Supplementary Figures

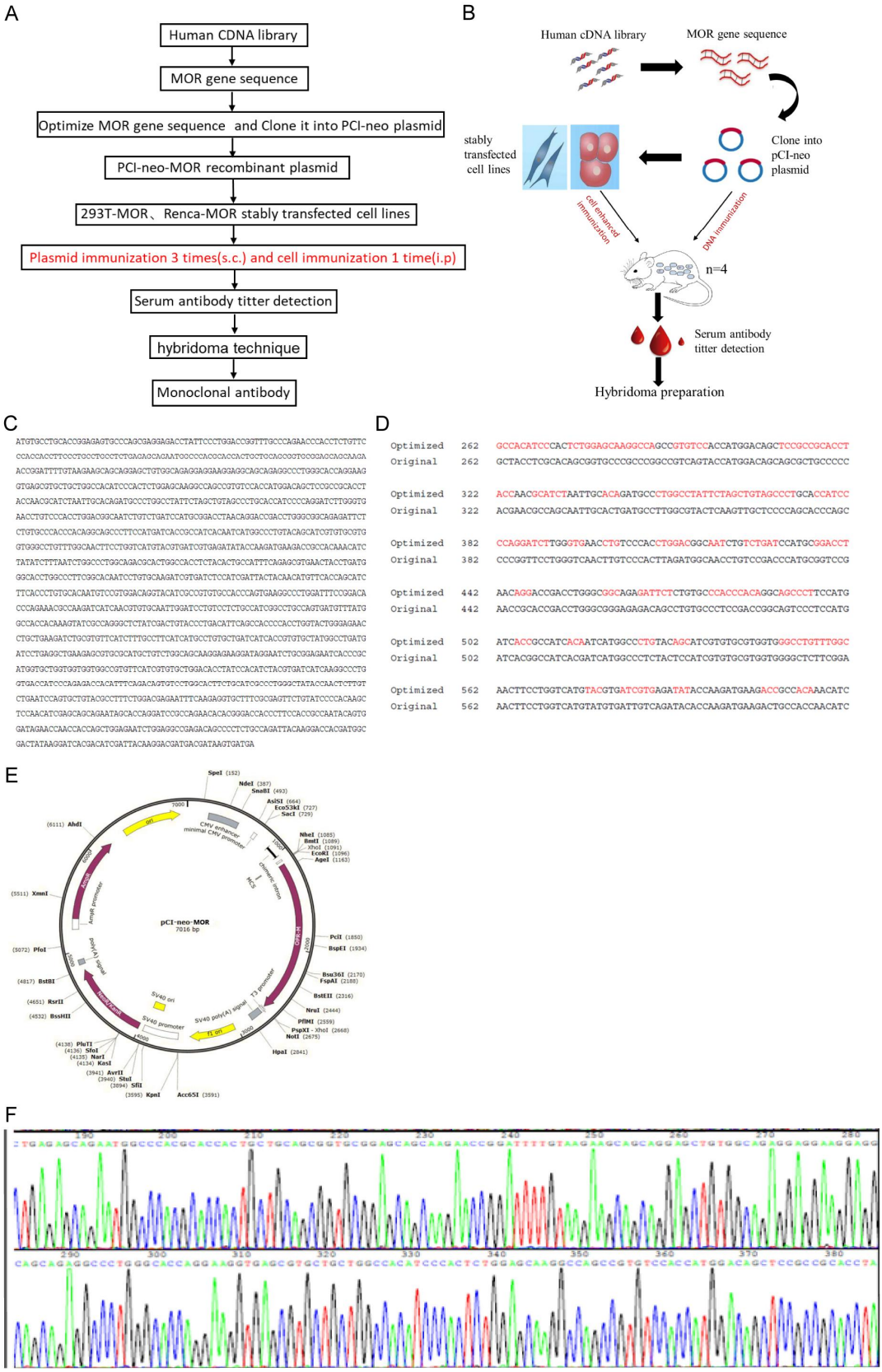


Fig. S1. Preparation and screening of monoclonal antibody (mAb) targeting mu-opioid receptor (MOR), part 1. (A) Experiment flowchart for preparation of mAb targeting MOR. (B) Schematic for the procedure of preparing mAb targeting MOR. (C) Gene sequence of human *OPRM*. (D) Partially

optimized sequence of *OPRM*, with bases marked with red color being the optimized bases. (E) Map information of recombinant pCI-neo-MOR vector. The human *OPRM* gene with partially optimized sequence was cloned into the pCI-neo vector between *EcoR* I and *Not* I restriction sites. (F) Deoxyribonucleic acid sequencing results showing that the *OPRM* sequence was correctly inserted into the pCI-neo vector.

