# Clathrin regulates the β-arrestin pathway regardless of ligand bias

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**Article**

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1 **Clathrin regulates the β-arrestin pathway regardless of ligand bias**

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8

**Abstract**

μ-Opioid receptors (MOPs) are considered to activate the G protein-mediated analgesic pathway and β-arrestin 2-mediated side effect pathway; however, ligands that only minimally recruit β-arrestin 2 to MOPs may also cause opioid side effects. Such side effects are also induced in mutant mice lacking β-arrestin 2 or expressing phosphorylation-deficient MOP that do not recruit β-arrestin 2. These findings raise the critical question as to whether β-arrestin 2 recruitment to MOP triggers side effects. Here, we show that β-arrestin 1 partially compensates for the lack of β-arrestin 2 in a neuronal cell line and, thus, might be involved in triggering such side effects in β-arrestin 2-null mice. Moreover, the magnitude of β-arrestin-mediated signals was not correlated with β-arrestin recruitment to MOP via phosphorylation of the carboxyl-terminal of MOP, which has long been used to evaluate the β-arrestin bias of a ligand. Instead, β-arrestin appears to activate downstream signals by binding with the clathrin heavy chain in the process of clathrin-coated pit formation. Our findings provide novel insight into G protein-coupled receptor-mediated signaling to overcome opioid side effects and further highlights an unexpected concept that the accumulation of molecules required for endocytosis is a key for activating intracellular signaling.

## Introduction

G protein-coupled receptors (GPCRs) recruit signal effectors and adaptor molecules such as G proteins and β-arrestins to convert extracellular stimuli into intracellular signals[1]. Upon μ-opioid receptor (MOP) activation, G proteins induce analgesia by regulating various ion channels, whereas β-arrestin 2 triggers lethal side effects such as respiratory depression[2]. Even though various opioids target MOP, the analgesic or adverse effects vary depending on the ligands. Differences in pharmacological effects, known as functional selectivity, have generally been understood in relation to ligand bias, as GPCR adopts a specific active conformation for each ligand[1,3,4]. Differences in the activated structure, observed mainly in intracellular regions[5], are presumed to result in qualitative differences in receptor signaling complexes[1,6]. Applying this principle, bias-tuned ligands, which are G protein-biased compounds that selectively activate the G protein-analgesic pathway while only minimally recruiting β-arrestin 2, were proposed as ideal opioids with an improved property[7,8]. However, in contrast to expectations, recent studies revealed that G protein-biased ligands might also produce classical opioid side effects at an analgesic dosage with limited functional selectivity[9,10]. These observations strongly suggest an important unresolved gap between the concept of biased agonism and functional selectivity. Furthermore, most recent studies reported that mutant mice lacking β-arrestin 2 or expressing phosphorylation-deficient MOP, which does not recruit β-arrestin 2, also presented with side effects following opioid administration[11-13]. These results critically raise the question as to whether recruitment of β-arrestin 2 to MOP is the true culprit that triggers opioid side effects[14].

β-arrestin plays multiple roles simultaneously. β-arrestins competitively repress the G protein pathway and activate G protein-independent signals[1], and also serve as

scaffolds for clathrin-coated pit formation for GPCR internalization[15]. Receptor endocytosis tunes down extracellular stimuli and resensitizes the receptors at endosomes while defining the spatiotemporal localization and physiological outcomes of the signal[16,17]. It is noteworthy that several hundred GPCRs are regulated by only two highly homologous non-visual arrestins: β-arrestin 1 and 2[18]. β-Arrestins are widely distributed in tissues throughout the body and are particularly abundant in the central nervous system. β-arrestin 1-deficient mice do not show any specific symptoms in response to opioid administration, whereas β-arrestin 2-deficient mice show enhanced analgesic effects and reduced adverse effects upon opioid administration[19-21]. Therefore, it has been assumed that β-arrestin 1 may not play a major role in the opioid system. However, there remain some unanswered questions such as whether β-arrestin 1 compensates for the lack of β-arrestin 2. As β-arrestin 1 and 2 are highly homologous in the amino acid sequence, and the simultaneous loss of both arrestins results in embryonic lethality[22], it can be assumed that β-arrestin 1 and 2 exhibit high functional homology and compensate for each other when one is missing. Another important question is how β-arrestin-mediated signals are activated. Several researchers have evaluated the recruitment of β-arrestin to MOP as a bias toward β-arrestin[2,7,8,23]. However, the correlation between β-arrestin bias and the strength of the downstream signal has not been thoroughly tested.

To resolve these conflicts, we aimed to explore why G protein-biased ligands or mutant mice that lack β-arrestin 2 or do not recruit β-arrestin 2 nevertheless present with opioid side effects. To address these questions, we used a CRISPR/Cas9-modified neuronal cell line endogenously expressing MOP. Our findings highlight a novel concept that a carrier component for endocytosis is a crucial regulator of intracellular signals, thus providing a basis for a strategy to more effectively overcome opioid side effects.

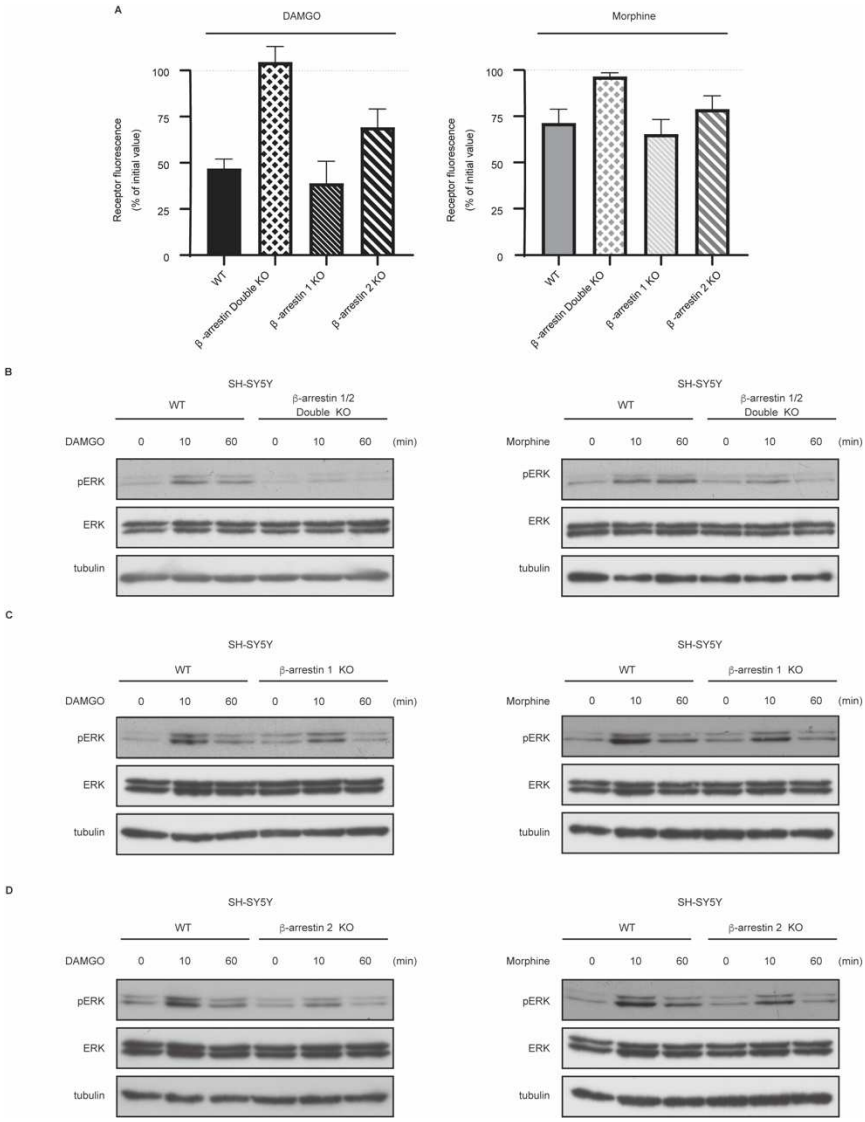
## Results

### β-arrestin 1 only partially compensates for β-arrestin 2

Initially, we focused on β-arrestin 1, whose amino acid sequence is highly homologous to that of β-arrestin 2 (Supplemental Table S1, Supplemental Figure S1A). We were particularly interested in determining whether β-arrestin 1 compensates for the lack of β-arrestin 2 in the nervous system. We used two ligands with different β-arrestin biases, [D-Ala$^2$, NMe-Phe$^4$-, Gly-ol$^5$] (DAMGO) and morphine, to consider the effect of ligand biases, with the former recruiting β-arrestins to MOP more robustly than the latter[24-26]. We further used CRISPR/Cas9 to genetically modify SH-SY5Y cells, a neuronal cell line physiologically expressing MOP, to establish cells lacking either β-arrestin 1, 2, or both simultaneously (Supplemental Figure S1B–E). Upon MOP activation, β-arrestin accumulates molecules as a scaffold to internalize the activated MOP and simultaneously drives G protein-independent signals[1,15]. Therefore, we investigated the functional differences and redundancy between β-arrestin 1 and 2 in terms of MOP internalization and signal activation.

The loss of both β-arrestin 1 and 2 almost completely abolished receptor internalization for both ligands; β-arrestin 1 loss had little effect on receptor internalization, whereas β-arrestin 2 loss comparatively reduced receptor internalization (Figure 1A). In line with previous reports suggesting that β-arrestin is involved in mitogen-activated protein kinase (MAPK) activation downstream of GPCRs[22], the phosphorylation of ERK, a hallmark of the MAPK pathway, was nearly abolished when both β-arrestin 1 and 2 were genetically deleted (Figure 1B). However, when either β-arrestin 1 or 2 was absent, the MAPK pathway was only slightly attenuated upon

stimulation with either ligand (Figure 1C and 1D). These results indicate that in the absence of β-arrestin 2, β-arrestin 1 compensates for MAPK activation comparatively but compensates for MOP internalization only partially.




