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ORIGINAL ARTICLE

CRISPR/Cas9 unveils the dynamics of the endogenous μ-opioid receptors on neuronal cells under continuous opioid stimulation

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Abstract

Long-term opioid use develops tolerance and attenuates analgesic effects. Upon activation, μ-opioid receptors (MOPs) are internalized and directed to either recycling or degradation pathway. Ligand stimulation also promotes de novo MOP synthesis. These processes collaboratively regulate MOP expression and play critical roles in tolerance development. However, there is limited understanding of how the endogenous MOP expression changes after prolonged opioid administration because previous analyses have focused on individual processes using overexpression systems, which ignored physiological regulation. Another fundamental problem is the unavailability of commercial antibodies to detect the low expression of endogenous MOP in neuronal systems. Here, we established a neuronal cell line to detect endogenous MOP with sufficient sensitivity using CRISPR/Cas9 technology. We incorporated the hemagglutinin sequence into the MOP gene of the SH-SY5Y cell. The genome-editing did not significantly impair MOP functions such as MOP internalization or the downstream signaling. The clone was differentiated into a state similar to the primary culture undergoing treatment with all-trans retinoic acid, followed by brain-derived neurotrophic factor. Upon continuous stimulation with MOP ligands, endogenous MOP constantly decreased up to 48 h. The expression level was maintained at a certain level following this period, depending on the ligand properties. DAMGO reduced MOP from the cell surface by about 70%, while morphine did so by 40%. Our results indicate that even a few days of opioid administration could significantly reduce the MOP expression level. Our cell line could be a potential tool to investigate the molecular mechanisms underlying the problems caused by long-term opioid use.

1 | INTRODUCTION

Opioids are potent analgesics widely used in surgical anesthesia, intensive care, and chronic pain management. However, their long-term use often results in tolerance toward opioids[1,2] or undesired symptoms, such as dependence and withdrawal (Ref. [3]; see Ref. [4] and references therein). Such problems stem from the gradual transformation of the nervous system upon prolonged stimulation of the μ-opioid receptors (MOPs). The MOP does not merely continue the conversion of the external stimulus to a set of signals upon sustained activation. MOPs usually undergo post-translational modifications or bind to various adaptor molecules to firmly modulate their functions upon ligand binding (see Refs. [5,6] and references therein). For example, an activated MOP is rapidly phosphorylated by G protein-coupled receptor (GPCR) kinases.[7] Phosphorylation induces MOP desensitization and internalization, which further tunes the stimulus (see Ref. [8] and references therein). The receptors are subsequently sorted into recycling or degradation pathways, thus contributing to the restoration of cellular sensitivity.[8] Simultaneously, MOP activates intracellular signals to trigger the de novo synthesis of MOP.[9,10] The aforementioned processes are coordinately involved in the tight regulation of MOP expression on the cell surface and in determining the sensitivity toward the extracellular environment. However, it is challenging to comprehensively analyze these processes under physiological conditions because of several problems.

For example, previous studies often used cell lines, such as human embryonic kidney 293 (HEK293) cells, with sufficiently high transfection efficiency. Thus, they could easily establish a MOP-expressing cell line.[11] However, it is doubtful if these ectopically expressed MOPs would undergo physiological regulation. This can be attributed to non-neuronal cell lines being not necessarily equipped with molecules to regulate MOP.[12,13] Moreover, the ectopically introduced MOP is regulated by strong expression promoters, such as human cytomegalovirus or human elongation factor-1 alpha, which are constitutively active regardless of the cellular status.[14] In addition, cultured cell lines are unsuitable for prolonged investigations because they divide more than twice as much in a few days. MOP knock-in mice fused with fluorescent proteins, such as green fluorescent protein to the MOP carboxyl-terminal have the potential to monitor the localization of MOP.[15] However, such fluorescent proteins with relatively large molecular weights at the carboxyl-terminal potentially interfere with MOP regulation, such as trafficking or intracellular signaling. Therefore, previous studies have not suitably analyzed MOP kinetics under physiological conditions for a relatively long period. One of the fundamental problems is the unavailability of commercial antibodies to detect the low expression of MOPs in neuronal systems with sufficient sensitivity.

In this study, we applied gene-editing technology using CRISPR/Cas9 to overcome the above-mentioned problems. We successfully knocked in the epitope-tag hemagglutinin (HA) sequence into the amino-terminal end of MOP in the genomic DNA of a neural cell line. We differentiated the cell line into a state similar to that of the primary culture and observed changes in the expression of endogenous MOP upon prolonged opioid stimulation. We proposed a potential strategy for analyzing the physiological regulation of the opioid system. Our methodology might help us unveil the molecular basis of clinical concerns regarding opioid use, such as tolerance, dependence, and withdrawal.

