Clathrin regulates the β-arrestin pathway regardless of ligand bias

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Article

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Abstract

μ-opioid receptors (MOP) are thought to activate the G protein-mediated analgesic pathway and β-arrestin 2-mediated side effect pathway[1]; however, ligands that recruit β-arrestin 2 only minimally to MOP may also cause opioid side effects[2,3]. 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[4-6]. These findings critically questioned whether β-arrestin 2 recruitment to MOP triggers side effects[7]. 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 is not correlated with β-arrestin recruitment to MOP via phosphorylation of the carboxyl-terminal of MOP, which has long been used to evaluate β-arrestin bias of a ligand. Instead, β-arrestin activates downstream signals by binding with the clathrin heavy chain in the process of clathrin-coated pit formation. Our findings provide not only a novel insight into G protein-coupled receptor-mediated signalling to overcome opioid side effects but also an unexpected concept that the accumulation of molecules required for endocytosis is a key for activating the intracellular signalling.

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[8]. 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[1]. Even though various opioids target MOP, the analgesic effects or adverse effects vary depending on the ligands. Differences in pharmacological effects, called functional selectivity, have been understood in relation to ligand bias, a concept that GPCR adopts a specific active conformation for each ligand[8-10]. Differences in activated structure, observed mainly in intracellular regions[11], are presumed to result in qualitative differences in receptor signalling complexes[8,12]. Applying this principle, bias-tuned ligands, that is, G protein-biased compounds that selectively activate the G protein-analgesic pathway while recruiting β-arrestin 2 only minimally, were proposed as ideal opioids with an improved property[13,14]. However, contrary to expectations, recent studies revealed that G protein-biased ligands might also produce classical opioid side effects at an analgesic dosage and have limited functional selectivity[2,3]. These observations strongly suggest that there is 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 do not recruit β-arrestin 2, also presented with such side effects following opioid administration[4-6]. These results critically questioned whether recruitment of β-arrestin 2 to MOP is the true culprit that triggers opioid side effects.

β-arrestin play multiple roles simultaneously. β-arrestins competitively repress the G protein pathway and activate G protein-independent signals[8] and also serve as

scaffolds for clathrin-coated pit formation for GPCR internalisation[15]. Receptor endocytosis tunes down extracellular stimuli and resensitises the receptors at endosomes while defining the spatiotemporal localisation 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-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]. Although these phenotypes suggest that β-arrestin 1 has no remarkable function in the opioid system, there are still 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 amino acid sequence, and the simultaneous loss of both resulted in embryonic lethality[22], it can be assumed that β-arrestin 1 and 2 share high functional homology and compensate for each other when one is missing. Another important question is how β-arrestin-mediated signals are activated. Researchers have evaluated β-arrestin recruitment to MOP as bias toward β-arrestin[1,13,14,23]. However, the correlation between β-arrestin bias and the strength of the downstream signal has not been thoroughly tested.

To resolve conflicts in this research area, we aimed to explore why G protein-biased ligands or mutant mice that lack β-arrestin 2 or do not recruit β-arrestin 2 disappointedly present with opioid side effects, by using 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 the basis for overcoming opioid side effects more effectively.

Results

Homology between β-arrestin 1 and 2

Initially, we focused on β-arrestin 1, whose amino acid sequence is highly homologous to that of β-arrestin 2 (Extended Data Fig. 1). We were especially 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, where the former recruits β-arrestins to MOP more robustly than the latter[24-26]. Furthermore, we used CRISPR/Cas9 to genetically modify SH-SY5Y cells, a neuronal cell line physiologically expressing MOP, to establish cells lacking either β-arrestin 1 or 2 or both simultaneously (Extended Data Fig. 2-4). On MOP activation, β-arrestin accumulates molecules as a scaffold to internalise the activated MOP and, simultaneously, drives G protein-independent signals[8,15]. Therefore, we investigated the functional differences and redundancy between β-arrestin 1 and 2 in terms of MOP internalisation and signal activation. First, the loss of both β-arrestin 1 and 2 almost completely abolished receptor internalisation for both ligands; β-arrestin 1 loss had little effect on receptor internalisation, whereas β-arrestin 2 loss comparatively reduced receptor internalisation (Fig. 1a, Extended Data Fig. 5). As previous reports suggested 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 (Fig. 1b). However, when either β-arrestin 1 or 2 was absent, the MAPK pathway was only slightly attenuated upon stimulation with either ligand (Fig. 1c,d). These results indicate that β-arrestin 1 compensates for MAPK

activation comparatively but compensates for MOP internalisation only partially, in the absence of β-arrestin 2.