107

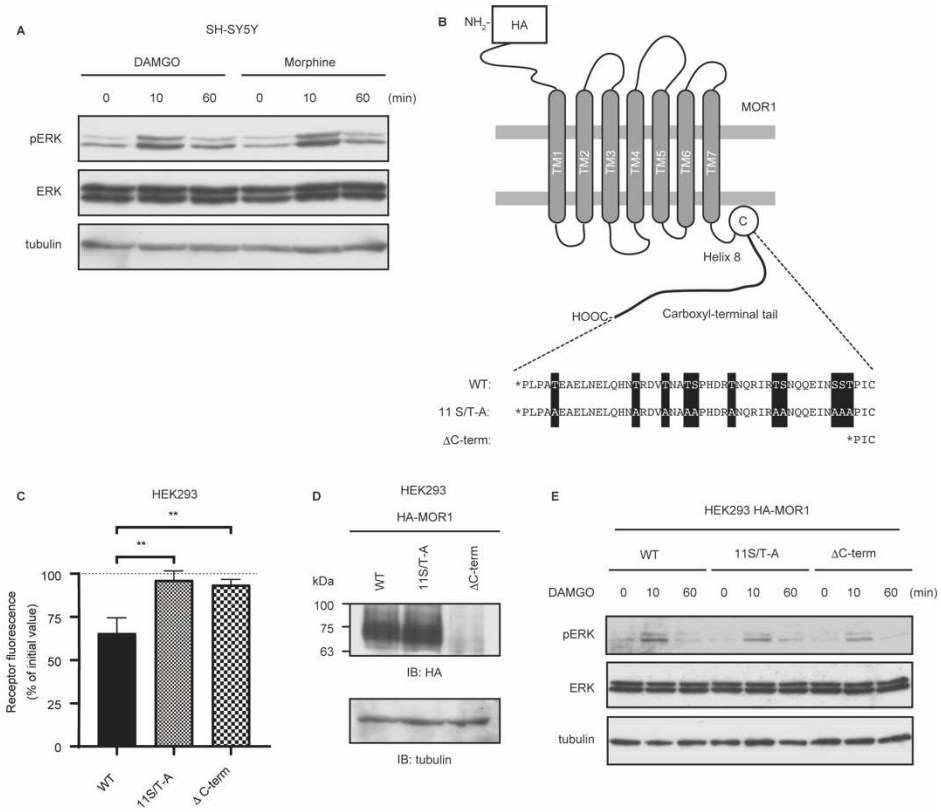
108 **Figure 1. β-arrestin 1 only partially compensates for β-arrestin 2. A.** Flow cytometry
109 analysis for MOP internalization under the loss of both β-arrestin 1 and 2 simultaneously,
110 or either one of the β-arrestins, in SH-SY5Y cells overexpressing hemagglutinin (HA)-
111 tagged MOR1 upon stimulation with 10 μM of DAMGO (left panel) and morphine (right
112 panel) for 60 min. The data represent the mean fluorescence intensity of the PE anti-HA
113 tag on the cell surface. **B–D.** Immunoblot analyses of MAPK activation in whole-cell
114 lysates from SH-SY5Y cells with the indicated genotypes following stimulation with 10
115 μM of DAMGO (left panel) and morphine (right panel), and the indicated time.

**β-arrestin-recruitment to MOP does not correlate with the intensity of the β-arrestin-mediated signal**

Previous studies have evaluated ligand bias toward β-arrestin based on the extent to which β-arrestin is recruited to MOP[2,7,8,23]. Therefore, we next investigated whether there is a direct correlation between β-arrestin recruitment and the β-arrestin-mediated signal intensity in the modified neuronal system. We tested SH-SY5Y cells endogenously expressing MOP using MOP ligands with different biases, DAMGO and morphine, and evaluated ERK phosphorylation as the hallmark of MAPK signaling originating from β-arrestin. Surprisingly, the magnitude of ERK phosphorylation was almost equal between ligands showing different biases (Figure 2A), indicating that there was no correlation between β-arrestin-recruitment toward MOP and the magnitude of β-arrestin-mediated downstream signals.

A previous study using phosphorylation-deficient MOP mutant mice assumed that the mutation prevented the recruitment of β-arrestin, and hence its downstream pathway would not be activated[13]. As our results differed from this assumption, we further evaluated β-arrestin-mediated signals upon stimulation with MOP mutants that do not recruit β-arrestin. First, we generated two MOP mutants lacking the scaffold for β-arrestin recruitment: a phosphorylation-deficient mutant (11S/T-A), in which all serine and threonine residues in the carboxyl-terminal regions are replaced by alanine, and a MOP mutant lacking the entire carboxyl-terminal region (ΔC-term) (Figure 2B). As expected, neither mutant internalized MOP upon stimulation with DAMGO (Figure 2C, Supplemental Figure S2A). Next, we assessed whether these MOP mutants activate the signaling pathway. We used HEK293 cells to test intracellular signals upon MOP activation because endogenous MOP in SH-SY5Y cells activated the signals too strongly,

making it difficult to evaluate the significance of the ectopically introduced MOP (Supplemental Figure S2B). Compared with that in the wild-type, MOP expression in the 11S/T-A mutant was almost the same, whereas the MOP expression level in the ΔC-term mutant was significantly decreased, likely owing to instability of the mutant structure (Figure 2D, Supplemental Figure S2C). Surprisingly, MAPK was almost fully activated in both MOP mutants (Figure 2E, Supplemental Figure S2D). Of note, even such a tiny amount of ΔC-term mutant fully activated MAPK. The activation appeared to require only contact but not a direct association with MOP. These results collectively revealed the absence of a correlation between β-arrestin recruitment to MOP and the β-arrestin-mediated signal intensity.




153 **Figure 2. Lack of correlation between β-arrestin-recruitment to MOP and the**
154 **intensity of β-arrestin-mediated signals. A.** Immunoblot analysis of MAPK activation
155 in whole-cell lysates from SH-SY5Y cells following stimulation with 10 μM of DAMGO
156 or morphine for the indicated times. **B.** Scheme describing the design of two MOP
157 mutants and the sequence of the wild-type: 11S/T-A mutant, in which all serine and
158 threonine residues in the carboxyl-terminal regions are replaced by alanine, and ΔC-term
159 mutant, in which the carboxyl-terminal regions are deleted. **C.** Receptor internalization
160 when wild-type or mutated MOP tagged with a hemagglutinin (HA) sequence were
161 introduced into the indicated cell lines and stimulated with 10 μM of DAMGO for 10 min
162 (**$P < 0.01$). **D.** Expression level of wild-type or mutated MOP in HEK293 cells
163 analyzed by immunoblotting. **E.** Immunoblotting analysis of MAPK activation in whole-
164 cell lysates from HEK293 cells stably expressing wild-type or mutated MOP tagged with
165 an HA sequence.

## β-arrestin activates MAPK signaling via association with clathrin heavy chain during clathrin-coated pit formation

Next, we questioned how β-arrestin maintains its activated state in the absence of an association with MOP. β-arrestin consists of an N-domain and a C-domain. In the basal state, the carboxyl-tail hooks onto amino-terminal sequences to stay in an inactivated form. Upon activation, the carboxyl-tail is unhooked from the amino-terminal region, and the angle formed by the two domains is slightly displaced[18,27-29]. The structural insight into β-arrestin activation suggested that there might be a factor preventing β-arrestins from reverting to the inactive state even when dissociated from MOP. Under physiological conditions, β-arrestins rapidly gather molecules to form clathrin-coated pits to internalize deactivated MOP[15]. During this process, β-arrestins are associated with various molecules with relatively large molecular weight, such as the AP2 adaptor complex, clathrin heavy chain[17,30]. We hypothesized that once activated by MOP, β-arrestin maintains its activated conformation by associating with such molecules during clathrin-coated pit formation and tested this hypothesis using various endocytosis inhibitors.

First, we used Dyngo-4a, a dynamin inhibitor that disrupts the final step of endocytosis to detach clathrin-coated pits from the plasma membrane. In the presence of Dyngo-4a at concentrations that sufficiently inhibited MOP internalization, DAMGO activated MAPK to the same extent as that found in the absence of Dyngo-4a (Figure 3A and 3B), indicating that the processes regulating β-arrestin activation occur prior to the dissociation of clathrin-coated pits. Next, we inhibited the association of β-arrestin, clathrin heavy chain, and the AP2 adaptor complex using barbadin[31]; pre-treatment with

barbadin at a concentration that sufficiently inhibited receptor internalization abolished the signal originating from β-arrestin (Figure 3C and 3D). The signal activated by the MOP 11S/T-A mutant was also successfully abolished by barbadin, indicating that the formation of clathrin-coated pits is vital for β-arrestin to drive downstream signals (Figure 3E).