2 | MATERIALS AND METHODS

2.1 | Cell culture

SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC CRL-2266) 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). They were supplemented with 10% fetal bovine serum (Cat# FB-1365/500; Biosera) in an incubator with 5% CO_2 at 37°C. HEK293 cells were provided by RIKEN BRC through the National BioResource Project of MEXT, Japan. These cells were cultured in D-MEM (high glucose) with L-glutamine, phenol red, and sodium pyruvate (Cat# 043-30085; FUJIFILM Wako), supplemented with 10% fetal bovine serum in an incubator with 5% CO_2 at 37°C.

2.2 | Plasmids and transfection

The HA epitope tag sequence was ligated into the amino-terminal of the open reading frame of human MOR1 and cloned into PB533A-2 (System Biosciences). The NEPA21 electroporator was used to transfect the plasmid into SH-SY5Y cells to establish SH-SY5Y cells overexpressing HA-MOR1 as a positive control (Figures 2 and 3).

2.3 | Gene editing with CRISPR/Cas9

To knock in the HA sequence at the amino-terminal of the MOP gene of SH-SY5Y cells, we transfected recombinant Cas9 nuclease protein NLS (316-08651; Nippon gene) and sgRNA with the designed

FIGURE 1 Strategy to establish a cell line with HA sequences incorporated into the MOP gene using CRISPR/Cas9. (A) Schema of MOP indicating the functions of transmembrane and intracellular regions. (B) Strategy for incorporating the HA sequence into the MOP gene. (C) Strategies for the efficient detection of clones that have undergone the desired genetic modification

2.4 | Cell lysis and immunoblotting
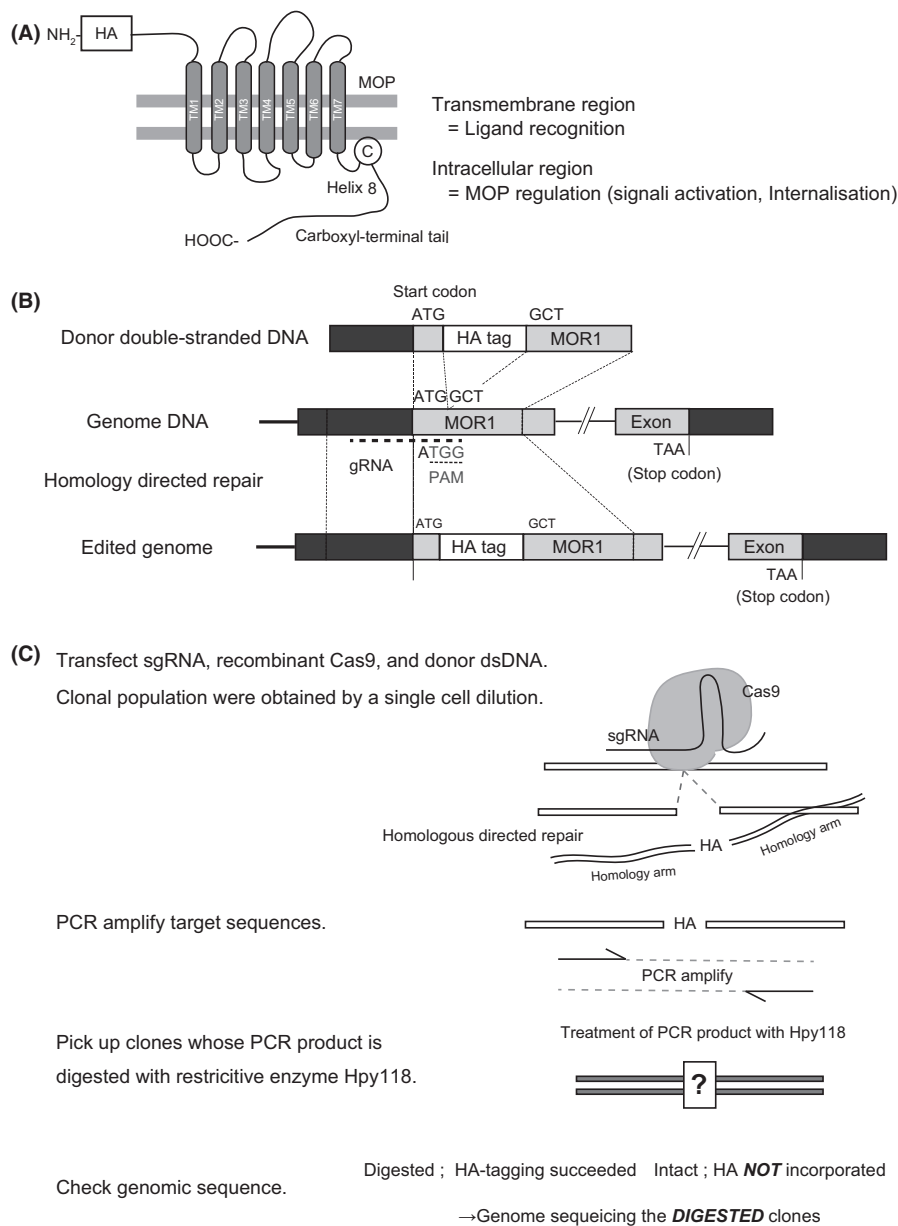
donor double-stranded DNA (dsDNA) purchased from FASMAC using NEPA21 electroporator (Nepagene). The guide RNA sequence was designed using the CHOPCHOP database.[16] Moreover, the sgRNA was synthesized in vitro using the CUGA® 7 gRNA synthesis Kit (314-08691; Nippon gene). Monoclonal cell lines were obtained using a limited dilution. The genome sequence of each clone was amplified using polymerase chain reaction (PCR). We selected clones comprising PCR products digested with the restriction endonuclease Hpy118 (New England BioLabs) (Figure 1). We selected the monoclonal cell line that was successfully incorporated into the HA sequence by sequencing the PCR product. Table 1 enlists the sequences of donor dsDNA designed for homology-directed repair to incorporate the HA sequence, oligo dsDNA used to prepare template DNA for sgRNA synthesis, and oligo DNA used for PCR to confirm the edited genome sequence.