Ligand bias and signal intensity

Previous studies have evaluated ligand bias toward β-arrestin by the extent to which β-arrestin is recruited to MOP[13,14,23]. We then addressed the question if there is a direct correlation between β-arrestin recruitment and β-arrestin-mediated signal intensity in the 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 intensity originating from β-arrestin. Surprisingly, the magnitude of ERK phosphorylation was almost equal between ligands showing different biases (Fig. 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 mutant did not recruit β-arrestin, and hence its downstream pathway would not be activated[6]. As our results differed from this assumption, we thus evaluated β-arrestin-mediated signals upon stimulation with MOP mutant that do not recruit β-arrestin to MOP. 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 whole carboxyl-terminal region (ΔC-term) (Fig. 2b). As expected, neither mutant internalised MOP upon stimulation with DAMGO (Fig. 2c). Next, we assessed whether these MOP mutants activate the signalling 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 (Extended Data Fig. 6). Compared with that in the wild-type, MOP expression in the 11S/T-A mutant was almost the same, whereas that in the ΔC-term mutant was significantly decreased, probably owing to the instability of the mutant structure (Fig. 2d, Extended Data Fig. 6). Surprisingly, MAPK was almost fully activated in both MOP mutants (Fig. 2e, Extended Data Fig. 7). Of note, such a tiny amount of ΔC-term mutant fully activated MAPK. The activation appeared to require only a contact, but not association, with MOP. These results collectively reveal the absence of correlation between β-arrestin recruitment to MOP and β-arrestin-mediated signal intensity.

Clathrin regulates the β-arrestin pathway

Next, we questioned how β-arrestin maintains its activated state in the absence of association with MOP. β-arrestin consists of an N-domain and a C-domain. In the basal state, the carboxyl-tail hooks on 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 something that prevents β-arrestins from reverting to the inactive state even when dissociated with MOP. Under physiological conditions, β-arrestins rapidly gather molecules to form clathrin-coated pits to internalise 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 hypothesised that once activated by MOP, β-arrestin maintains its activated conformation by associating with such molecules in 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 internalisation, DAMGO activated MAPK to the same extent as that in the absence of Dyngo-4a (Fig. 3a, b), indicating that the processes regulating β-arrestin activation occurs 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 internalisation abolished the signal originating from β-arrestin (Fig. 3c, d). 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 (Fig. 3e). To further elucidate the molecular mechanism and identify the molecular target to block the β-arrestin pathway, we used CRISPR/Cas9 to genetically deplete the β2-adaptin subunit of AP2 adaptor complex and clathrin heavy chain, which β-arrestin binds to[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 internalisation and did not affect β-arrestin-mediated signals (Extended Data Fig. 8, Fig. 3f, g). Next, we attempted to establish a cell line lacking clathrin heavy chain. However, despite designing gRNAs 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 roles such as cell division or survival. However, we found that some of the mutated clones of clathrin heavy chain, in which one allele was null but the other had three-base fold deletion, expressing a much lower amount of clathrin heavy chain than the cell culture obtained by the knockdown method (Extended Data Fig. 9). Although MOP internalisation in the mutant cells was almost comparable to that in wild-type cells, β-arrestin-mediated signals were significantly attenuated (Fig. 3h, i). Because the reduction of clathrin heavy chain

attenuated β-arrestin-mediated signals more than expected, we speculated that clathrin heavy chain plays an essential role in the β-arrestin pathway. As we were unable to establish a clathrin-deficient strain, we next used Pitstop 2, a reagent that inhibits the association of clathrin heavy chains with β-arrestin, to evaluate the involvement of clathrin heavy chain in β-arrestin mediated signals. At concentrations that sufficiently inhibit MOP internalisation, Pitstop 2 diminished the activation of the β-arrestin pathway (Fig. 3j, k). Furthermore, we pre-treated a cell line lacking β2-adaptin with Pitstop2 and found that Pitstop 2 successfully abolished the β-arrestin pathway in the β2-adaptin-null cells (Fig. 3l). These results collectively indicate that the β-arrestin pathway is activated in association with clathrin heavy chain during clathrin-coated pit formation.