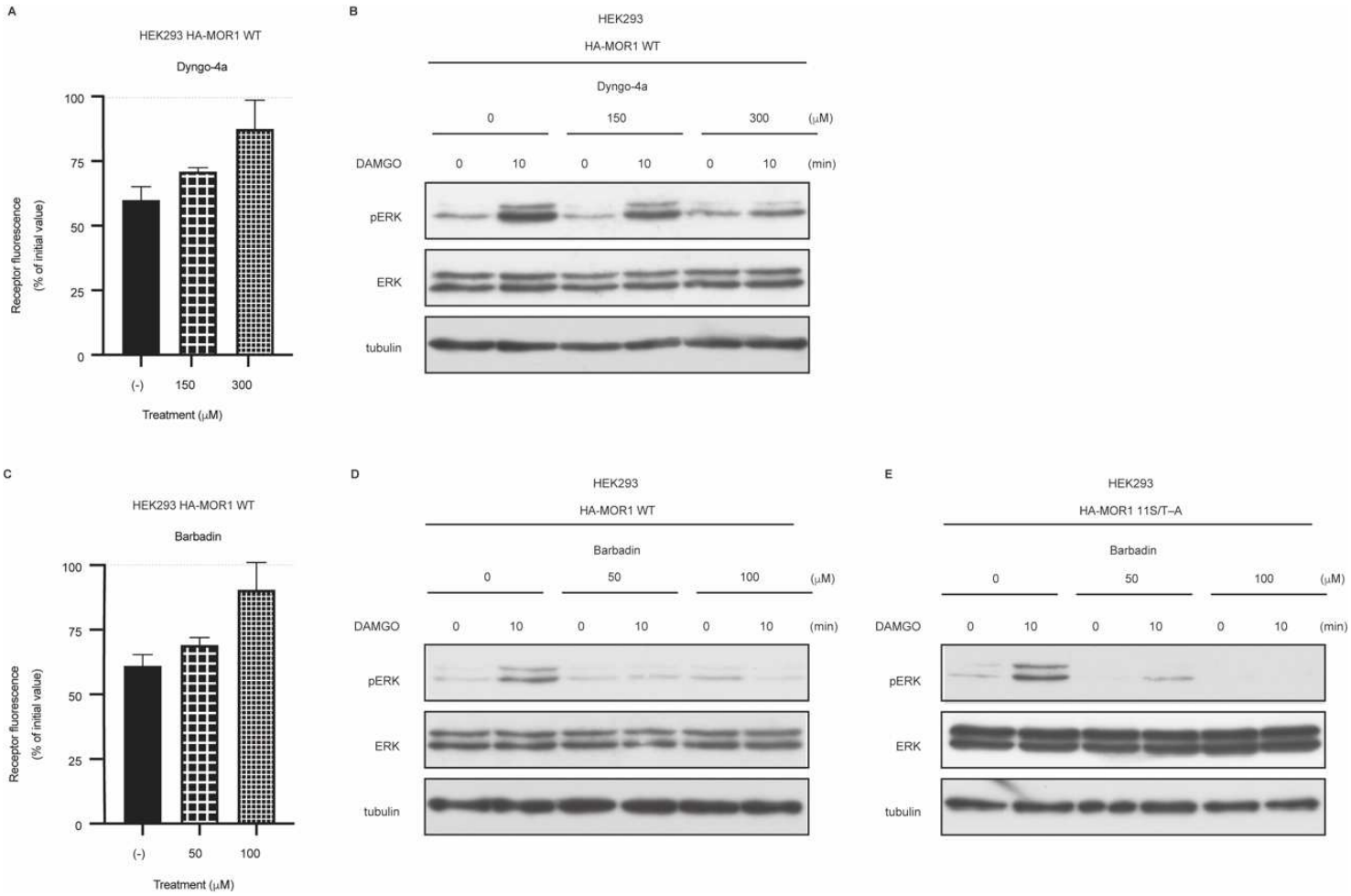



**Figure 3. Formation of clathrin-coated pits is vital for β-arrestin to drive downstream signals. A and B.** Effects of inhibiting clathrin-coated pits detachment by a dynamin inhibitor, Dyngo-4a, on receptor internalization **(A)** and MAPK activation for the indicated times **(B)**, followed by stimulation with 10 μM of DAMGO in HEK293 cells expressing MOR1 tagged with a hemagglutinin (HA) sequence. **C–E**. Effects of inhibiting clathrin-coated pits formation by barbadin on receptor internalization **(C)** and MAPK activation, followed by stimulation with 10 μM of DAMGO in HEK293 cells expressing wild-type MOR1 **(D)** or MOR1 with the 11S/T-A mutation **(E)** for the indicated times.

To further elucidate the molecular mechanism and identify the molecular target that blocks the β-arrestin pathway, we used CRISPR/Cas9 to genetically deplete the β2-adaptin subunit of the AP2 adaptor complex and the clathrin heavy chain to which β-arrestin binds[32]. First, we established a cell line lacking the β2-adaptin subunit of the AP2 adaptor complex. Loss of the β2-adaptin subunit only slightly impaired MOP internalization and did not affect β-arrestin-mediated signals without an apparent difference between the ligands (Figure 4A–D, Supplemental Figure S3A and S3B). Next, we attempted to establish a cell line lacking clathrin heavy chain. However, despite designing guide RNAs targeting multiple exons, we could not establish a clone completely depleted of clathrin heavy chain. We presumed that the gene encoding clathrin heavy chain might be an essential gene, as it regulates various vital processes such as cell division or survival. However, some of the mutated clones of clathrin heavy chain, in which one allele was null but the other had a three-base fold deletion, expressed a much lower amount of clathrin heavy chain than found in the cell culture obtained by the knockdown method (Figure 4E–H, Supplemental Figure S3C and S3D). Although MOP internalization in the mutant cells was almost comparable to that in wild-type cells, β-arrestin-mediated signals were significantly attenuated regardless of the ligands (Fig 4E–H).

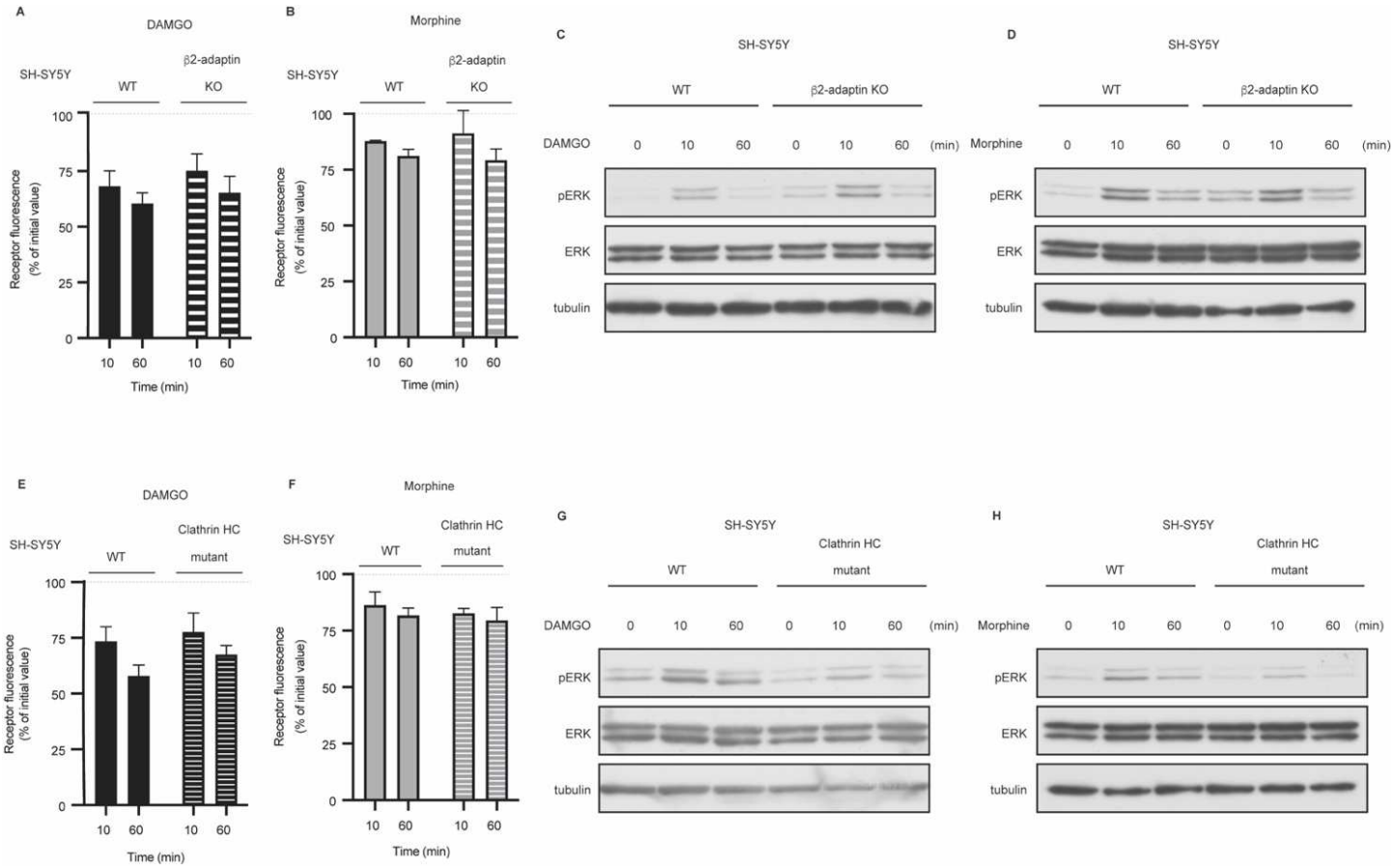



**Figure 4. β-arrestin-mediated signals in β2-adaptin-depleted or clathrin heavy chain mutant SH-SY5Y cells. A–D.** MOP internalization (A and B) and MAPK activation (C and D) in β2-adaptin-depleted SH-SY5Y cells upon stimulation with 10 μM DAMGO or morphine for the indicated times. **E–H.** MOP internalization (E and F) and MAPK activation (G and H) in clathrin heavy chain (HC)-mutated SH-SY5Y cells upon stimulation with 10 μM DAMGO or morphine for the indicated times.

