dose–response curves shown for several candidate compounds are poorly resolved, with irregular spacing of concentration points and inconsistent baseline fluorescence. Replicates are either not shown or appear duplicated, making it impossible to assess the reproducibility of the primary screen. The chemical structures presented are not accompanied by purity verification or analytical confirmation, leaving uncertainty regarding compound identity. The screening throughput, hit selection criteria, and statistical cutoffs are inadequately described. Without information on Z'-factor values or quality control parameters for the assay plates, the reliability of the screening results is questionable. **ED Figure 1** thus provides an insufficient foundation for establishing CCK-X as a credible pharmacological hit.

11.2. ED Figure 2: Signaling Kinetics, Temporal Resolution, and Replicability

ED Figure 2 attempts to provide kinetic resolution for G_q and β-arrestin signaling, yet the data appear noisy and incomplete. Calcium traces show baseline drift and high-frequency fluctuations characteristic of experimental instability rather than biologically meaningful signaling. Arrestin recruitment traces lack early timepoints, preventing assessment of transient binding. The authors do not specify whether signals were calibrated against internal standards or whether background subtraction was performed consistently. Several curves appear to share identical noise patterns, suggesting potential duplication or insufficient biological replication. Because kinetic profiling is essential to distinguishing true bias from asynchronous pathway activation, the deficiencies in **ED Figure 2** significantly undermine the interpretation of signaling dynamics.

11.3. ED Figure 3: Cryo-EM Processing Workflow and Resolution Validation

ED Figure 3 presents the cryo-EM processing pipeline, including particle selection, 2D classifications, and 3D refinements. However, the images shown reveal signs of preferred orientation, with particles appearing clustered in specific angular distributions. This phenomenon often reduces effective resolution and introduces directional artifacts. The authors do not provide unmasked Fourier shell correlation curves, preventing evaluation of overfitting during model refinement. Local resolution maps appear artificially smoothed, obscuring heterogeneity that would normally be present in flexible receptor regions. The absence of particle heterogeneity analysis or 3D variability maps further limits interpretive value. Taken together, the processing pipeline shown in **ED Figure 3** lacks the transparency required to validate the structural conclusions drawn in the main figures.

11.4. ED Figure 4: Ligand Density and Binding Pocket Geometry

ED Figure 4 presents ligand density maps intended to support the precise positioning of CCK-X within the receptor binding pocket. However, the ligand density appears anomalously uniform and well defined compared to surrounding receptor regions, suggesting possible model bias or aggressive density sharpening. The authors do not provide half-map correlation analyses or omit maps that would allow independent assessment of ligand fitting. Several areas of the ligand density appear to align more closely with the refined model than with raw map features, raising concerns about overfitting. Because ligand binding geometry underpins the mechanistic narrative of biased agonism, the ambiguous density presentation in **ED Figure 4** substantially weakens the credibility of the proposed binding pose.

11.5. ED Figure 5: β-Arrestin and G-Protein Pathway Assays

ED Figure 5 attempts to expand pathway analysis, but the arrestin data are limited to single timepoint assays without kinetic resolution, and the G-protein assays do not include calibration controls. Baseline noise is inconsistent, and dynamic range appears compressed. In several panels, error bars are absent or disproportionately small, suggesting either low variability or insufficient replicates. The absence of comparisons to known biased agonists or pathway inhibitors prevents contextualization of the data. The limited methodological detail in **ED Figure 5** prevents it from contributing meaningfully to the claim of selective pathway activation.

11.6. ED Figure 6: Neuronal Calcium Imaging Controls

ED Figure 6 provides supplemental neuronal calcium imaging data, but the traces appear highly variable and lack standardized normalization. $\Delta F/F$ calculations are not described, and the regions of interest appear inconsistently selected across trials. Photobleaching correction, motion correction, and drift compensation are not mentioned, despite being essential for interpreting live-neuron imaging. The spatial patterns of calcium signals shown in heatmap form exhibit uneven brightness and unnatural clustering, raising questions about image processing. Without robust controls, the neuronal calcium imaging data remain ambiguous.

11.7. ED Figure 7: Neuronal Morphology and Imaging Fidelity

ED Figure 7 attempts to validate neuronal morphology findings by presenting additional spine imaging and quantification. However, several dendritic segments appear visually similar across different panels, raising concerns about potential duplication or reuse of images. The brightness and contrast differ between treatment groups in ways that could bias spine detection. Three-dimensional reconstructions are not provided, and dendritic sampling strategies remain unspecified. Quantification of spine density is presented without global or regional distribution analysis, making it unclear whether the observed differences are systematic or due to selection bias. These limitations compromise the reliability of morphological claims.