Discussion

We revealed the mechanisms underlying the activation of the β-arrestin pathway. β-arrestin 1 has not been considered important in MOP-mediated signals 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 lead to comprehensive understanding of the various discrepancies among reports describing whether or not β-arrestin 2-null mice presented with respiratory depression upon opioid administration. Here, we found that β-arrestin 1 activated β-arrestin-mediated signals almost comparably to β-arrestin 2, while it only partially compensates for MOP internalisation. That is, β-arrestin 1 can compensate for β-arrestin 2 in the absence of β-arrestin 2, 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, resulted from strain differences, as pointed out previously[4], and that β-arrestin 1 might have compensated for β-arrestin 2-mediated signals.

It was surprising to us that the ligands with different β-arrestin-bias activated β-arrestin-mediated signals almost equally. Our results were contrary to what previous studies had assumed. These results strongly motivated us to further investigate molecular mechanisms underlying the activation of the β-arrestin pathway. Although bias toward β-arrestins had been evaluated as the potency of ligands to recruit β-arrestins to MOP, presumably via phosphorylated residues in the carboxyl-terminal of MOP[1,11,13,14], we showed that neither phosphorylation-deficient MOP nor carboxyl-terminal-deficient MOP impaired β-arrestin-mediated signals. Of note, MOP mutant lacking carboxyl-

terminal fully activated the downstream signal, though to a lower extent than wild-type MOP. Our results are consistent with the concept proposed by previous studies showing that β2-adrenergic receptor, a member of GPCRs, activates β-arrestin just as an enzyme catalyses the substrate [33,34]. These results may collectively explain why G protein-biased ligands, which recruit β-arrestins only minimally to MOP, presented with classic opioid side effects.

Most importantly, we found that β-arrestins remained activated by binding with clathrin heavy chain during clathrin-coated pit formation. Our findings are consistent with previous crystallographic data showing that clathrin heavy chain binds to fit between the N-domain and C-domain of β-arrestin[35,36]. The binding to clathrin heavy chain might help β-arrestins remain activated and be essential for activating β-arrestin-mediated signals. This is a novel concept 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.

In conclusion, we revealed that two β-arrestins co-ordinately activated the downstream signalling 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.