241 Because the reduction of clathrin heavy chain attenuated β-arrestin-mediated
242 signals more than expected, we speculated that clathrin heavy chain plays an essential
243 role in the β-arrestin pathway. As we were unable to establish a clathrin-deficient strain,
244 we next used Pitstop 2 to evaluate the involvement of clathrin heavy chain in β-arrestin-
245 mediated signals. Pitstop 2 is a reagent that binds to amino acids R64, Q89, and F91 of
246 clathrin heavy chains and inhibits their binding to molecules such as β-arrestin[33]. At
247 concentrations that sufficiently inhibited MOP internalization, Pitstop 2 diminished
248 activation of the β-arrestin pathway regardless of the ligand type (Figure 5A–C). Amino
249 acids Q89 and F91, which are targets of Pitstop 2, are also the binding sites of clathrin
250 to β2-adaptin[34]. Therefore, we tested whether activation of the β-arrestin pathway was
251 inhibited by Pitstop 2 in cells lacking β2-adaptin to confirm that the binding of clathrin
252 to β-arrestin, rather than clathrin to β2-adaptin, is important for activation of β-arrestin-
253 mediated signaling. After pre-treatment of the cell line lacking β2-adaptin with Pitstop2,
254 the β-arrestin pathway was completely abolished (Figure 5D). These results collectively
255 indicate that the β-arrestin pathway is activated in association with clathrin heavy chain
256 during clathrin-coated pit formation.

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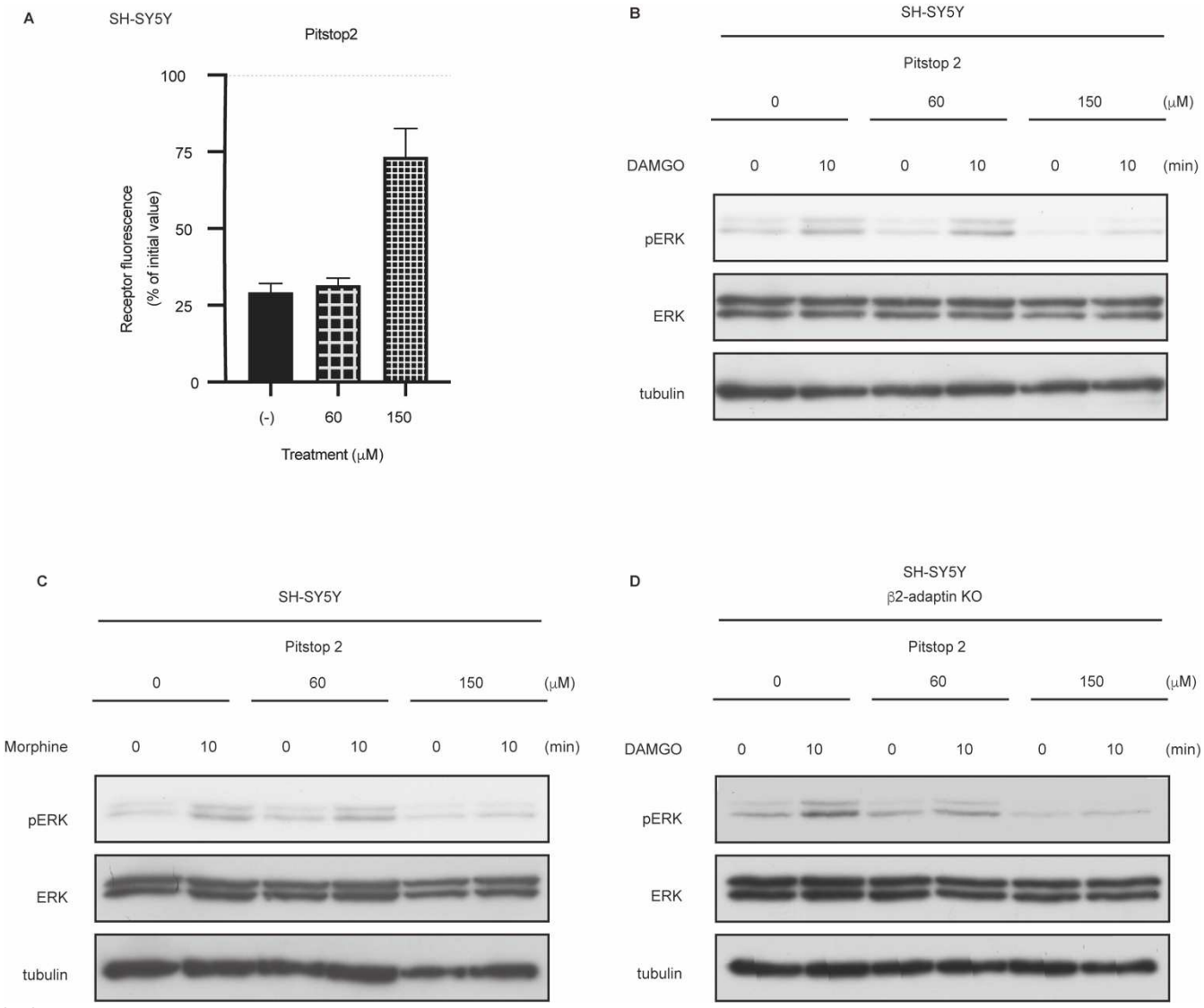


**Figure 5. β-arrestin activates MAPK signaling via association with clathrin heavy chain during clathrin-coated pit formation. A.** Effects of the clathrin inhibitor Pitstop 2 on receptor internalization in wild-type SH-SY5Y cells upon stimulation with 10 μM of DAMGO. **B and C**. Immunoblot analysis of MAPK activation, followed by stimulation with 10 μM of DAMGO or morphine. **D.** Effects of Pitstop 2 on MAPK activation tested in β2-adaptin-depleted cells followed by stimulation with 10 μM of DAMGO.

## Discussion

We revealed the mechanisms underlying activation of the β-arrestin pathway in a neuronal cell line. Although previous studies mainly used non-neuronal cell lines in which MOPs are expressed ectopically, such as human embryonic kidney cells 293 (HEK293) or Chinese hamster ovary cells, these non-neuronal cell lines might not be appropriate for analyses of MOP-mediated signaling. Zaworski et al.[35] demonstrated that the recombinant human D3 dopamine receptor expressed in SH-SY5Y cells triggered greater G protein activation than observed in HEK293 cells, indicating the importance of selecting neuronal cell lines in investigations on neuronal receptors. Therefore, we chose a neuronal cell line for this study, SH-SY5Y cells, which endogenously express MOPs.

β-arrestin 1 has not been considered to play an important role in MOP-mediated signaling to date, because β-arrestin 1-null mice do not show remarkable phenotypes in response to opioid administration[21]. Considering that β-arrestin 1 and 2 share high homology and loss of both simultaneously resulted in embryonic lethality[22], we expected that investigations on the functional redundancy and differences between β-arrestin 1 and 2 would provide comprehensive understanding of the various discrepancies among reports describing whether or not β-arrestin 2-null mice presented with respiratory depression upon opioid administration. In the present study, we established β-arrestin 1 or 2-deficient SH-SY5Y cells with the CRISPR/Cas9 approach. These constructs helped to overcome the problems in conventional experimental systems such as knockdown or overexpression, enabling investigation of the functional redundancy and differences between β-arrestin 1 and 2. We found that β-arrestin 1 activated β-arrestin-mediated signals almost comparably to β-arrestin 2, whereas it only partially compensated for the

lack of β-arrestin 2 in MOP internalization. Thus, β-arrestin 1 can compensate for the loss of β-arrestin 2 function, although not fully. We speculate that the discrepancy in the development of classic opioid side effects among reports describing β-arrestin 2-null mice might, in part, be due to strain differences, as pointed out previously[12], and that β-arrestin 1 might have compensated for β-arrestin 2-mediated signals.

Surprisingly, ligands with different β-arrestin-bias activated β-arrestin-mediated signals almost equally. These results are in contrast to the assumptions of previous studies, in which the bias toward β-arrestin was assessed by the degree of recruitment to the receptor without testing whether the recruitment correlated with the strength of the downstream signal.[2,7,8,23] We found that no correlation between β-arrestin recruitment and signaling. These results strongly motivated us to further investigate the molecular mechanisms underlying the activation of the β-arrestin pathway. Although bias toward β-arrestins had been evaluated based on the potency of ligands to recruit β-arrestins to MOP, presumably via phosphorylated residues in the carboxyl-terminal of MOP[2,5,7,8], we showed that neither phosphorylation-deficient MOP nor carboxyl-terminal-deficient MOP impaired β-arrestin-mediated signals. Of note, the MOP mutant lacking the carboxyl-terminal fully activated the downstream signal, although to a lower extent than found for wild-type MOP. Our results are consistent with the previously proposed concept that β2-adrenergic receptor, a member of GPCRs, activates β-arrestin similar to the manner in which an enzyme catalyzes the substrate[36,37]. These results may collectively explain why G protein-biased ligands, which recruit β-arrestins only minimally to MOP, result in classic opioid side effects. The conformational change of the receptor core upon activation, which varies according to the ligand, seems to be particularly important for determining the state of β-arrestin; thus, understanding the role of the receptor core may

help to discover ideal opioids without side effects.

Most importantly, we found that β-arrestins remained activated by binding with clathrin heavy chain during clathrin-coated pit formation. This finding is consistent with previous crystallographic data showing that clathrin heavy chain binds to fit between the N-domain and C-domain of β-arrestin[38,39]. In its inactive state, β-arrestin has a structure in which the C-terminus interact with the N-domain. Upon activation, the interaction is disrupted, and the C-domain twists by about 20° relative to the N-domain[18,28,29]. β-arrestin has a clathrin-binding site on the C-terminus, and once clathrin heavy chain binds to this site, the C-terminus is not likely to interact with the N-domain again. In other words, clathrin is important for maintaining the activated structure of β-arrestin without returning to the inactive state. The binding to clathrin heavy chain might help β-arrestins remain activated and could thus be an essential step for activating β-arrestin-mediated signals. Traditionally, endocytosis has been considered to be responsible for the uptake of extracellular molecules into the cell, and the subsequent transmission of the molecules and their information to organelles. However, the present study showed that the accumulation of molecules for endocytosis, rather than their physical distribution, is important for signal formation. This is a novel concept indicating that the accumulation of molecules required for endocytosis is a key for activating β-arrestin-mediated downstream signals. Furthermore, our results comprehensively explain why the β-arrestin pathway seemed to be activated by a G protein-biased agonist *in vivo* and in mutant mice that do not recruit β-arrestin 2.