11.8. ED Figure 8: Behavioral Trajectories and Navigational Metrics

ED Figure 8 displays raw swim-path traces used to support Morris water maze results. However, the trajectories appear unusually smooth and do not reflect the variability typically observed in rodent behavior. Several patterns appear highly similar across animals, raising the possibility of excessive smoothing or image reuse. The authors do not show unprocessed trajectory data or provide velocity–time curves that would allow evaluation of motor function. The heatmaps lack scale bars and color normalization, preventing interpretation of search strategies. Because behavioral trajectories provide critical evidence for cognitive rescue, the anomalies in Extended Data Figure 8 raise significant concerns regarding data authenticity and interpretive validity.

11.9. ED Figure 9: ELISA Standards and Quantitative Reliability

ED Figure 9 presents standard curves for Aβ and tau ELISAs, but the curves exhibit irregular shapes and poor fits, with saturation evident at high concentrations. The absence of replicates or residual plots prevents evaluation of curve robustness. Several sample points fall into regions of nonlinearity, meaning small measurement

errors could produce disproportionately large concentration errors. Without technical replicates, plate controls, or spike–recovery tests, the quantitative reliability of the ELISA data is questionable. These issues directly affect interpretation of pathological burden and undermine the study's central biochemical claims.

11.10. ED Figure 10: Single-Cell RNA-seq Quality Metrics

ED Figure 10 attempts to validate transcriptomic quality by showing QC metrics, yet mitochondrial read percentages of 20–30 percent suggest cellular stress or degradation. High mitochondrial content typically disqualifies samples from differential expression analysis. The absence of cell-level filtering criteria, such as minimum gene counts or UMI thresholds, makes it unclear whether low-quality cells were appropriately removed. The clustering patterns shown lack consistency and do not align with expected neuronal or glial identities. Without thorough QC filtering and lineage validation, the scRNA-seq dataset lacks interpretive value.

11.11. ED Figure 11: Proteomics QC and Dataset Depth

ED Figure 11 provides proteomics validation but reveals low peptide-spectrum matches and incomplete coverage of neuronal proteins. The figure lacks essential QC plots such as distribution of missing values, coefficient of variation across replicates, or normalization diagnostics. The reported proteome depth is insufficient for reliable pathway analysis and does not align with state-of-the-art mass spectrometry capabilities. Without rigorous QC demonstration, the proteomic dataset cannot substantiate claims of synaptic restoration.

12. Analysis of Supplementary Figures (SFs)

12.1. SF1: Chemical Synthesis Workflow and Compound Validation

SF1 attempts to outline the synthetic route used for producing CCK-X, yet the level of detail is inadequate for confirming the identity or purity of the compound. The schematic representation of the synthetic steps lacks crucial experimental information such as reagent equivalents, yields, reaction atmospheres, purification methods, and chromatographic conditions. No nuclear magnetic resonance (NMR) spectra, mass spectrometry data, elemental analysis, or high-performance liquid chromatography purity traces accompany the synthesis. As a result, the chemical identity of CCK-X remains insufficiently verified. The absence of spectroscopic data is particularly concerning given that conclusions about receptor binding and pharmacological efficacy rely on precise structural features of the ligand. Without comprehensive chemical characterization, the possibility of impurities, structural misassignment, or batch-to-batch variability cannot be excluded. This omission

undermines confidence in the reproducibility and reliability of downstream biochemical and behavioral results.

12.2. SF2: Stability Assessments and Degradation Profiles

SF2 presents limited stability data for CCK-X, but the experimental design and interpretation appear incomplete. The data focus on short-term stability under unspecified conditions, and no information is provided regarding solvent systems, pH, temperature cycles, or photostability. The compound's stability in plasma, brain homogenate, or microsomal preparations is not examined, leaving major gaps in understanding its metabolic fate. Stability assessments are essential for determining whether the observed pharmacological effects are attributable to the parent compound or to active or inactive metabolites. Without kinetic degradation profiles, half-life calculations, or metabolite identification, the stability dataset is too superficial to support conclusions about the compound's suitability for *in vivo* use. The lack of orthogonal stability assays significantly limits the interpretive depth of this supplementary figure.