The samples were treated as described previously.[17] Briefly, 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 comprising a protease inhibitor cocktail (160-26071; FUJIFILM Wako). The lysates were clarified by centrifugation at 15 000 rpm for 20 min at 4°C. We resolved the samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred them to a polyvinylidene difluoride membrane. Following blocking in Tris-buffered saline containing 0.1% Tween 20% and 5% (wt/vol) non-fat dry milk (31149-75; Nacalai), the membranes were immunoblotted with appropriate primary antibodies. The bound antibodies were visualized via enhanced chemiluminescence following incubation with horseradish peroxidase-conjugated

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TABLE 1 Synthetic oligos for preparing double-stranded DNA to serve as donor DNA. HA sequences are indicated as capital letters

Forward:
5'-ctcggtgctcctggctacctcgcacagcggtgcccgcccggccgtcagtaccatgTACCCATACGATGTTCCA
GATTACGCTgacagcagcgctgcccccacgaacgccagcaattgcactgatgccttggcg -3'
Reverse:
5'- cgccaaggcatcagtgcaattgctggcgttcgtgggggcagcgctgctgtcAGCGTAATCTGGAACATCGT
ATGGGTAcatggtactgacggccgggcgggcaccgctgtgcgaggtagccaggagcaccgag -3'
The guide RNA sequences targeting MOP
To generate template DNA for synthesize gRNA, oligos described below were PCR amplified as manufacture's instruction.
5'- CTAATACGACTCACTATAGCCGCCCGGCCGTCAGTACCAG -3'
5'- AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATT
TTAACTTGCT ATTTCTAGCTCTAAAAC -3'
5'- AAAAGCACCGACTCGGTGCCAC -3'
Sequencing primer pair for confirming incorporation of HA sequence
Forward: 5'- atgtcagatgctcagctcgg -3'
Reverse: 5'- acaagttgacccaggaaccg -3'

secondary antibodies against mouse or rabbit IgG. Immunoblotted protein bands were visualized and analyzed using X-ray films.

2.5 | Antibodies

We obtained the following antibodies from commercial sources and used them at the indicated dilutions for western blot analysis: anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (extracellular regulated kinase (ERK)1/2) (1:5000, Cat# 9101; Cell Signaling Technology), anti-p44/42 MAPK (ERK1/2) (1:2000, Cat# 9102; Cell Signaling Technology), anti-α-tubulin (1:5000, Cat# 3873; Cell Signaling Technology), purified anti-HA.11-epitope tag (1:2000, Cat# 901501; Biolegend), and anti-MOP UMB3 (1:1000, Abcam). For flow cytometry analysis, we used phycoerythrin (PE) anti-HA.11 epitope tag antibody (1:100, 901518; BioLegend) PE anti-mouse IgG1, and κ Isotype Ctrl Antibody (1:100, 400111; BioLegend).

2.6 | Flow cytometry analysis

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

2.7 | Quantitative real-time PCR analyses

For quantitative real-time PCR (qPCR), total RNA from the parental or gene-edited SH-SY5Y cells was isolated using ISOGEN II (311-07361; Nippon gene) according to the product's instruction. The RNA was reverse transcribed into cDNA by using a high-Capacity cDNA Reverse Transcription Kit (4368814; Thermo Fisher). qPCR was performed with PowerUp™ SYBR™ Green PCR Master Mix

(A25742; Thermo Fisher) with the use of Thermal Cycler Dice® Real-Time System (TP-900, TaKaRa). Each experiment was normalized to GAPDH expression. Sequence-specific primers were designed as follows: human OPRM1, 5'- cgtcagaacactagagaccacc -3' (sense) and 5'- cttggtgaaggtcggaatggca -3' (anti-sense); human GAPDH, 5'- gtctcctctgacttcaacagcg -3' (sense); 5'- accaccctgttgctgtagccaa -3' (anti-sense).