However, we should note that molecules other than β-arrestin also activate the MAPK pathway. In particular, it is considered that G-protein activates the MAPK pathway in balance with β-arrestin[40,41]. This balance seems to explain the slight difference

in the degree of attenuation of MAPK signaling between DAMGO and morphine observed in the present study in both β-arrestin 1- and 2-lacking SH-SY5Y cells. However, the loss of β-arrestin did not enhance MAPK signaling even though β-arrestin is known to suppress G protein pathway, suggesting that β-arrestin predominantly contributes to the MAPK pathway. The significance of MAPK signaling on opioid side effects is not yet clear[42,43]. MAPK signaling at MOP has also been implicated in opioid tolerance and dependence. However, it is unknown how inhibition of the MAPK pathway by disrupting the association between β-arrestin and clathrin will affect opioid effects *in vivo*. Our findings may help to provide a new strategy to suppress signals related to the adverse effects of opioids selectively. However, the main difficulty remains in that clathrin plays an essential role in the endocytosis of any molecule; thus, inhibiting the association of β-arrestin with clathrin may interfere with the system that widely regulates GPCRs.

In conclusion, we revealed that two β-arrestins coordinately activated downstream signaling via binding to clathrin heavy chain. Our findings may provide a molecular basis for overcoming opioid side effects more effectively and selectively. Moreover, we uncovered part of the regulatory mechanisms of the large GPCR family that may be widely shared by cell-surface receptors whose activation leads to endocytosis.

## Materials and Methods

### Cell culture

SH-SY5Y cells were obtained from American Type Culture Collection (ATCC CRL-2266; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium (D-MEM)/Ham's F-12 with L-glutamine and phenol red (Cat# 048-29785; FUJIFILM Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Cat# FB-1365/500; Biosera,

Nuaille, France) in an incubator with 5% $CO_2$ at 37 °C. HEK293 cells were provided by RIKEN BRC, Tsukuba, Japan (Cat# RCB1637) through the National BioResource Project of MEXT, Japan, and cultured in D-MEM (high glucose) with L-Glutamine, phenol red, and sodium pyruvate (Cat# 043-30085; FUJIFILM Wako, Osaka, Japan) supplemented with 10% fetal bovine serum in an incubator with 5% $CO_2$ at 37 °C. The Lenti-X™ 293T cell line (HEK293T) was obtained from Clontech, Mountain View, CA, USA (Cat# 632180) and cultured in the same manner as described above for HEK293 cells.

**Lentiviral expression**

For lentiviral expression, CS2-CMV-MCS-IRES2-Bsd was provided by RIKEN BRC, Tsukuba, Japan (Cat# RDB04385) through the National BioResource Project of MEXT, Japan. For genome editing with CRISPR/Cas9, we used the lentiCRISPR v2 vector (Plasmid 52961; Addgene, Watertown, MA, USA)[44], and the guide RNA sequence designed for the target gene was cloned according to the manufacturer's instruction. LentiCRISPR v2 was obtained as a gift from Fen Zhang. Lenti-X™ 293T cells were transfected with the appropriate vector along with Lentiviral Hight Titer Packaging Mix (Cat# 6955; TaKaRa, Kusatsu, Japan) using TransIT-293 Transfection Reagent (MIR2704; Mirus, Madison, WI, USA) according to the manufacturer's instructions. After 48 h of incubation, the lentivirus-containing supernatant was collected and filtered through a 0.45-μm polyvinylidene difluoride filter (Cat# SLHVR33RS; Merck, Darmstadt, Germany). The clarified lentiviral supernatant was concentrated with Lenti-X Concentrator (Cat# 631231; Clontech, Mountain View, CA, USA), and then used to infect SH-SY5Y cells or HEK293 cells in the culture medium containing 9 μg/ml of polybrene (Cat# H9268; Sigma Aldrich, St. Louis, MO, USA). The stably transduced cells were selected using blasticidin (10 μg/ml) or puromycin (3 μg/ml).

**Gene editing with CRISPR/Cas9**

To edit the genome of SH-SY5Y cells targeting *ARRB1* (β-arrestin 1), *ARRB2* (β-arrestin 2), *AP2B1* (β2-adaptin), and *CTCL* (clathrin heavy chain), guide RNA sequences for the respective target genes were cloned into the lentiCRISPR v2 vector. SH-SY5Y cells were infected with the resultant viruses, followed by selection with puromycin. Monoclonal cell lines were obtained by limited dilution. Gene knockout in the obtained cell lines was verified by checking the genome sequence and confirming the loss of expression of the targeted protein by western blotting.

The guide RNA sequences targeting each gene were as follows: human *ARRB1* exon3, TGTGGACCACATCGACCTCG; human *ARRB2* exon3, CGGGACTTCGTAGATCACC; human *AP2B1* exon 6, TGCCCAGGGAAATTACTCGG; human *CLTC* exon 7, TGCCCAGGGAAATTACTCGG. For genomic PCR, the primer pairs used were as follows: human *ARRB1* exon 3 Fwd 5′-ATAAGGGTTAGGGATGGGTCTG-3′, Rev 5′-GAGCTGAGAGCTATTTCTGGGA-3′; human *ARRB2* exon 3 Fwd 5′-GCTCAAAAGCCTAAAGGTCCAC-3′, Rev 5′-GCGGTCCTTCAGGTAGTCAG-3′; human *AP2B1* exon 6 Fwd 5′-ATTAATAAGCTGCTGACAGCCC-3′, Rev 5′-CAATATCTACCCCAGAAGCCAC-3′; human *CLTC* exon 7 Fwd 5′-ATAACTTAGCCGGTGCTGAAGA-3′, Rev 5′-CCAAATCTAGAGGCATTGACAAG-3′.

**Knockdown of clathrin heavy chain**

To knockdown the *CLTC* gene, SH-SY5Y cells were transfected with small interfering RNA (sense, GCAAUGAGCUGUUUGAAGATT) for 48 h[33]. As a negative control, a universal negative control small interfering RNA (NIPPON GENE, Toyama, Japan) was

409 prepared. We used TransIT-TKO Transfection Reagent (MIR2152; Mirus, Madison, WI,
410 USA) according to the manufacturer's instructions.

411 **Plasmids**

412 The hemagglutinin (HA) epitope tag sequence was ligated into the amino terminal of the
413 open reading frame of human *MOR1* and then cloned into the retroviral expression vector.
414 The site-directed mutations were introduced by designing primers and using polymerase
415 chain reaction. To generate phosphorylation-deficient MOR1, all 11 serine and threonine
416 residues in the carboxyl-terminal (11S/T-A) were replaced by alanines (T356A, S357A,
417 S358A, S365A, T366A, T372A, S377A, T378A, T381A, T385A, T396A). To generate
418 the MOR1 mutant lacking the carboxyl-terminal (ΔC-term), we introduced the stop codon
419 after the codon encoding amino acid P402. cDNAs were ligated into the retroviral
420 expression vector described above.

421 **Cell lysis and immunoblotting**

422 The samples were treated as described previously[45]. In brief, cells were lysed with lysis
423 buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium
424 dodecyl sulfate, and 0.5% sodium deoxycholate containing protease inhibitor cocktail
425 (160-26071; FUJIFILM WAKO, Osaka, Japan). The lysates were clarified by
426 centrifugation at 15,000 rpm for 20 min at 4 °C. Samples were resolved by sodium
427 dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene
428 difluoride membranes. After blocking in Tris-buffered saline containing 0.1% Tween 20
429 and 5% (wt/vol) non-fat dry milk (31149-75; Nacalai, Kyoto, Japan), the membranes were
430 immunoblotted with the appropriate primary antibodies. The bound antibodies were
431 visualized via enhanced chemiluminescence after incubation with horseradish
432 peroxidase-conjugated secondary antibodies against mouse or rabbit IgG. Immunoblotted

protein bands were visualized and analyzed using X-ray films.

**Antibodies**

The following antibodies were obtained from commercial sources and used at the indicated dilutions for western blotting analysis: anti-phospho-p44/42 MAPK (ERK1/2) (1:5,000, Cat# 9101; Cell Signaling Technology, Danvers, MA, USA), anti-p44/42 MAPK (ERK1/2) (1:2,000, Cat# 9102; Cell Signaling Technology, Danvers, MA, USA), anti-β-arrestin 1/2 (1:2,000, Cat# 4674; Cell Signaling Technology, Danvers, MA, USA), anti-AP2B1 (1:2,000, Cat# 15690-1-AP; Proteintech, Rosemont, IL, USA), anti-clathrin heavy chain (1:2,000, Cat# sc-12734; Santa Cruz Biotechnology, Dallas, TX, USA), anti-α-tubulin (1:5,000, Cat# 3873; Cell Signaling Technology, Danvers, MA, USA), and purified anti-HA.11-epitope tag (1:2000, Cat# 901501; Biolegend, San Diego, CA, USA). For flow cytometry analysis, PE anti-HA.11 Epitope Tag Antibody (1:100, 901518; BioLegend, San Diego, CA, USA) and PE Mouse IgG1, κ Isotype Ctrl Antibody (1:100, 400111; BioLegend, San Diego, CA, USA) were used.