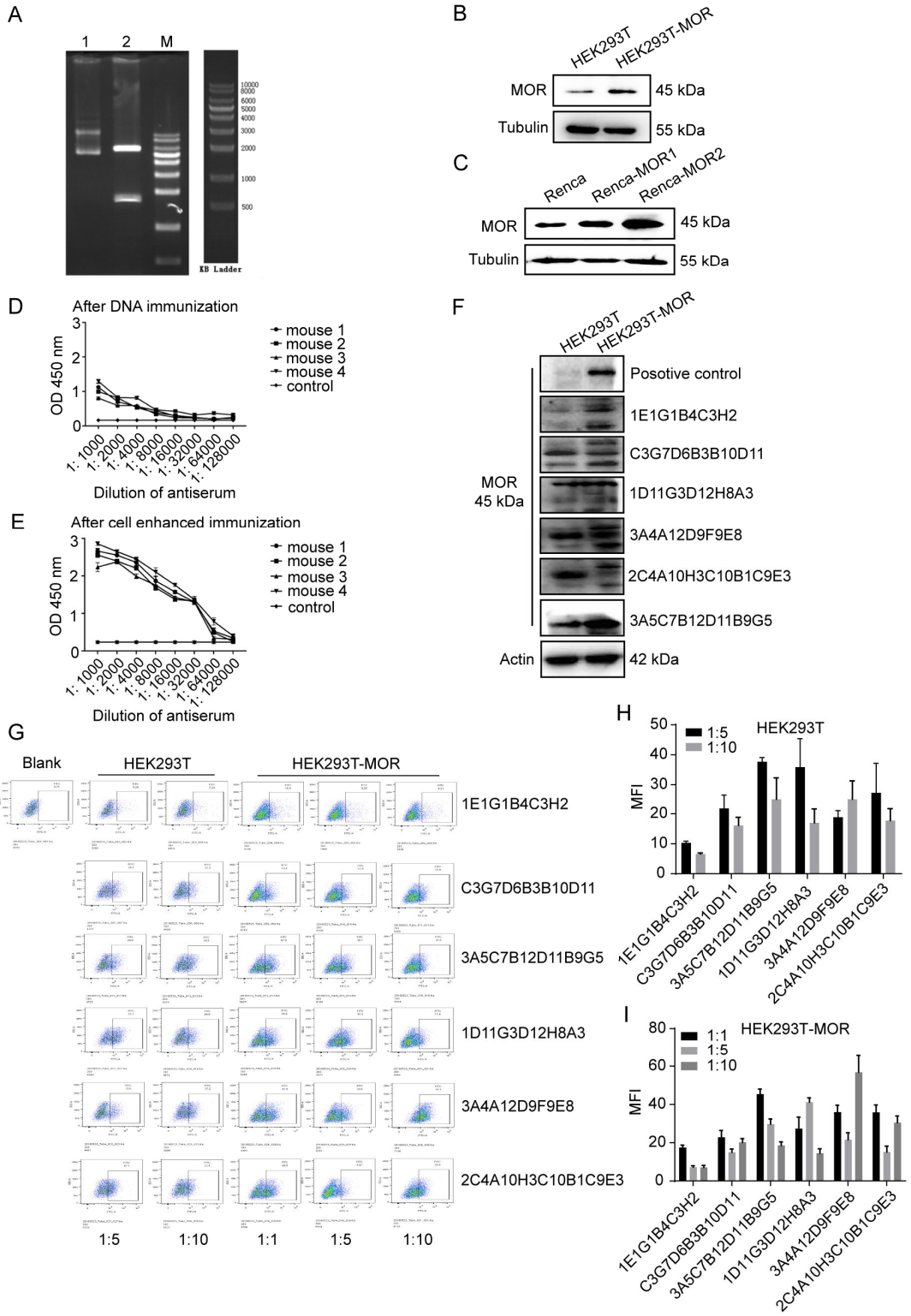


Fig. S2. Preparation and screening of monoclonal antibody (mAb) targeting mu-opioid receptor (MOR), part 2. (A) Identification of recombinant pCI-neo-MOR vector by nucleic acid electrophoresis. Lane 1: undigested pCI-neo-MOR vector; lane 2: pCI-neo-MOR vector digested by *EcoR* I and *Not* I; lane M: nucleic acid electrophoresis marker ladder. The band after electrophoresis of the recombinant vector was about 7000 bp and a fragment of about 1500 bp was removed after the vector was digested with *EcoR* I and *Not* I, which indicated that the pCI-neo-MOR recombinant vector was successfully