2.8 | MOP ligands, chemicals, and cytokines for the differentiation of SH-SY5Y cells

We purchased the following MOP-targeting ligands: [D-Ala^2, NMe-Phe^4-, Gly-ol^5] (DAMGO) (Abcam), and morphine hydrochloride (Takeda Pharmaceutical Company). Brain-derived neurotrophic factor (BDNF) and all-trans retinoic acid (ATRA) were purchased from FUJIFILM Wako. We altered the medium every 24 h for prolonged treatment with ATRA or BDNF or prolonged stimulation with a MOP ligand.

2.9 | Statistical analyses

Statistical significance was analyzed by *t*-test (in Figures 3C, 4B,C) and two-way repeated-measures ANOVA (in Figure 5B) using Prism 9.0 (GraphPad Software). We used ligands and time as the main factors for two-way repeated-measures ANOVA. The *p*-values or adjusted *p*-values below .05 were considered significant.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.

FIGURE 2 Genomic and amino acid sequences obtained by genome editing with CRISPR/Cas9. (A) The chromatogram showing the fluorescence intensity peaks of the genome sequence around the MOP start codon following gene editing. The genome sequence displays successful incorporation of the HA sequence immediately after the MOP start codon. (B) Comparison of the obtained gene and amino acid sequences with that of the parental cell. Ten amino acids immediately after the HA tag sequence are incorporated as a repeat. The codon has not been affected. (C) The 50 base sequences were unintendedly inserted upstream of the HA-MOP gene

(A)

Start codon — HA tag sequence — OPRM1 sequence

T C A G T A C C A T G T A C C C A T A C G A T G T T C C A G A T T A C G C T G A C A G C A G C G C T G

(B)

NH₂- HA

MOP

HOOC-

Parental genomic sequence

GAC AGC AGC CGT GCC | CCC ACG AAC GCC AGC AAT TGC ACT GAT GCC | TTG GCG TAC TCA

Obtained genomic sequences

GAC AGC AGC CGT GCC | CCC ACG AAC GCC AGC AAT TGC ACT GAT GCC **CCC ACG AAC GCC**

GCC AGC AAT TGC ACT GAT GCC | TTG GCG TAC TCA

Parental amino acid sequence

DSSAA PTNASNCTDA LAYSSCSPAPSPGSW...

Obtained amino acid sequence

DSSAA PTNASNCTDA **PTNASNCTDA** LAYSSCSPAPSPGSW...

(C)

Start codon
ATG   GCT
HA tag   MOR1

Edited genome sequence

Parental genomic sequence

CACAGCGG TGCCCGCCCGGCCGTCAGTACCATG
Start codon

Obtained genomic sequences

CACAGCGG **TGCCCGCCCGGCCGTCAGTACTCGGTGCTCCTGGCTACCTCGCACAGCGA** TGCCCG

org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[18] and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[19]

3 | RESULTS

3.1 | The establishment of a cell line incorporating HA sequences into the MOP gene using CRISPR/Cas9

We designed to knock-in HA sequences in the amino-terminal of MOP (Figure 1A). The location of the amino-terminal end of MOP in the extracellular region enables the detection of only MOP on the cell surface, without detecting those inside the cell by internalization with flow cytometry. In addition, MOP recognizes ligands by the transmembrane region and binds to intracellular signal transducers and adaptor molecules by intracellular regions, such as the carboxyl-terminal.[20] Therefore, the modification of the amino-terminal part would exert a minimal effect on MOP function. We designed a sgRNA to bring a double-strand break by Cas9 near the MOP start codon and double-stranded homology donor arm comprising HA sequences as a donor for the double-strand breaks to repair[21] (Figure 1B). We co-transfected in vitro-synthesized sgRNA, recombinant Cas9, and double-stranded DNA into SH-SY5Y cells by electroporation. This was followed by obtaining monoclonal clones using the limiting dilution method and selecting a clone in which both alleles were edited as desired. The expected frequency of genetic modification was as low as 7% according to the product's instructions (Nippon gene, Code No. 319-08641). Therefore, we adopted the following measures to efficiently select clones incorporated with HA sequences (Figure 1C). First, the genomic region containing the edited sequence was amplified by PCR. The estimated

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FIGURE 3 Incorporation of the HA sequence allows the detection of endogenous MOP in SH-SY5Y cells with a commercially available anti-HA tag antibody. (A) Immunoblotting analyses display that endogenous MOPs are detected at a similar band height as the positive control using a commercially available anti-HA antibody. Anti-MOP antibody (UMB3) can detect MOP only when overexpressed. (B) Flow cytometry analyses displaying HA-MOP is appropriately positioned on the cell membrane. (C) RT-PCR analysis of MOP transcription level in gene-edited or parental SH-SY5Y cells. GAPDH is used as a control