12.3. SF3: Behavioral Controls Beyond Cognitive Measures

SF3 provides additional behavioral data intended to support the conclusions of improved cognitive function following CCK-X treatment. However, the figure focuses narrowly on cognitive endpoints without presenting essential behavioral controls such as locomotor activity, anxiety-like behavior, or sensorimotor function. Measures such as open-field exploration, elevated-plus-maze performance, or rotarod endurance are necessary to distinguish cognitive improvement from generalized behavioral alterations. Without these data, enhanced performance in memory tasks could reflect changes in arousal, anxiety, motivation, or motor capacity rather than genuine cognitive rescue. Examination of the figure reveals inconsistencies in sample sizes and a lack of clear statistical reporting, further limiting interpretive strength. The absence of these fundamental controls undermines the validity of the behavioral claims.

12.4. SF4: Cryo-EM Classifications and Structural Heterogeneity

SF4 displays additional cryo-EM classifications intended to support the structural conclusions of the study, but the data do not adequately address the issue of conformational heterogeneity. Many of the classes shown contain very small particle counts, with distributions that are not statistically robust. Such low-population classes often represent noise or misaligned particles rather than genuine alternative conformations. The authors do not provide variability analyses or 3D conformational exploration, which are essential for assessing whether the receptor

exists in multiple distinct states when bound to CCK-X. The lack of reproducibility across independent reconstructions is also concerning. Without demonstrating consistent conformational differences across multiple particle subsets, the structural heterogeneity observed cannot be interpreted meaningfully. As a result, the supplementary classification data do not strengthen the structural argument for biased agonism.

12.5. SF5: Functional Assays in Alternative Cell Lines

SF5 presents additional functional assays in a second heterologous cell line, presumably to demonstrate that the pharmacological profile of CCK-X is not cell-line–specific. However, the results in this figure do not replicate the pronounced bias reported in the main text. The calcium responses appear diminished, and the β-arrestin engagement shows partial activity that contradicts the claim of minimal arrestin recruitment. Differences in EC_{50} values between HEK293 and CHO or other host cells raise concerns about receptor density, signaling machinery, or endogenous GPCR cross-talk influencing results. Without quantifying receptor expression or controlling for assay sensitivity, discrepancies across cell lines cannot be interpreted mechanistically. This figure therefore introduces additional uncertainty regarding the robustness of CCK-X's signaling properties rather than reinforcing the conclusions of the main experiments.

12.6. SF6: Pharmacokinetic and ADME Characterization

SF6 presents a limited set of pharmacokinetic data intended to complement the main figures, yet the figure lacks essential components required for evaluating absorption, distribution, metabolism, and excretion (ADME) properties. The concentration–time curves include few timepoints and do not extend sufficiently to capture elimination phases. No replicate variability is shown, and the reported data lack clarity regarding whether values represent mean concentrations or individual measurements. Without calculating area under the curve, clearance, or volume of distribution, the PK interpretation remains superficial. The figure also lacks metabolite identification, plasma protein binding assays, or permeability assessments, all of which are critical for understanding brain penetration and *in vivo* efficacy. Overall, **SF6** does not provide the depth or rigor needed to substantiate claims of suitable pharmacokinetic properties for CNS targeting.

13. Data Integrity, Reproducibility, and Transparency

13.1. Consistency of Experimental Replicates and Internal Data Coherence

Across the study, inconsistencies in experimental replicates raise persistent concerns regarding data integrity. Many figures, both in the main text and

supplementary sections, present results without showing individual data points, error bars, or descriptions of biological versus technical replicates. Several datasets, particularly those involving calcium imaging, LTP recording, and proteomic quantification, contain traces or numerical values that are overly uniform or identical in appearance, suggesting potential duplication or heavy preprocessing. Additionally, discrepancies in sample sizes across similar experiments are not explained, creating ambiguity regarding how animals, cells, or tissue samples were assigned or excluded. Internal coherence is further weakened by mismatches between text descriptions and graphical data, such as EC_{50} values that do not align with curves or inconsistent fold changes across independent analyses. The lack of raw data files, including electrophysiological traces, sequencing datasets, and unprocessed western blots, prevents thorough verification. These issues collectively undermine confidence in the internal consistency of the study's findings.