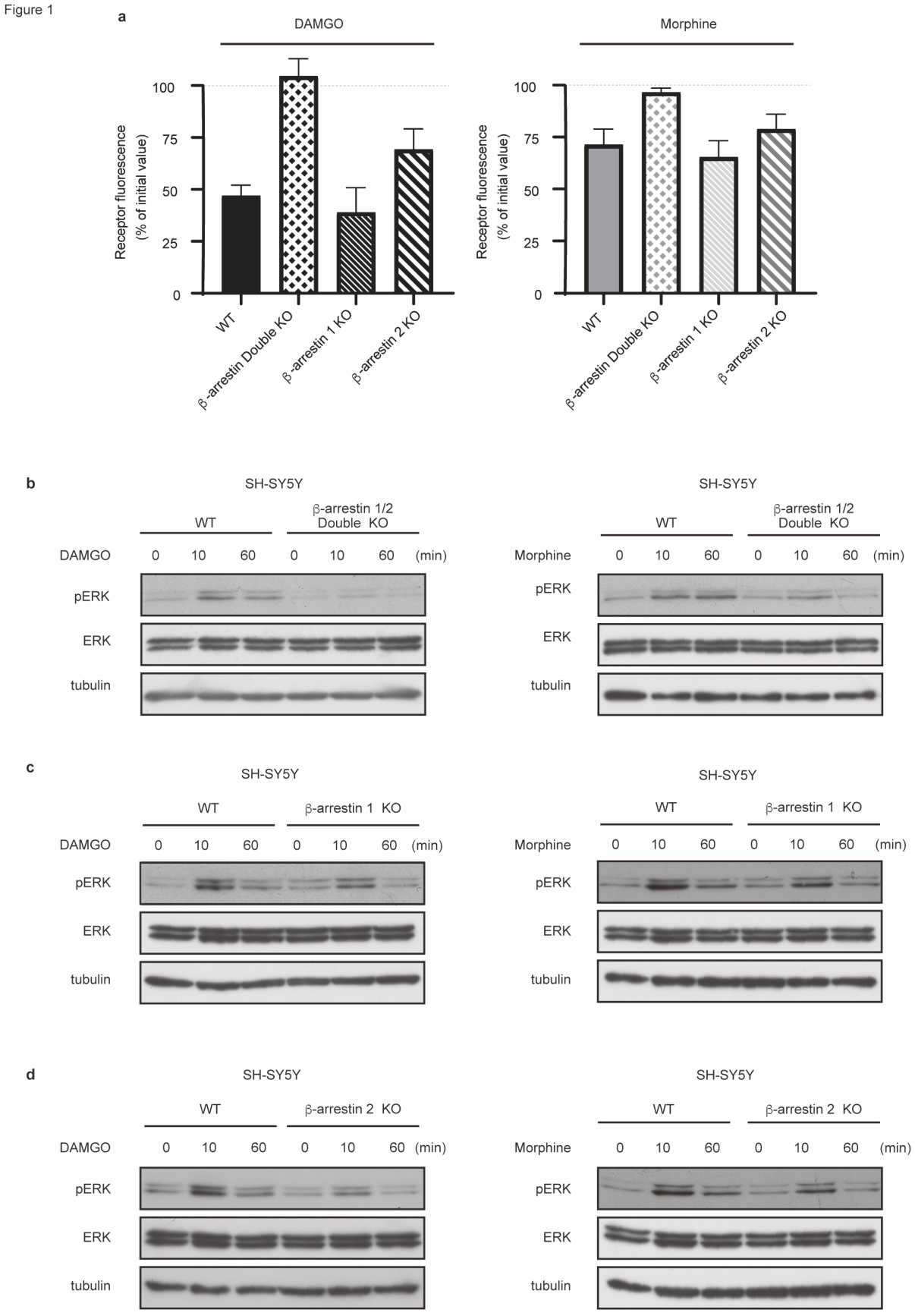

Figure 1. β-arrestin 1 compensates for β-arrestin 2, although not completely.

a. MOP internalisation under the loss of both β-arrestin 1 and 2 simultaneously, or either one of the β-arrestins, in SH-SY5Y cells overexpressing hemagglutinin (HA)-tagged MOR1 upon stimulation with 10 μM of DAMGO (left panel) and morphine (right panel) for 60 min. b-d. Immunoblot analyses of MAPK activation in whole-cell lysate from SH-SY5Y cells with the indicated genotypes following stimulation with the indicated ligand and indicated time.