PCR product would be 27 bases higher than that obtained from the parental cells on being successfully incorporated with the HA sequences. Considering the difficulty to distinguish a slight difference by electrophoresis, we treated the PCR products with the restriction enzyme Hpy118, which only cleaves PCR products containing HA sequences. We analyzed 41 clones. Of these clones while one had both alleles containing the HA sequence, two clones comprised only one allele containing the HA sequence. Subsequently, we analyzed the genomic sequences of the PCR products with completely cleaved Hpy118 to confirm the appropriate incorporation of the clone with the HA sequence. We eventually obtained the clone successfully incorporated with the HA sequence, immediately after the start codon (Figure 2A). However, a 30 bp sequence, same as a part of the amino-terminal region of MOP, was repeated downstream of the start codon (Figure 2B). In addition, a 50 bp undesigned sequence of unknown origin had been unintendedly incorporated upstream of the start codon (Figure 2C). Despite the extracellular region becoming 10 amino acids longer, the sequences did not interfere with codons. We speculated that unexpected insertions of these gene sequences occurred during the Cas9-mediated repair of double-strand breaks. Similar sequence insertions during the repair

of double-strand breaks cannot be avoided as the previous report indicated.[22] Moreover, the frequencies of appropriately incorporating HA sequences are as low as 7%. Therefore, we decided to use the clone for subsequent analyses. Hereafter, we will describe the novel established cell line as HA-MOP SH-SY5Y cells.

3.2 | The introduction of the HA sequence enabled the detection of MOP by commercially available antibodies

We evaluated the ability of a commercially available anti-HA epitope tag antibody to detect endogenous MOP, compared to commercially available anti-MOP antibodies, principally used to detect MOP in overexpression systems using SH-SY5Y cells. We established SH-SY5Y cells overexpressing MOR1, an isoform of MOP, often used in analyzing MOP functions as a positive control. We successfully detected endogenous MOP using the anti-HA antibody in HA-MOP SH-SY5Y cells by immunoblotting, displaying a smear-like band around 75–100 kDa as observed in a positive control overexpressing HA-MOR1 in SH-SY5Y cells (Figure 3A). However, MOPs must