13.2. Potential Image Integrity and Data Presentation Irregularities

Several figures raise questions regarding image integrity. Western blots appear cropped tightly around bands, preventing assessment of non-specific signals or background patterns. Some loading controls display near-identical band shapes across different blots, suggesting potential reuse or duplication. Dendritic spine images in morphology analyses show brightness and contrast differences between treatment groups that may bias interpretation, and in a few cases, segments of dendrites appear suspiciously similar across different panels. Behavioral trajectory maps in the Morris water maze appear unnaturally smooth or repetitive, inconsistent with typical rodent navigation patterns. Cryo-EM density maps display ligand density that appears overly consistent between reconstructions, which is unusual given inherent variability across cryo-EM datasets. These irregularities do not constitute definitive evidence of manipulation, but they raise enough concern to warrant the release of raw, unprocessed images for independent review. Transparent data presentation is essential for maintaining scientific credibility, and the study falls short in this regard.

13.3. Missing Raw Data, Metadata, and Required Methodological Details

Reproducibility depends on access to raw data and detailed methodological descriptions, both of which are insufficiently provided. Key datasets—including full cryo-EM micrographs, raw sequencing reads, LC–MS/MS files, behavioral video recordings, and electrophysiological traces—are not included in supplementary material nor deposited in public repositories. Metadata such as animal identifiers, age, sex, housing conditions, and treatment logs are absent. Critical laboratory

parameters, such as antibody lot numbers, imaging acquisition settings, plate reader calibration metrics, and electrophysiology holding potentials, are not described. These omissions prevent independent reproduction of results and violate emerging standards in neuroscience and structural biology that emphasize open-data practices. Because the study's major claims span multiple disciplines, transparency in raw datasets and methodological detail is essential. The lack of such information significantly limits reproducibility.

13.4. Statistical Reporting, Multiple Testing, and Analytical Rigor

The statistical analyses throughout the study exhibit several shortcomings. Many figures present mean values without individual data points, obscuring variability and distributional properties. The specific statistical tests used are not consistently stated, nor are assumptions regarding normality or variance documented. Adjustments for multiple comparisons, which are essential for transcriptomic, proteomic, and behavioral analyses, are inconsistently applied or omitted entirely. Confidence intervals are not presented, and p-values are often reported only as generalized thresholds rather than exact values. In some cases, statistical outcomes described in the text do not align with visual interpretations of the data. Without appropriate statistical rigor, the significance of the reported findings remains uncertain, and the potential for Type I or Type II errors is substantial. Proper statistical transparency is particularly vital when claims rely on subtle differences in signaling, behavior, or molecular phenotypes.

13.5. Overall Assessment of Reproducibility and Scientific Transparency

Taken together, the issues identified throughout the study reflect systemic limitations in reproducibility and transparency. The lack of raw data, incomplete statistical reporting, and potential image irregularities undermine the interpretive strength of the experimental findings. These concerns are amplified by the study's broad mechanistic claims, which require exceptionally strong evidence across multiple levels of analysis. Without full disclosure of data and methods, independent investigators cannot confirm or challenge the conclusions, limiting the scientific value of the work. The study illustrates the importance of cross-disciplinary rigor when proposing mechanistic frameworks that span pharmacology, structural biology, neurophysiology, behavior, and systems biology. Addressing these limitations would not only strengthen the current findings but also contribute to improving the reliability of complex, multi-modal research in Alzheimer's disease and GPCR signaling.

14. Broader Scientific and Mechanistic Implications

14.1. Prospects and Pitfalls of GPCR Biased Agonism in Neurodegenerative Disease

The concept of using biased GPCR agonism to modulate neuronal function has significant theoretical appeal, particularly for disorders such as AD where synaptic dysfunction, circuit disintegration, and neurotransmitter imbalance contribute to progressive cognitive decline. Biased agonism promises to fine-tune receptor signaling by amplifying beneficial pathways while suppressing maladaptive ones. For CCKBR, the idea that selective G_q activation might promote synaptic resilience while minimizing arrestin-dependent desensitization or stress responses is an intriguing possibility. However, the broader GPCR literature underscores the complexity of such strategies. GPCR signaling is profoundly context-dependent, shaped by receptor localization, interacting proteins, cell type–specific machinery, and temporal patterns of activation. A pathway deemed beneficial in one neuronal population may produce pathological effects in another. Arrestin pathways, which Wang *et al*. implicitly characterize as detrimental, have established roles in synaptic maturation, homeostatic plasticity, and neuroprotective signaling. Reducing arrestin recruitment could therefore introduce unintended consequences. Moreover, biased agonism in neurons differs fundamentally from biased agonism in peripheral tissues because neuronal signaling integrates both biochemical and electrical activity. The mechanistic oversimplification observed in the study risks overstating the therapeutic potential of targeting a single receptor node without appreciating the emergent complexity of neuronal systems. The promise of biased agonism in Alzheimer's disease should be approached with caution, grounded in mechanistic rigor and validated through high-resolution analyses of neuronal circuit consequences.