**Flow cytometry analysis**

Flow cytometry data were collected using a BD FACS Calibur flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA) and analyzed by FCSalyzer ver.09.15-alpha (Slashdot Media, San Diego, CA, USA).

**MOP ligands and endocytosis inhibitors**

The following ligands targeting MOP were purchased: [D-Ala$^2$, NMe-Phe$^4$-, Gly-ol$^5$] (DAMGO) (Cat# ab120674; Abcam, Cambridge, UK) and morphine hydrochloride (Takeda Pharmaceutical Company, Tokyo, Japan). Reagents targeting specific points of endocytosis were commercially obtained: barbadin (Acon2774; Axon Medchem, Reston, VA, USA), Dyngo-4a (ab120689; Abcam, Cambridge, UK), and Pitstop 2 (ab120687;

Abcam, Cambridge, UK).

**Statistical analysis**

Quantitative data are expressed as the mean, and error bars represent the standard error of the difference between means, unless indicated otherwise. Statistical significance was analyzed by one-way analysis of variance with Dunnett's multiple comparison test using Prism 9.0 (GraphPad Software, San Diego, CA, USA) ($\alpha = 0.05$). All experiments showing representative data were repeated independently at least three times with similar results.

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**Author contributions**

A.S. and S.S. conceived and designed the project, performed the experiments, analyzed the data, and wrote the manuscript.

**Competing interests**

The authors declare that no conflicts of interest exist.

## Additional information

We have materials transfer agreements with Addgene for lentiCRISPRv2 vector and pSpCas9(BB)-2A-Puro(PX459) vector, and with RIKEN BRC for CSII-CMV-MCS-IRES2-Bsd vector. Correspondence and requests for materials should be addressed to Satoshi Shimizu.

## References


1 Wootten, D., Christopoulos, A., Marti-Solano, M., Babu, M. M., Sexton, P. M. Mechanisms of signalling and biased agonism in G protein-coupled receptors. *Nat Rev Mol Cell Biol* **19**, 638-653, doi:10.1038/s41580-018-0049-3 (2018).

2 Schmid, C. L., Kennedy N. M., Ross, N. C., Lovell, K. M., Yue, Z., Morgenweck, J., Cameron, M. D., Bannister, T. D., Bohn, L. M. Bias factor and therapeutic window correlate to predict safer opioid analgesics. *Cell* **171**, 1165-1175.e1113, doi:10.1016/j.cell.2017.10.035 (2017).

3 Raehal, K. M., Schmid, C. L., Groer, C. E., Bohn, L. M. Functional selectivity at the μ-opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol Rev* **63**, 1001-1019, doi:10.1124/pr.111.004598 (2011).

4 Zhou, L., Bohn, L. M. Functional selectivity of GPCR signaling in animals. *Curr Opin Cell Biol* **27**, 102-108, doi:10.1016/j.ceb.2013.11.010 (2014).

5 Huang, W., Manglik, A., Venkatakrishnan, A. J., Laeremans, T., Feinberg, E. N., Sanborn, A. L. Kato, H. E., Livingston, K. E., Thorsen, T. S., Kling, R. C., *et al.* Structural insights into mu-opioid receptor activation. *Nature* **524**, 315-321, doi:10.1038/nature14886 (2015).

6 Lemos Duarte, M., Devi, L. A. Post-translational modifications of opioid receptors. *Trends Neurosci* **43**, 417-432, doi:10.1016/j.tins.2020.03.011 (2020).

7 DeWire, S. M., Yamashita, D. S., Rominger, D. H., Liu, G., Cowan, C. L., Graczyk, T. M., Chen, X. T., Pitis, P. M., Gotchev, D., Yuan, C., *et al.* A G protein-biased ligand at the mu-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J Pharmacol Exp Ther* **344**, 708-717, doi:10.1124/jpet.112.201616 (2013).

8 Manglik, A., Lin, H., Aryal, D. K., McCorvy, J. D., Dengler, D., Corder, G., Levit, A., Kling, R. C., Bernat, V., Hübner, H., *et al.* Structure-based discovery of opioid analgesics with reduced side effects. *Nature* **537**, 185-190, doi:10.1038/nature19112 (2016).

9 Hill, R. Disney, A., Conibear, A., Sutcliffe, K., Dewey, W., Husbands, S., Bailey, C., Kelly, E., Henderson, G. The novel mu-opioid receptor agonist PZM21 depresses respiration and induces tolerance to antinociception. *Br J Pharmacol* **175**, 2653-2661, doi:10.1111/bph.14224 (2018).

10 Viscusi, E. R., Skobieranda, F., Soergel, D. G., Cook, E., Burt, D. A., Singla, N. APOLLO-1: a randomized placebo and active-controlled phase III study investigating oliceridine (TRV130), a G protein-biased ligand at the mu-opioid receptor, for management of moderate-to-severe acute pain following bunionectomy. *J Pain Res* **12**, 927-943, doi:10.2147/JPR.S171013 (2019).

11 Bachmutsky, I., Wei, X. P., Durand, A. Yackle, K. ß-arrestin 2 germline knockout does not attenuate opioid respiratory depression. *Elife* **10**, e62552. doi:10.7554/eLife.62552 (2021).

12 Kliewer, A. Gillis, A., Hill, R., Schmiedel, F., Bailey, C., Kelly, E., Henderson, G.,

Christie, M. J., Schulz, S. Morphine-induced respiratory depression is independent of beta-arrestin2 signalling. *Br J Pharmacol* **177**, 2923-2931, doi:10.1111/bph.15004 (2020).

13 Kliewer, A., Schmiedel, F., Sianati, S., Bailey, A., Bateman, J. T., Levitt, E. S., Williams, J. T., Christie, M. J., Schulz, S. Phosphorylation-deficient G-protein-biased mu-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nat Commun* **10**, 367, doi:10.1038/s41467-018-08162-1 (2019).

14 Gillis, A., Kliewer, A. Kelly, E., Henderson, G., Christie, M. J., Schulz, S., Canals, M. Critical assessment of G protein-biased agonism at the mu-opioid receptor. *Trends Pharmacol Sci* **41**, 947-959, doi:10.1016/j.tips.2020.09.009 (2020).

15 Weinberg, Z. Y., Puthenveedu, M. A. Regulation of G protein-coupled receptor signaling by plasma membrane organization and endocytosis. *Traffic* **20**, 121-129, doi:10.1111/tra.12628 (2019).

16 Pavlos, N. J., Friedman, P. A. GPCR Signaling and trafficking: the long and short of it. *Trends Endocrinol Metab* **28**, 213-226, doi:10.1016/j.tem.2016.10.007 (2017).

17 von Zastrow, M., Sorkin, A. Mechanisms for regulating and organizing receptor signaling by endocytosis. *Annu Rev Biochem* **90,** 709-737, doi:10.1146/annurev-biochem-081820-092427 (2021).

18 Chen, Q., Iverson, T. M., Gurevich, V. V. Structural basis of arrestin-dependent signal transduction. *Trends Biochem Sci* **43**, 412-423, doi:10.1016/j.tibs.2018.03.005 (2018).

19 Bohn, L. M., Lefkowitz, R. J., Gainetdinov, R. R., Peppel, K., Caron, M. G., Lin,

F. T. Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* **286**, 2495-2498, doi:10.1126/science.286.5449.2495 (1999).

20 Raehal, K. M., Walker, J. K., Bohn, L. M. Morphine side effects in beta-arrestin 2 knockout mice. *J Pharmacol Exp Ther* **314**, 1195-1201, doi:10.1124/jpet.105.087254 (2005).

21 Robins, M. T., Chiang, T., Berry, J. N., Ko, M. J., Ha, J. E., van Rijn, R. M. Behavioral characterization of beta-arrestin 1 knockout mice in anxiety-like and alcohol behaviors. *Front Behav Neurosci* **12**, 54, doi:10.3389/fnbeh.2018.00054 (2018).

22 Pierce, K. L., Lefkowitz, R. J. Classical and new roles of β-arrestins in the regulation of G-PROTEIN-COUPLED receptors. *Nat Rev Neurosci* **2**, 727-733, doi:doi:10.1038/35094577 (2001).

23 Kelly, E. Ligand bias at the μ-opioid receptor. *Biochem Soc Trans* **41**, 218-224, doi:10.1042/BST20120331 (2013).

24 Doll, C., Konietzko, J., Pöll, F., Koch, T., Höllt, V., Schulz, S. Agonist-selective patterns of μ-opioid receptor phosphorylation revealed by phosphosite-specific antibodies. *Br J Pharmacol* **164**, 298-307, doi:10.1111/j.1476-5381.2011.01382.x (2011).

25 Just, S., Illing, S., Trester-Zedlitz, M., Lau, E. K., Kotowski, S. J., Miess, E., Mann, A., Doll, C., Trinidad, J. C., Burlingame, A. L., *et al.* Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol Pharmacol* **83**, 633-639, doi:10.1124/mol.112.082875 (2013).

26 Miess, E., Gondin, A. B., Yousuf, A., Steinborn, R., Mösslein, N., Yang, Y., Göldner, M., Ruland, J. G., Bünemann, M., Krasel, C., *et al.* Multisite

phosphorylation is required for sustained interaction with GRKs and arrestins during rapid mu-opioid receptor desensitization. *Sci Signal* **11**, eaas9609, doi:10.1126/scisignal.aas9609 (2018).

27 Zhuo, Y., Vishnivetskiy, S. A., Zhan, X., Gurevich, V. V., Klug, C. S. Identification of receptor binding-induced conformational changes in non-visual arrestins. *J Biol Chem* **289**, 20991-21002, doi:10.1074/jbc.M114.560680 (2014).