Figure 2

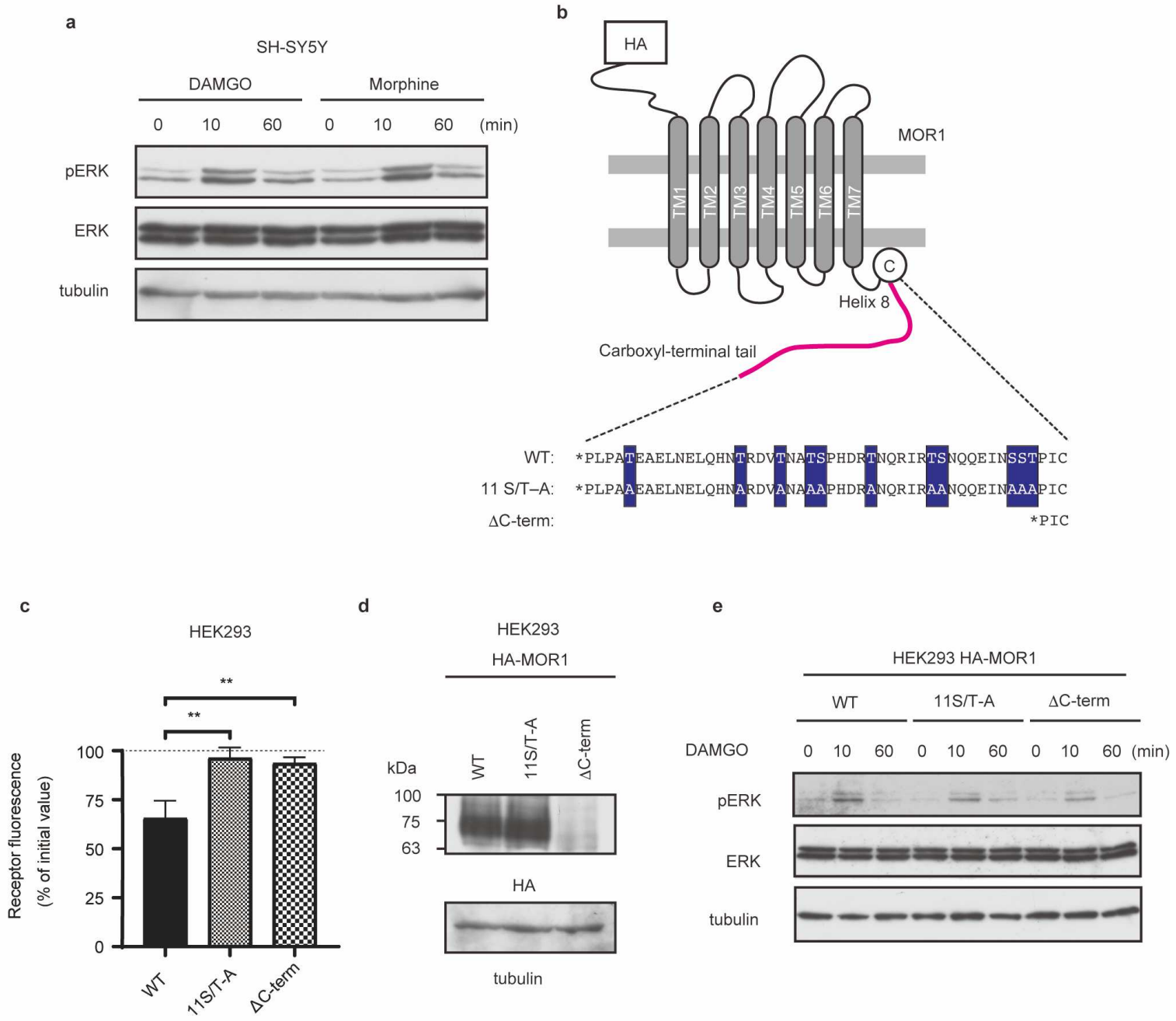

Figure 2. There is no correlation between β-arrestin-recruitment to MOP and the intensity of β-arrestin-mediated signals.

a. Immunoblot analysis of MAPK activation in whole-cell lysates from SH-SY5Y cells following stimulation with 10 μM of DAMGO or morphine for the indicated time. b. The scheme is describing the design of two MOP mutants and the sequence of the wild-type: 11S/T-A mutant, in which all serine and threonine residues in the carboxyl-terminal regions are replaced by alanine; and ΔC-term mutant, in which the carboxyl-terminal regions are deleted. c. Receptor internalisation when wild-type or mutated MOP tagged with HA sequence were introduced into the indicated cell line and stimulated with 10 μM of DAMGO for 10 min (**$P<0.01$). d. The expression level of wild-type or mutated MOP expressed in HEK293 cells was analysed by immunoblotting. e. Immunoblotting analysis of MAPK activation in whole-cell lysates from HEK293 cells stably expressing wild-type or mutated MOP tagged with HA sequence.