14.2. Alternative Explanations for Observed Phenotypes and Data Patterns

The effects attributed to CCK-X in the study may arise from mechanisms unrelated to biased CCKBR agonism. Several experimental results invite alternative explanations. Behavioral improvements could reflect anxiolytic or psychostimulant properties rather than cognitive enhancement, especially given CCKBR's known involvement in anxiety and emotion regulation. Changes in amyloid or tau pathology might result from indirect effects on metabolic pathways, proteostasis, inflammation, or glial function rather than direct modulation of neuronal signaling. Neuronal survival improvements may stem from off-target interactions or general cytoprotective effects unrelated to G_q-specific pathways. The transcriptomic and proteomic changes could be driven by stress responses, batch effects, or systemic

alterations rather than synaptic restoration. The structural data do not convincingly establish a unique conformation, leaving open the possibility that conformational differences reported are processing artifacts or reflect partial receptor activation rather than bias. Considering these alternative explanations is essential for contextualizing the study within the larger landscape of AD research, where many candidate mechanisms initially appeared promising but did not withstand rigorous scrutiny. Without excluding confounding mechanisms, the causal narrative proposed by the authors remains speculative.

14.3. Off-Target and Polypharmacological Considerations in the CNS Context

Central nervous system pharmacology presents unique challenges due to the dense receptor landscape, complex neurochemical interplay, and high potential for off-target interactions. The limited off-target profiling performed by Wang *et al*.[4] does not adequately rule out polypharmacology, particularly for a synthetic ligand whose physicochemical properties remain incompletely characterized. Off-target GPCR interactions could influence locomotor activity, arousal, emotionality, endocrine function, or autonomic responses, all of which may alter behavioral performance in AD models independent of cognitive rescue. Potential interactions with ion channels, neurotransmitter transporters, or intracellular enzymes remain unexplored. The possibility that CCK-X alters glial signaling or inflammatory pathways through mechanisms unrelated to CCKBR further complicates interpretation. In the CNS, even moderate off-target affinity can produce substantial physiological effects due to amplification through neural networks. Without a comprehensive pharmacological profile, claims of CCKBR-specific biased agonism remain insecure. A rigorous evaluation of off-target binding, functional screening across multiple receptor families, and chemogenomic profiling is necessary to distinguish specific from nonspecific effects. The study's translational relevance is therefore limited until these pharmacological gaps are addressed.

14.4. Alignment with Existing Literature and Implications for Future Research

When placed within the broader scientific literature, the findings reported by Wang *et al*.[4] align only partially with established knowledge of CCKBR function and GPCR biased agonism. Prior studies highlight the pleiotropic nature of CCKBR signaling, including its roles in anxiety, pain modulation, learning, and neuroendocrine regulation. The proposition that selective G_q activation alone drives cognitive improvement contradicts evidence that balanced signaling, including arrestin pathways, contributes to synaptic adaptation. Furthermore, most successful biased agonists characterized in the GPCR field rely on extensive structural, kinetic, and

functional validation far beyond what is presented in the current study. The lack of robust mutagenesis experiments, limited cryo-EM resolution, insufficient kinetic data, and weak pharmacological profiling indicate that the mechanistic insights remain preliminary. Nevertheless, the concept of targeting CCKBR remains worthy of further exploration provided that future research incorporates comprehensive structural validation, rigorous operational model analysis, meticulous behavioral controls, and transparent multi-omics integration. The study highlights the urgent need for deeper mechanistic dissection of GPCR signaling in AD, along with more systematic exploration of how receptor conformational states shape neuronal and cognitive outcomes. By addressing these scientific gaps, future investigations could clarify whether biased GPCR agonism indeed represents a viable therapeutic avenue in neurodegenerative disease.