FIGURE 4 Genome-editing of HA-tagging did not impair MOP functions. (A) Immunoblot analysis of phosphorylation of ERK in whole-cell lysates from HA-MOP SH-SY5Y cells and parental SH-SY5Y cells, following stimulation with 10 μM of DAMGO for the indicated time. (B, C) Internalization of HA-MOR1 overexpressed in SH-SY5Y cells (B) or HA-MOP in HA-MOP SH-SY5Y cells (C) upon stimulation with 10 μM of DAMGO (left, black bar) and morphine (right, grey bar) for 10–15 min. Error bars indicate the standard error
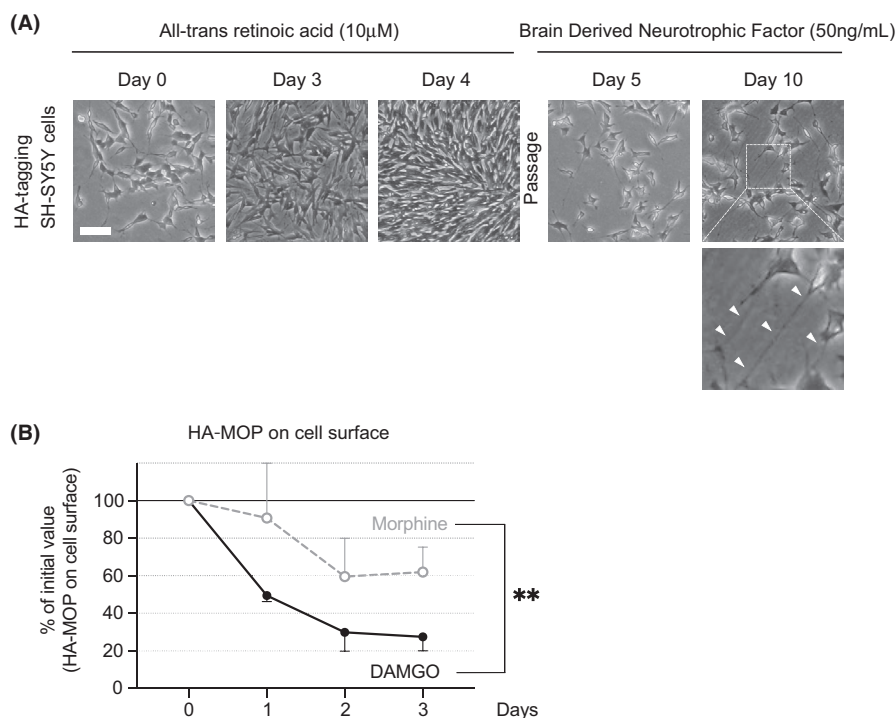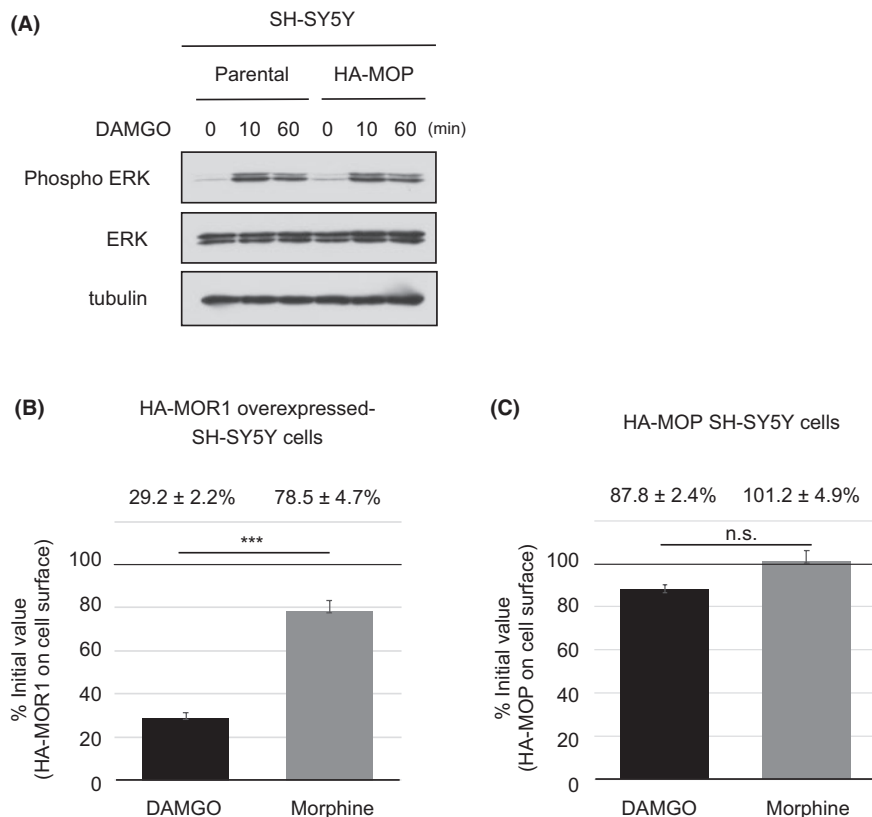
FIGURE 5 Differentiation of HA-MOP SH-SY5Y cells and subsequent analyses of MOP expression level upon stimulation with MOP ligands for 72 h. (A) Morphological changes in HA-MOP SH-SY5Y cells stimulated with 10 μM all-trans Retinoic Acid for 5 days, followed by 50 ng/ml of BDNF for 5 days. The cells are passaged once on Day 4. Treatment with BDNF stopped cell division and induced characteristic morphological changes, indicated by the arrowhead in the rightmost panel. Scale bar in the leftmost panel indicates 100 μm. (B) The differentiated HA-MOP SH-SY5Y cells are treated with 10 μM of DAMGO or morphine. The medium containing either chemical for differentiation or ligand for prolonged stimulation has been changed every 24 h. The line graph depicts the change in MOP expression level on the cell surface. Error bars indicate the standard error

be appropriately located on the plasma membrane even after genetic manipulation. Therefore, we checked if HA-tagged MOP could be detected on the cell membrane by flow cytometry. Despite no commercially available antibodies for detecting endogenous MOP for flow cytometry analysis targeting the extracellular region as an epitope, the molecule that reacted with the anti-HA antibody appeared, albeit sparingly, on the plasma membrane of HA-MOP SH-SY5Y cells. Nonetheless, it was not detected in the parental SH-SY5Y cells (Figure 3B). We then evaluated the expression level of HA-MOP with qPCR. The transcription level of HA-MOP was not weakened compared to that of MOP in the parental cells, indicating that genomic modification did not impair the HA-MOP expression (Figure 3C).