Figure 3

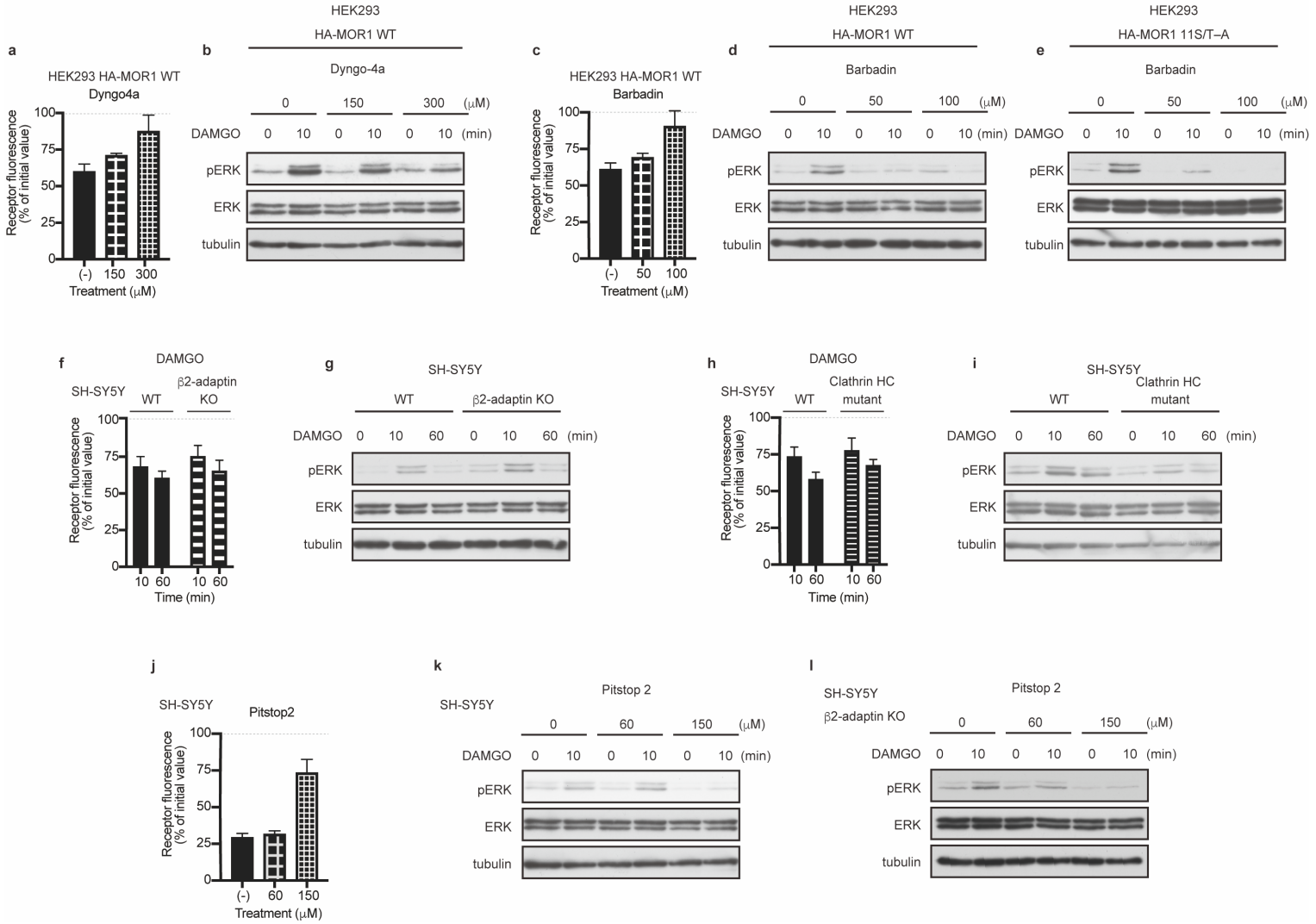

Figure 3. β-arrestin activates MAPK signalling via association with clathrin heavy chain during clathrin-coated pit formation.

a and b. Analyses of the effects of inhibiting CCP-detachment by a dynamin inhibitor, Dyngo-4a, on receptor internalisation (a) and MAPK activation for the indicated time (b), followed by stimulation with 10 μM of DAMGO in HEK293 cells introduced with MOR1 tagged with HA sequence. c. Analyses of the effects of inhibiting CCP formation by barbadin on receptor internalisation (c) and MAPK activation, followed by stimulation with 10 μM of DAMGO in HEK293 cells introduced with wild-type MOR1 (d) or MOR1 with 11S/T-A mutation (e) for the indicated time. f-i. MOP internalisation (f and h) and MAPK activation (g and i) in wild-type, β2-adaptin-depleted, or clathrin HC mutant SH-SY5Y cells upon stimulation with 10 μM of DAMGO for the indicated time period. j-l. Analyses of effects of the clathrin inhibitor Pitstop 2 on receptor internalisation in wild-type SH-SY5Y cells (j) and MAPK activation (k), followed by stimulation with DAMGO. Effects of Pitstop 2 on MAPK activation was tested in β2-adaptin-depleted cells (l).

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Methods

Cell culture

SH-SY5Y cells were obtained from ATCC (ATCC CRL-2266) and cultured in D-MEM/Ham's F-12 with L-Glutamine and Phenol Red (Cat# 048-29785; FUJIFILM Wako, Japan) supplemented with 10% foetal bovine serum (Cat# FB-1365/500; Biosera, France) in an incubator with 5 % CO_2 at 37 °C. HEK293 cells were provided by RIKEN BRC, 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, Japan) supplemented with 10% foetal bovine serum in an incubator with 5% CO_2 at 37 °C. Lenti-X™ 293T Cell Line (HEK293T) were obtained from Clontech (Cat# 632180) and cultured in the same way as HEK293 cells.