constructed. (B) Immunoblot demonstrating the construction and identification of HEK293T cell lines stably overexpressing MOR (denoted as HEK293T-MOR). (C) Immunoblot demonstrating the construction and identification of Renca cell lines stably overexpressing MOR (denoted as Renca-MOR1 and Renca-MOR2 respectively). HEK293T and Renca cell lines were transfected with pCI-neo-MOR vector using lipofectamine 2000, and the transfected cells were selected with neomycin after 48 h for about 30 days. Notably, the second Renca stable cell (Renca-MOR2) worked better, so it was selected for subsequent experiments. (D) Identification of serum antibody titer after three times of plasmid immunization using cellular enzyme-linked immunosorbent assay (ELISA). (E) Identification of serum antibody titer after one time of subsequent cell enhanced immunization using cellular ELISA. HEK293T-MOR cell was used as coating antigens. Sera from four immunized mice were used as primary antibodies. And sera from unimmunized mice were used as controls. The horizontal axis is the serum antibody dilution and the vertical axis is the value of optical density at 450 nm (OD 450 nm). Because the fourth mouse had the highest serum antibody titer (1:32000), hybridomas were prepared from spleen cells of this mouse. (F) Western blot detecting the antibody production in the supernatant of 6 monoclonal cell lines, with commercially available antibody as positive control. After multiple fusions and clonal screenings, six monoclonal strains were obtained and the antibody produced from the strain numbered 3A5C7B12D11B9G5 has the optimal binding ability. (G) Flow cytometry and quantification of HEK293T cells (H) and HEK293T-MOR cells (I) to detect the antibody production in the supernatant of 6 monoclonal cell lines. The dilution ratios of the cell supernatant were 1:1,1:5 and 1:10, respectively. Consistent with results of western blot, the strain numbered 3A5C7B12D11B9G5 produced the antibody with the optimal binding ability. Data were presented as mean ± standard error of mean. MFI: mean fluorescence intensity.