15. Conclusions and Recommendations

15.1. Summary of Core Concerns Across Experimental Domains

Across all levels of analysis, from ligand characterization and signaling assays to structural biology, neuronal physiology, behavioral testing, biochemistry, and multi-omics profiling, the study by Wang *et al*.[4] presents recurring methodological and interpretative limitations. The central claim of G_q-selective biased agonism at CCKBR is undermined by incomplete operational model quantification, use of non-matched assay conditions, insufficient kinetic profiling, and inconsistent EC50 and Emax reporting. The cryo-EM structures, presented as mechanistic evidence of distinct receptor conformations, do not reach the resolution or validation rigor required to support claims of bias-encoded structural states. Neuronal assays, including calcium imaging, dendritic spine quantification, and long-term potentiation recordings, lack essential controls, biological replication, and data transparency. Behavioral experiments in Alzheimer's disease mouse models exhibit insufficient blinding, inconsistent sample sizes, and anomalies in raw trajectory data. Biochemical assays, including ELISAs and western blots, show issues with quantitative reliability and data presentation. Transcriptomic and proteomic datasets suffer from batch effects, limited depth, and insufficient mechanistic linkage to receptor signaling. The accumulation of these issues calls into question the strength and validity of the overarching mechanistic narrative proposed by the study.

15.2. Clarification Required for Mechanistic Claims

The mechanistic interpretation that CCK-X stabilizes a unique CCKBR conformation leading to selective G_q engagement, synaptic rescue, and cognitive improvement remains speculative in the absence of rigorous supporting evidence. To substantiate

such a mechanism, future studies must incorporate comprehensive bias quantification using operational pharmacological models, matched assay conditions, and orthogonal kinetic assays. Structural insights must be validated by higher-resolution cryo-EM data, ligand-free controls, model-free density assessments, and mutagenesis experiments that test structural predictions. Neuronal physiology experiments require enhanced methodological rigor, including validation of receptor expression, replication across animals, and causal dissection using pathway-specific inhibitors. Behavioral studies must be conducted under blinded, randomized conditions with thorough motor and emotional controls to distinguish cognitive rescue from confounding performance factors. Pathological and biochemical results must be anchored in validated assays with transparent standard curves and reproducible detection. The study's mechanistic claims can only be substantiated when these methodological deficits are corrected and when causal pathways linking receptor-level events to systems-level outcomes are clearly demonstrated.

15.3. Recommendations for Strengthening Evidence and Improving Transparency

To advance the field and ensure the reliability of claims involving complex, multi-level mechanisms, the research community must prioritize transparency, methodological rigor, and reproducibility. Future work on CCKBR-biased agonism should include full disclosure of raw electrophysiological traces, unprocessed cryo-EM maps, behavioral video files, and quantitative QC metrics for transcriptomic and proteomic datasets. Standardized reporting of replicate numbers, sample exclusion criteria, and statistical tests is essential. A comprehensive assessment of compound identity, purity, metabolic stability, off-target interactions, and pharmacokinetics will be required before drawing conclusions about therapeutic relevance. Multi-omics data must undergo rigorous batch correction, replicate validation, and integration through established network-based frameworks. The field would benefit from comparative studies involving multiple biased ligands, CCKBR mutants, and genetic manipulation of pathway components to establish causal relationships. By adhering to these standards, future research can produce more reliable and interpretable findings that contribute substantively to understanding GPCR signaling in neurodegeneration.

15.4. Relevance for Alzheimer's Disease Therapeutics and Future Directions

Despite the limitations identified throughout this commentary, the pursuit of GPCR-targeted therapies for AD remains conceptually important and potentially transformative. CCKBR represents a biologically intriguing target given its

involvement in synaptic modulation, neuromodulatory integration, and neuronal excitability. The possibility that biased agonism could mitigate detrimental signaling pathways while enhancing beneficial ones merits careful investigation. However, claims of therapeutic efficacy must be grounded in robust mechanistic evidence and validated across independent experimental systems. The study by Wang *et al*. provides provocative but preliminary observations that require substantial refinement before informing translational strategies. Going forward, integrating advanced structural methodologies, high-resolution signaling assays, rigorous behavioral paradigms, and systems-level analyses will be essential for determining whether biased GPCR agonism can meaningfully alter the trajectory of neurodegenerative decline. Through such rigorous and collaborative efforts, the field may eventually determine whether CCKBR agonists can form part of a credible therapeutic framework for AD.

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