3.3 | Genome-editing of HA-tagging did not significantly impair MOP functions

MOP activates intracellular signals upon ligand binding.[6] In contrast, activated MOP gets phosphorylated by GPCR kinases to be desensitized.[7] The desensitized receptors are subsequently internalized by binding to β-arrestin.[8] MOP endocytosis leads the receptors to the re-sensitization and recycling pathway or the degradation pathway, which plays a crucial role in maintaining cellular sensitivity. Therefore, HA-tagged MOP does not get functionally impaired on assessing if it activates intracellular signals and the activated form is appropriately internalized. First, we evaluated the phosphorylation of ERK, a hallmark of MAPK activation, which is often assessed as a signal activated by MOP ligands. Upon stimulation with 10 μM of DAMGO, HA-MOP phosphorylated ERK almost equally to parental SH-SY5Y cells (Figure 4A). We then assessed if HA-MOP could be properly internalized using two classical MOP ligands, namely DAMGO and morphine. Previous studies have reported that DAMGO strongly phosphorylates multiple serine and threonine residues in the carboxyl-terminal region of MOP.[23] Therefore, DAMGO internalized MOP more strongly than morphine, which could weakly phosphorylate the similar region of MOP. We evaluated if HA-MOP would display a similar tendency during internalization as the overexpressed HA-MOR1 used in previous studies for analysis upon stimulation with these classical MOP ligands. We hypothesized that HA-MOP underwent proper phosphorylation if the degree of internalization differed depending on the ligand. According to previous reports,[23] while HA-MOR1 overexpressed in SH-SY5Y cells was robustly internalized by DAMGO, morphine weakly internalized only HA-MOR1 (Figure 4B). Although the internalization of MOP appeared more profound in the overexpression system, HA-MOP also showed ligand-bias on receptor internalization upon stimulation with DAMGO and morphine (Figure 4C). Although the genome-editing did not significantly impair MOP functions such as MOP internalization or the downstream signal activation, Figure 4 collectively indicate that HA incorporation might have some interference in the receptor function, although not significantly, such as ligand binding.

3.4 | Continuous opioid administration lowered MOP expression on the cell surface at a constant and ligand-specific level

We further analyzed changes in the expression level of endogenous MOP by sustained ligand stimulation with HA-MOP SH-SY5Y cells. SH-SY5Y cells are tumor cells that proliferate approximately twofold in 2–3 days. Hence, it is inappropriate to evaluate the changes in MOP expression levels over days in their intact states. Therefore, we treated the HA-MOP SH-SY5Y cell line with all-trans retinoic acid for 5 days, followed by BDNF treatment for 5 days, which differentiated SH-SY5Y cells into a state similar to a primary culture (Figure 5A).[24,25] Upon differentiation, SH-SY5Y cells became enriched with prolonged neurites, whose morphological characteristics resembled those of the primary culture (arrowheads on the rightmost panel in Figure 5A).

The endogenous MOP on the differentiated cell surface constantly decreased for approximately 48 h, presumably because of robust endocytosis, although not statistically significant (Figure 5B). Interestingly, after 48 h of stimulation, while the decrease in the MOP expression on the cell surface subsided, the amount of MOP on the cell surface became almost constant. The reduction level depends on the ligand potency to induce internalization, as observed in a relatively short period of response. While DAMGO reduced MOP from the cell surface by approximately 70%, morphine did so by approximately 40% (DAMGO vs. Morphine; $p < .005$). Our results indicated that MOP expression on the cell surface was regulated in a specific range as the sum of recycling, degradation, and de novo synthesis under prolonged opioid stimulation.

4 | DISCUSSION

In this study, we effectively monitored the dynamics of endogenous MOP on the neuronal cell surface upon prolonged opioid administration using CRISPR/Cas9, without impairing MOP function significantly (Figures 4 and 5). Interestingly, MOPs on the cell surface decreased to approximately 30% of the initial amount upon stimulation with DAMGO for 3 days, whose ligand properties are similar to that of fentanyl. With morphine, the expression of MOPs decreased to approximately 40% of the initial level. Our results indicated that MOPs on the neuronal cell surface might be considerably reduced to a substantially lesser extent than that expected upon continuous use. Furthermore, the reduction rate of MOP on the cell surface principally depended on ligand properties to internalize MOPs observed during the acute phase of stimulation (Figure 4B,C). For example, DAMGO was previously described to internalize MOPs more robustly than morphine. Our results indicated that a similar tendency was observed in the prolonged phase (Figure 5B).

Our experimental model could be a potential tool to advance our understanding of the development of opioid tolerance. For example, investigations on the rate at which residual MOPs on the cell surface activate the analgesic pathway following prolonged stimulation

with MOP ligands might help us understand the alteration of cellular response upon long-term opioid use. Our cell line might help us understand the basis of opioid rotation by investigating the alteration of intracellular signaling upon opioid switching. Further analyses of MOP kinetics may provide more sophisticated strategies for controlling severe pain.

Withdrawal syndromes are an essential concern in both clinical and social settings.[4] During opioid discontinuation, despite not returning to the situation before opioid administration, the patients present with severe clinical symptoms. In other words, continuous opioids administration supposedly activates some signaling pathways by discontinuation. Our experimental model might allow us to address critical issues that remain unanswered.

Furthermore, genome editing the gene of interest is a potential tool for monitoring molecules, which cannot be detected with sufficient sensitivity. A similar strategy could be applied to opioid receptor families, such as δ-and κ-opioid receptors or nociceptin receptors. Moreover, none of the receptors could be detected with commercially available antibodies. Using the aforementioned methods to those opioid receptor families might reveal their unknown physiological significance. It is also possible to establish mutant mice that enable us to monitor MOP by applying similar genetic manipulation to fertilized eggs and obtaining offspring with the desired gene in a few months.[26] A previous study reported on post-translational modifications of MOP by mass spectrometry using whole-brain tissue.[27] Such mutant mice might provide information on the physiological regulation of endogenous MOP. These mutant mice may also clarify the localization of MOP, further advancing our understanding of MOP transportation.