28 Sente, A., Peer, R., Srivastava, A., Baidya, M., Lesk, A. M., Balaji, S., Shukla, A. K., Babu, M. M., Flock, T. Molecular mechanism of modulating arrestin conformation by GPCR phosphorylation. *Nat Struct Mol Biol* **25**, 538-545, doi:doi:10.1038/s41594-018-0071-3 (2018).

29 Latorraca, N. R., Wang, J. K., Bauer, B., Townshend, R. J. L., Hollingsworth, S. A., Olivieri, J. E., u, E., Sommer, M. E., Dror, R. O. Molecular mechanism of GPCR-mediated arrestin activation. *Nature* **557**, 452-456, doi:10.1038/s41586-018-0077-3 (2018).

30 Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., Caron, M. G. The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. *J Biol Chem* **275**, 23120-23126, doi:10.1074/jbc.M002581200 (2000).

31 Beautrait, A., Paradis, J. S., Zimmerman, B., Giubilaro, J., Nikolajev, L., Armando, S., Kobayashi, H., Yamani, L., Namkung, Y., Heydenreich, F. M., *et al.* A new inhibitor of the β-arrestin/AP2 endocytic complex reveals interplay between GPCR internalization and signalling. *Nat Commun* **8**, 15054, doi:10.1038/ncomms15054 (2017).

32 Goodman, O. B. Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B.,

Gagnon, A. W., Keen, J. H., Benovic, J. L. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* **383**, 447-450, doi:10.1038/383447a0 (1996).

33 von Kleist, L., Stahlschmidt, W., Bulut, H., Gromova, K., Puchkov, D., Robertson, M. J., MacGregor, K. A., Tomilin, N., Pechstein, A., Chau, N., *et al.* Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. *Cell* **146**, 471-484, doi:10.1016/j.cell.2011.06.025 (2011).

34 Goodman, O. B., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., Keen, J. H. Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. *J Biol Chem* **272**, 15017-15022, doi:10.1074/jbc.272.23.15017 (1997).

35 Zaworski, P. G., Alberts, G. L., Pregenzer, J. F., Im, W. B., Slightorn, J. L., Gill, G. S. Efficient functional coupling of the human D3 dopamine receptor to G(o) subtype of G proteins in SH-SY5Y cells. *Br J Pharmacol* **128**, 1181-1188, doi:10.1038/sj.bjp.0702905 (1999).

36 Eichel, K., Jullie, D. & von Zastrow, M. beta-Arrestin drives MAP kinase signalling from clathrin-coated structures after GPCR dissociation. *Nat Cell Biol* **18**, 303-310, doi:10.1038/ncb3307 (2016).

37 Eichel, K., Jullié D., Barsi-Rhyne, B., Latorraca, N. R., Masureel, M., Sibarita, J. B., Dror, R. O., von Zastrwo, M. Catalytic activation of beta-arrestin by GPCRs. *Nature* **557**, 381-386, doi:10.1038/s41586-018-0079-1 (2018).

38 Goodman, O. B. Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., Keen, J. H. Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. *J Biol Chem* **272**, 15017-15022,

doi:10.1074/jbc.272.23.15017 (1997).

39 Kang, D. S., Kern, R. C., Puthenveedu, M. A., von Zastrwo, M., Williams, J. C., Benovic, J. L. Structure of an arrestin2-clathrin complex reveals a novel clathrin binding domain that modulates receptor trafficking. *J Biol Chem* **284**, 29860-29872, doi:10.1074/jbc.M109.023366 (2009).

40 Luttrell, L. M., Wang, J., Plouffe, B., Smith, J. S., Yamani, L., Kaur, S., Jean-Charles, P. Y., Gauthier, C., Lee, M. H., Pani, B., *et al.* Manifold roles of β-arrestins in GPCR signaling elucidated with siRNA and CRISPR/Cas9. *Sci Signal* **11**, eaat7650, doi:10.1126/scisignal.aat7650 (2018).

41 Grundmann, M., Merten, N., Malfacini, D., Inoue, A., Preis, P., Simon, K., Rüttiger, N., Ziegler, N., Benkel, T., Schmitt, N. K., *et al.* Lack of beta-arrestin signaling in the absence of active G proteins. *Nat Commun* **9**, 341, doi:10.1038/s41467-017-02661-3 (2018).

42 Bobeck, E. N., Ingram, S. L., Hermes, S. M., Aicher, S. A., Morgan, M. M. Ligand-biased activation of extracellular signal-regulated kinase 1/2 leads to differences in opioid induced antinociception and tolerance. *Behav Brain Res* **298**, 17-24, doi:10.1016/j.bbr.2015.10.032 (2016).

43 Gutstein, H. B., Rubie, E. A., Mansour, A., Akil, H., Woodgett, J. R. Opioid effects on mitogen-activated protein kinase signaling cascades. *Anesthesiology* **87**, 1118-1126, doi:10.1097/00000542-199711000-00016 (1997).

44 Sanjana, N. E., Shalem, O., Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* **11**, 783-784, doi:10.1038/nmeth.3047 (2014).

45 Shimizu, S., Fujita, H., Sasaki, Y., Tsuruyama, T., Fukuda, K., Iwai, K. Differential

involvement of the Npl4 zinc finger domains of SHARPN and HOIL-1L in linear ubiquitin chain assembly complex-mediated cell death protection. *Mol Cell Biol* **36**, 1569-1583, doi:10.1128/MCB.01049-15 (2016).

653 **Supplementary Material**

654

| | | Percent Identity | E value |
|---|---|---|---|
| beta-arrestin-1 Isoform A | beta-arrestin-2 Isoform 1 | 76.14% | 0 |
| | 2 | 73.25% | 0 |
| | 3 | 72.48% | 0 |
| | 4 | 80.46% | $8.00\times10^{-150}$ |
| | 5 | 74.00% | 0 |
| | 6 | 71.19% | 0 |
| | 7 | 73.09% | $4.00\times10^{-112}$ |

| | | Percent Identity | E value |
|---|---|---|---|
| beta-arrestin-1 Isoform B | beta-arrestin-2 Isoform 1 | 77.64% | 0 |
| | 2 | 74.69% | 0 |
| | 3 | 73.83% | 0 |
| | 4 | 80.46% | $9.00\times10^{-153}$ |
| | 5 | 75.42% | 0 |
| | 6 | 72.55% | 0 |
| | 7 | 75.81% | $4.00\times10^{-115}$ |

655

656 **Table S1. Amino acid sequence homology between β-arrestin 1 and 2, as searched by**

657 **BLAST in the NCBI database.**

658

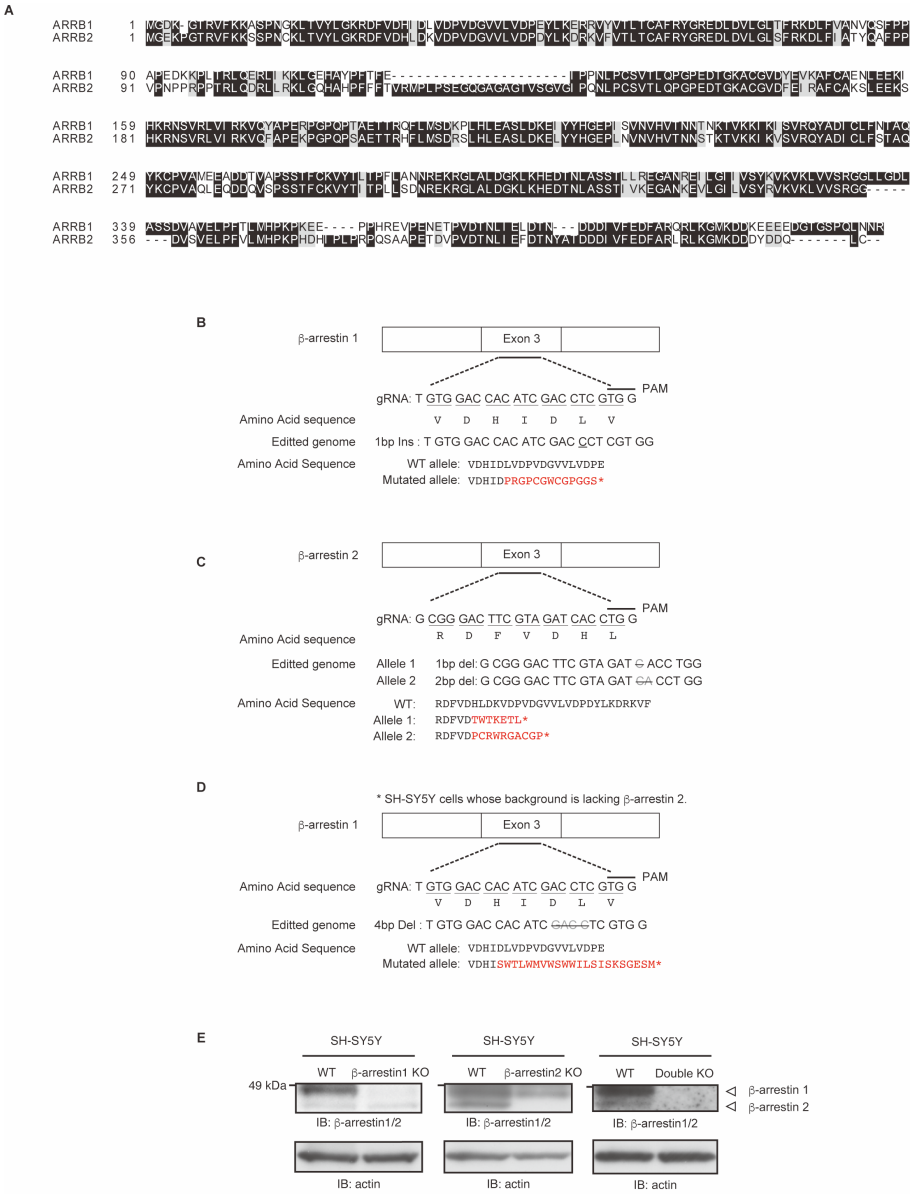


**Figure S1. Amino acid sequence homology between β-arrestin 1 and 2, and the design for genetic depletion of β-arrestins with CRISPR/Cas9 in SH-SY5Y cells.**

A. Homology in amino acid sequence between β-arrestin 1 and 2, as searched by BLAST in the NCBI database. Alignment search between β-arrestin 1 isoform A and β-arrestin 2 isoform 3 as a representative diagram. B-D. The gRNA design as well as obtained genomic and amino acid sequence for establishing knockout cell lines using CRISPR/Cas9. E. Western blotting analysis was conducted to confirm the loss of the targeted β-arrestin.