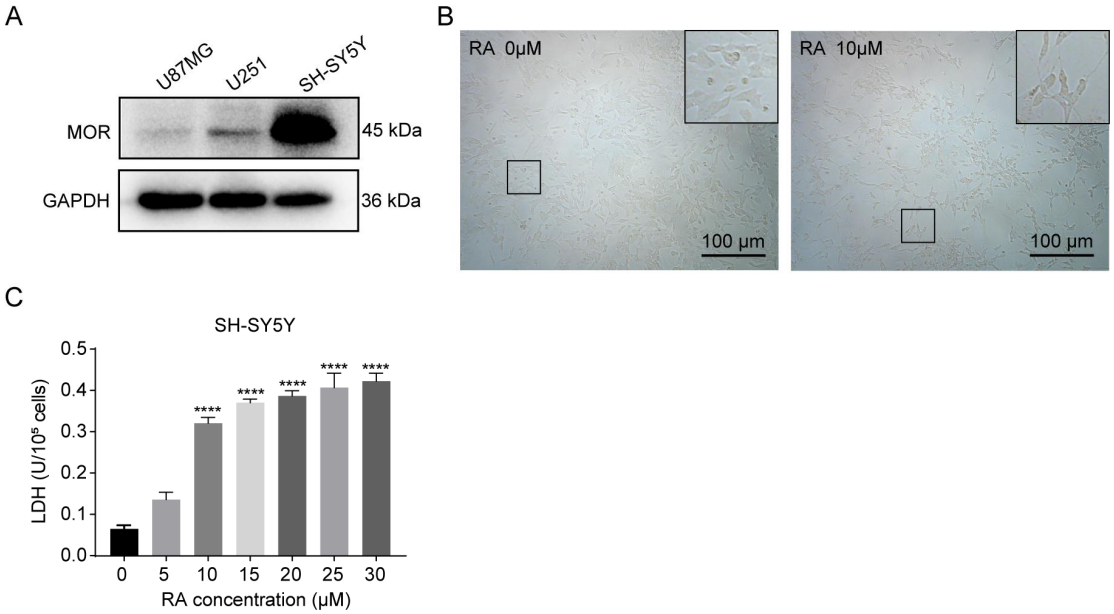
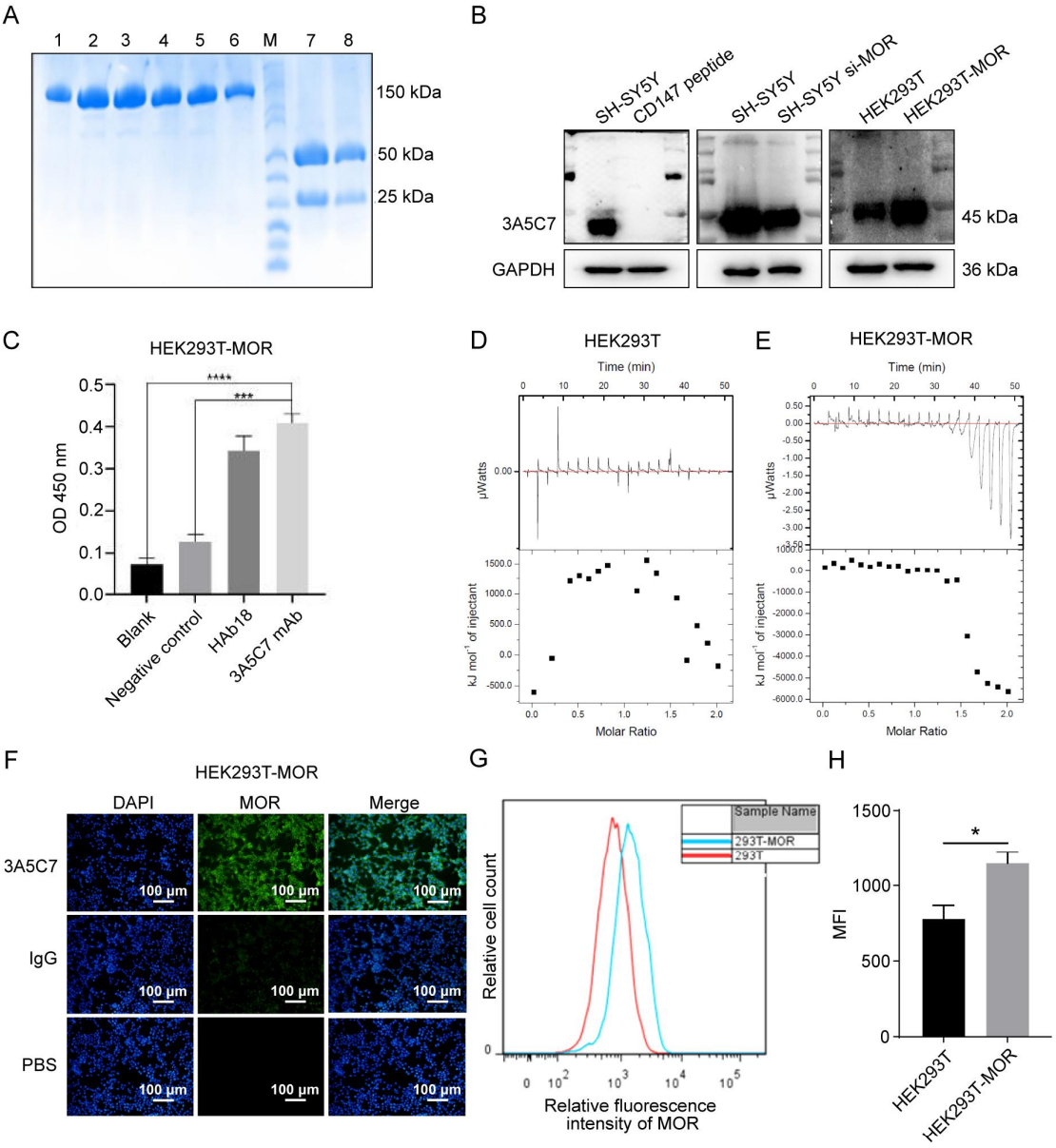