Lentiviral expression

For lentiviral expression, CS2-CMV-MCS-IRES2-Bsd was provided by RIKEN BRC (Cat# RDB04385) through the National BioResource Project of MEXT, Japan. For genome editing with CRISPR/Cas9, we used lentiCRISPR v2 vector (Plasmid 52961; Addgene, U.S.A.)[37], 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 Cell Line were transfected with the appropriate vector along with Lentiviral Hight Titer Packaging Mix (Cat# 6955; TaKaRa, Japan) using TransIT-293 Transfection Reagent (MIR2704; Mirus, U.S.A.) according to the manufacturer's instruction. After 48 h of incubation, the lentivirus-containing supernatant was collected and filtered through a 0.45-μm polyvinylidene difluoride filter (Cat# SLHVR33RS; Merck, Germany). The clarified lentiviral supernatant was concentrated with a Lenti-X

Concentrator (Cat# 631231; Clontech, U.S.A.), and then used to infect SH-SY5Y cells or 293 cells in the culture medium containing 9 μg/ml of polybrene (Cat# H9268; Sigma Aldrich, U.S.A.). 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 exon6 (TGCCCAGGGAAATTACTCGG)

human CLTC exon7 (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 CLTC gene, SH-SY5Y cells were transfected with siRNA (sense, GCAAUGAGCUGUUUGAAGATT) for 48 h[33]. As a negative control, a universal negative control siRNA (NIPPON GENE, Japan) was prepared. We used TransIT-TKO Transfection Reagent (MIR2152; Mirus, U.S.A.) according to the manufacturer's instruction.

Plasmids

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

Cell lysis and immunoblotting

The samples were treated as described previously[38]. In brief, cells were lysed with lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% Sodium Dodecyl Sulfate and 0.5% sodium deoxycholate containing protease inhibitor cocktail (160-26071; FUJIFILM WAKO, Japan). The lysates were clarified by centrifugation at 15,000 rpm for 20 min at 4 °C. Samples were resolved by sodium

dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane. After blocking in Tris-buffered saline containing 0.1% Tween 20 and 5% (wt/vol) non-fat dry milk (31149-75; Nacalai, Japan), the membranes were immunoblotted with the appropriate primary antibodies. The bound antibodies were visualised via enhanced chemiluminescence after incubation with horseradish peroxidase-conjugated secondary antibodies against mouse or rabbit IgG. Immunoblotted protein bands were visualised and analysed 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, U.S.A.), anti-p44/42 MAPK (ERK1/2) (1:2,000, Cat# 9102; Cell Signaling Technology, U.S.A.), anti-β-arrestin 1/2 (1:2,000, Cat# 4674; Cell Signaling Technology,U.S.A.), anti-AP2B1 (1:2,000, Cat# 15690-1-AP; Proteintech, U.S.A.), anti-clathrin heavy chain (1:2,000, Cat# sc-12734; Santa Cruz, Germany), anti-α-tubulin (1:5,000, Cat# 3873; Cell Signaling Technology, U.S.A.), and purified anti-HA.11-epitope tag (1:2000, Cat# 901501; Biolegend, U.S.A.). For flow cytometry analysis, PE anti-HA.11 Epitope Tag Antibody (1:100, 901518; BioLegend, U.S.A.) and PE Mouse IgG1, κ Isotype Ctrl Antibody (1:100, 400111; BioLegend, U.S.A.) were used.

Flow cytometry analysis

Flow cytometry data were collected using a BD FACS Calibur flow cytometer (BD Bioscience, U.S.A.) and analysed by FCSalyzer ver.09.15-alpha (Slashdot Media, U.S.A.).

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, U.K.) and morphine hydrochloride (Takeda Pharmaceutical Company, Japan). Reagents targeting specific points of endocytosis were commercially obtained: barbadin (Acon2774; Axon Medchem, U.S.A.), Dyngo-4a (ab120689; Abcam U.K.), and Pitstop 2 (ab120687; Abcam U.K.).

Statistical analysis

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

Methods References

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Competing interests

The authors declare that no conflicts of interest exist.

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