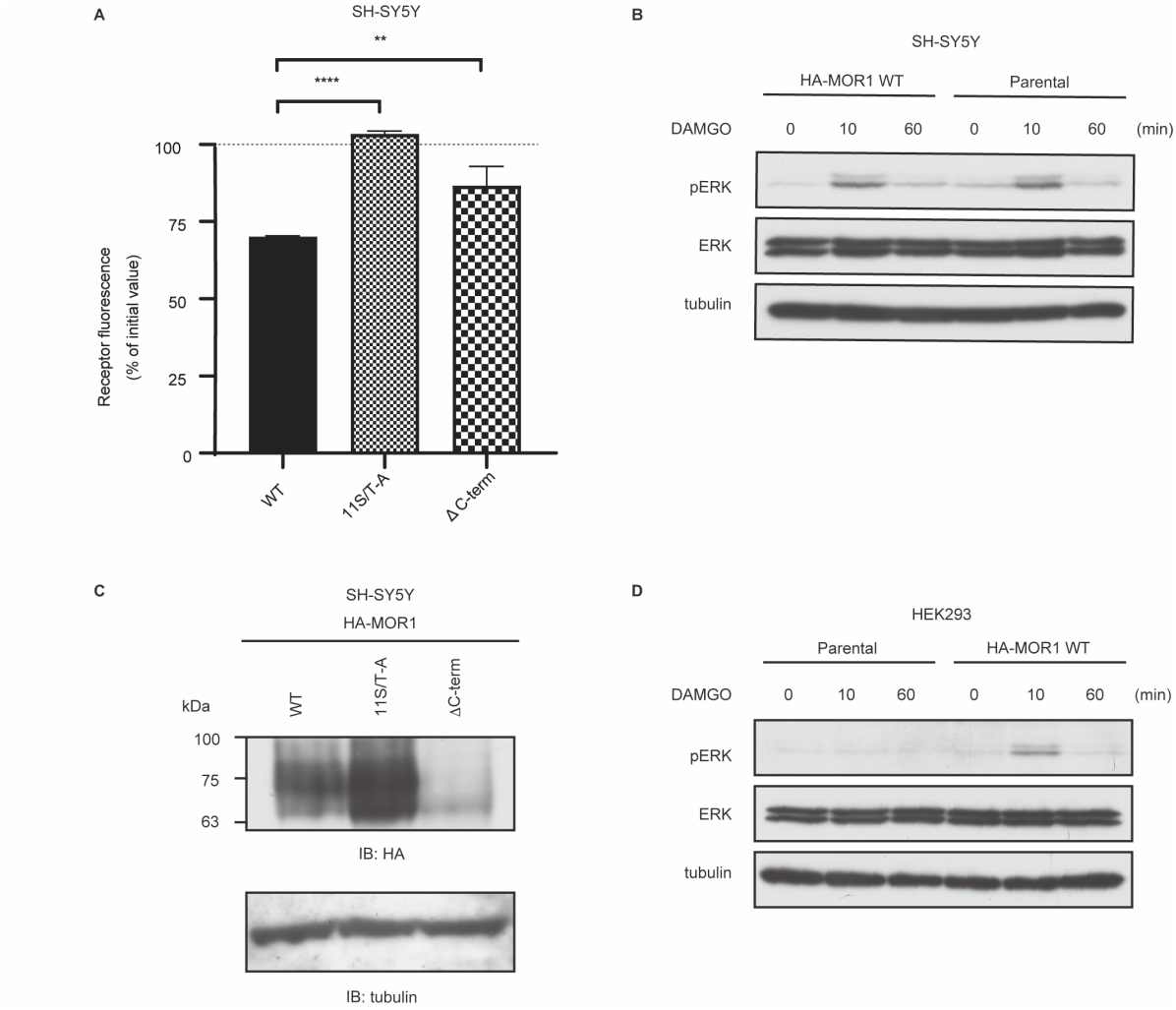


**Figure S2. Control experiments for evaluating MOP mutants introduced in SH-SY5Y and HEK293 cells.**

A. Receptor internalization when wild-type or mutated MOP tagged with an HA sequence were transfected into SH-SY5Y cells and stimulated with 10 μM of DAMGO for 10 min (**$P < 0.01$, ****$P < 0.0001$). B. Immunoblot analysis of MAPK activation in whole-cell lysates from SH-SY5Y cells stably expressing wild-type MOP (left) in comparison with the parental cells (right). C. Expression level of wild-type or mutated MOP expressed in SH-SY5Y cells was analyzed by immunoblotting. D. Immunoblot analysis of MAPK activation in whole-cell lysates from HEK293 cells stably expressing wild-type MOP (right) in comparison with the parental cells (left).

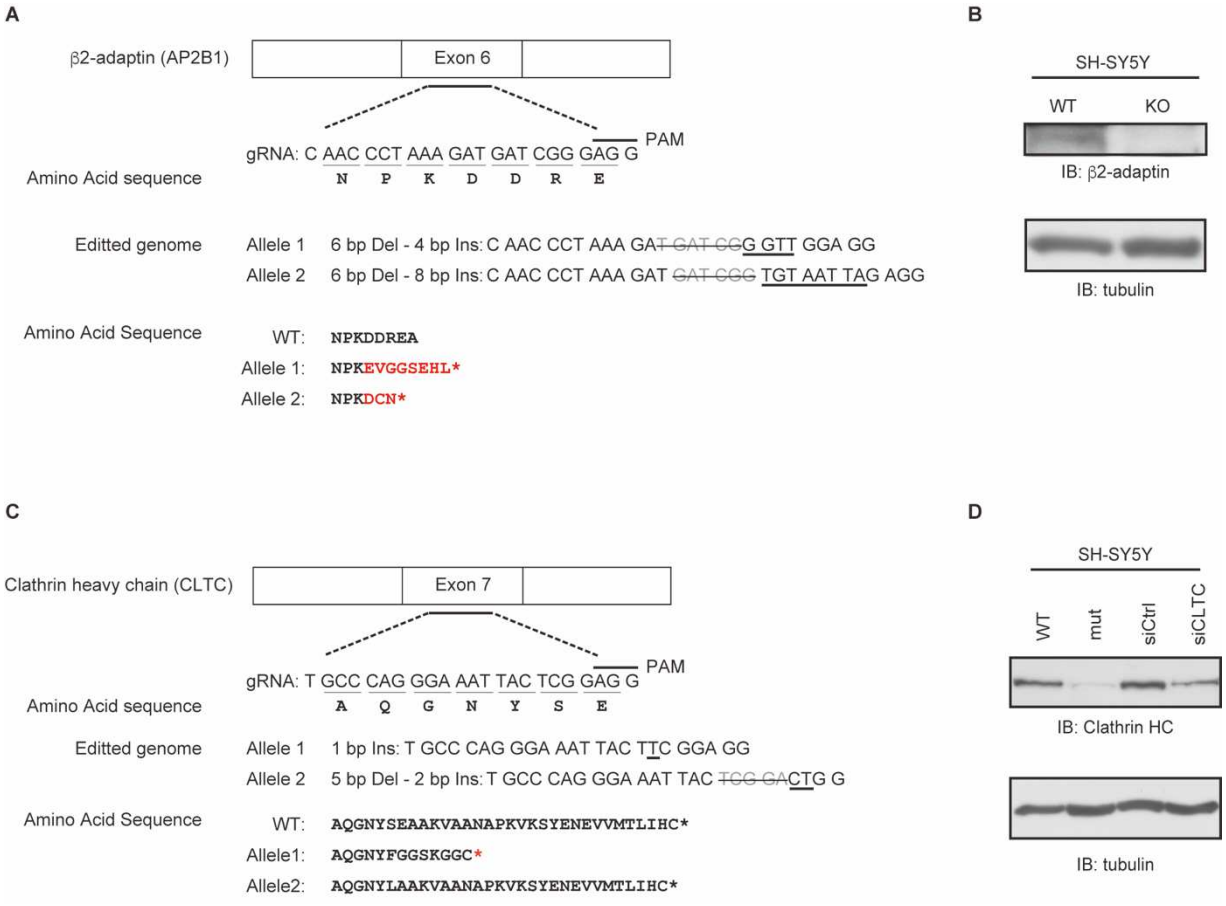


**Figure S3. Gene-editing on β2-adaptin and clathrin heavy chain with CRISPR/Cas9 in SH-SY5Y cells.**

A. The gRNA design as well as obtained genomic and amino acid sequence for establishing β2-adaptin null-cell lines using CRISPR/Cas9. B. Western blotting analysis was conducted to confirm the loss of expression of the β2-adaptin subunit of the AP2 adaptor complex. C. The gRNA design as well as obtained genomic and amino acid sequence for introducing mutation on CLTC gene encoding clathrin heavy chain (clathrin HC) using CRISPR/Cas9. D. Western blotting analysis was conducted to confirm the reduction of the expression level of the clathrin HC. Sample of cell cultures obtained by the knockdown method were used as references.

# Supplementary Files

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