There were several limitations in the interpretation of our results. First, SH-SY5Y cells are a tumor cell-line, despite being differentiated into a state similar to that of primary culture. For example, it is unknown if the amount of MOP expressed in SH-SY5Y cells is comparable to the amount of MOP expressed by neurons in vivo. The expression level of MOP was extremely low, particularly before differentiation. Therefore, it could be barely detected by flow cytometry and demonstrated with a relatively large error bar, despite using an affinity-purified antibody labeled with a high fluorescent protein, such as phycoerythrin (Figures 3B, 4B). Therefore, we need to expand culture cells to a substantial number than studies using an overexpression system. This will facilitate detailed analyses, such as post-translational modifications or associated molecules of MOP by mass spectrometry. Second, the genetic manipulation to incorporate the HA sequence immediately after the start codon might have affected the MOP expression and function, although not significantly. For example, the transcription level of MOP was upregulated in HA-MOP cells than in parental SH-SY5Y cells (Figure 3C). Considering the quantitative relationship between the receptor and signaling, the signal from HA-MOP might be weaker than that from endogenous MOP in parental SH-SY5Y cells. In addition, the MOP internalization appeared reduced compared to the overexpression system (Figure 4B,C). These data indicated that HA-tagging to the N-terminus might interfere with the receptor function, although tag-sequenced genes have been widely accepted in conventional overexpression systems. Third, the incorporation of sequences immediately after the start codon might impair the Kozak sequence, which determines the translation efficiency.[28] Specifically, the original genomic sequence of the MOP gene, accATGG, was altered to accATGT, which potentially reduced the translation efficiency (Figure 2A). The increase in transcriptional activity (Figure 3C) might compensate for the loss of translational efficiency due to the impairment of the Kozak sequence. We could have avoided this concern by incorporating the tag sequence into the carboxyl end of the gene of interest. However, tagging the amino-terminal end of MOP enabled us to discern MOP on the cell surface using flow cytometry. In addition, we avoided editing the intracellular region, which is vital for MOP transport and signal transduction (Figure 1A). Furthermore, the CRISPR/Cas9 system had several limitations. Despite successfully incorporating HA into the MOP gene (Figure 2B), we identified an unexpected 50 bp insertion upstream of the start codon. The insertion made the distance to the promoter, which might have altered the transcription efficiency (see[29] and references therein). In addition, a 30-bp repeat sequence was incorporated into the extracellular domain (Figure 2B). This sequence was repeated in a similar region of MOP and appeared to exert a minimal impact on its function. However, gene editing with CRISPR/Cas9 is always associated with risks, such as unexpected genetic alterations.[22] Despite these limitations, the aforementioned cell line could be established within a short duration (approximately 1 month). In addition, our cell line enables us to easily expand the scale for various kinds of subsequent analyses. In addition, several treatments for differentiation enabled us to use a cell line as a state similar to the primary culture. In contrast, a conventional method would have required several years to establish genetically modified mice to obtain the primary culture (Figure 5A). Nevertheless, primary cultures obtained from mice might not be identical to that of humans. Thus, the observed experimental effects may not occur identically in humans. In addition, our cell line is free from ethical concerns or is maintained at a considerably lower cost than primary neurons. Therefore, our cell line would be a promising tool to further investigate MOP regulatory mechanisms, such as molecules associated with MOP or post-translational modifications, or to identify subcellular localization upon prolonged stimulation.

In conclusion, we analyzed the endogenous MOP dynamics on the cell surface using a genome-edited neural cell line, which was subsequently induced to differentiate into a state similar to that of primary culture. Further biochemical analyses, such as mass spectrometry analyses of post-translational modification or adaptor molecules, might help us understand the alteration of MOP under physiological conditions.

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DISCLOSURE

No conflicts of interest exist, including financial relationships that could have influenced its outcome.

AUTHOR CONTRIBUTIONS

S.S. and A.S. equally contributed to this work. *Participated in research design*: Shimizu and Shiraki. *Conducted experiments*: Shimizu and Shiraki. *Performed data analysis*: Shimizu and Shiraki. *Wrote or contributed to the writing of the manuscript*: Shimizu and Shiraki.

ETHICS APPROVAL STATEMENTS

The study was approved by the Committee for Safe Handling of Living Organisms in Kyoto University (Permission number: 180126) and conducted in accordance with the ethical standards of the committee.

DATA AVAILABILITY STATEMENT

The data and materials that support the findings of this work are available from the corresponding author upon reasonable request.

ORCID

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