Fig. S3. Retinoic acid (RA)-induced differentiation of human neuronal SH-SY5Y cells. (A) Western blot showing MOR expression in different nerve cell lines. (B) Photographs of SH-SY5Y cells treated with 10 μM RA for 6 days (right) and without RA (left). (C) Assays of released lactate dehydrogenase (LDH) of SH-SY5Y cells after incubation with different concentrations of RA (0, 5, 10, 15, 20, 25, 30 μM) for 6 days. Student's *t* tests were used to analyze data. $n = 3$ independent experiments. Data were presented as mean ± standard error of mean. ****$P < 0.0001$ versus control cells (RA 0 μM) in (C).



I

VH

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act tca gtg aag atg tcc tgc aag gct tct gga tac aca tcc act 90
agt aat gtt gtc cac tgg gtg aag cag aag cct ggg cag ggc ctt 135
gag tgg att gga tat att agt cct atc aat gaa gga act aag tac 180
aat gag aaa ttc aaa ggc aag gcc aca ctg act gca gac aaa tcc 225
tcc agc aca gcc ttc atg gag ctc agc agc ctg acc tct gag gac 270
tct gcg gtc tat tac tgt gca gat ggt aac tat aac ttt gct atg 315
gac ttc ggg ggt caa gga acc tca ctc acc gtc tcc tca 354

J

VL

aac att atg atg aca cag tcg cca tca ttt ctg gct gtg tct gca 45
gga gaa aag gtc act ata aat tgt aag tcc agt caa agt gtt tta 90
tat agt tca aat cag aag aac tac ttg gcc tgg tac cag cag aaa 135
cca gga cag tct cct aaa ctg ctg atc tac tgg gca tcc act agg 180
gaa tct ggt gtc cct gat cgc ttc aca ggc agt gga tct ggg aca 225
tat ttt act ctg acc atc agc aat gta cag gct gaa gac ctg gca 270
gtt tat tac tgt cat caa tac ttc tcc tca tac acg ttc gga ggg 315
ggg acc aag ctg gaa att aaa 336

Fig. S4. Characteristics of 3A5C7 monoclonal antibody (mAb). The mAb 3A5C7 was obtained from the monoclonal strain numbered 3A5C7B12D11B9G5 via enrichment (ascites amplification) and purification. (A) Coomassie blue staining of reduced and non-reduced 3A5C7 mAb. Lanes 1–6: nonreduced antibodies; lanes 7–8: reduced antibodies; lane M: protein electrophoresis marker ladder. The size of the nonreduced antibody was 150 kDa, and the size of the heavy chain and the light chain were separately 50 kDa and 25 kDa, suggesting that the 3A5C7 mAb was an immunoglobulin G subtype. (B) Immunoblots demonstrating the identification of the specificity of 3A5C7 mAb. Cluster of

Differentiation (CD)147 peptide was used as an unrelated peptide. si-MOR was small interfering ribonucleic acid targeting mu-opioid receptor (MOR). SH-SY5Y si-MOR was SH-SY5Y with MOR being knocked down with si-MOR. (C) Cellular enzyme-linked immunosorbent assay (ELISA) showing the affinity of 3A5C7 mAb. (D, E) Isothermal titration calorimetry showing the affinity of 3A5C7 mAb. In ELISA, phosphate buffer saline (PBS) was used as blank control; mouse IgG was used as negative control; and HAb18 mAb and its antigen HAb18G were used as positive control. One-way Analysis of Variance with Bonferroni's *post hoc* tests were used for statistical analysis. ***$P < 0.001$, ****$P < 0.0001$. (F) Immunofluorescence staining showing the ability of 3A5C7 mAb to bind to antigen with spatial conformation. (G) Flow cytometry and quantification (H) showing the ability of 3A5C7 mAb to bind to antigen with spatial conformation. Student's *t* tests were used for statistical analysis. *$P < 0.05$. (I) Heavy chain variable region gene sequences of anti-human MOR mAb 3A5C7. (J) Light chain variable region gene sequences of anti-human MOR mAb 3A5C7. $n = 3$ independent experiments. Data were presented as mean ± standard error of mean. OD 450nm: optical density at 450 nm; MFI: mean fluorescence intensity; VH: variable region of heavy chain; VL: variable region of light chain.

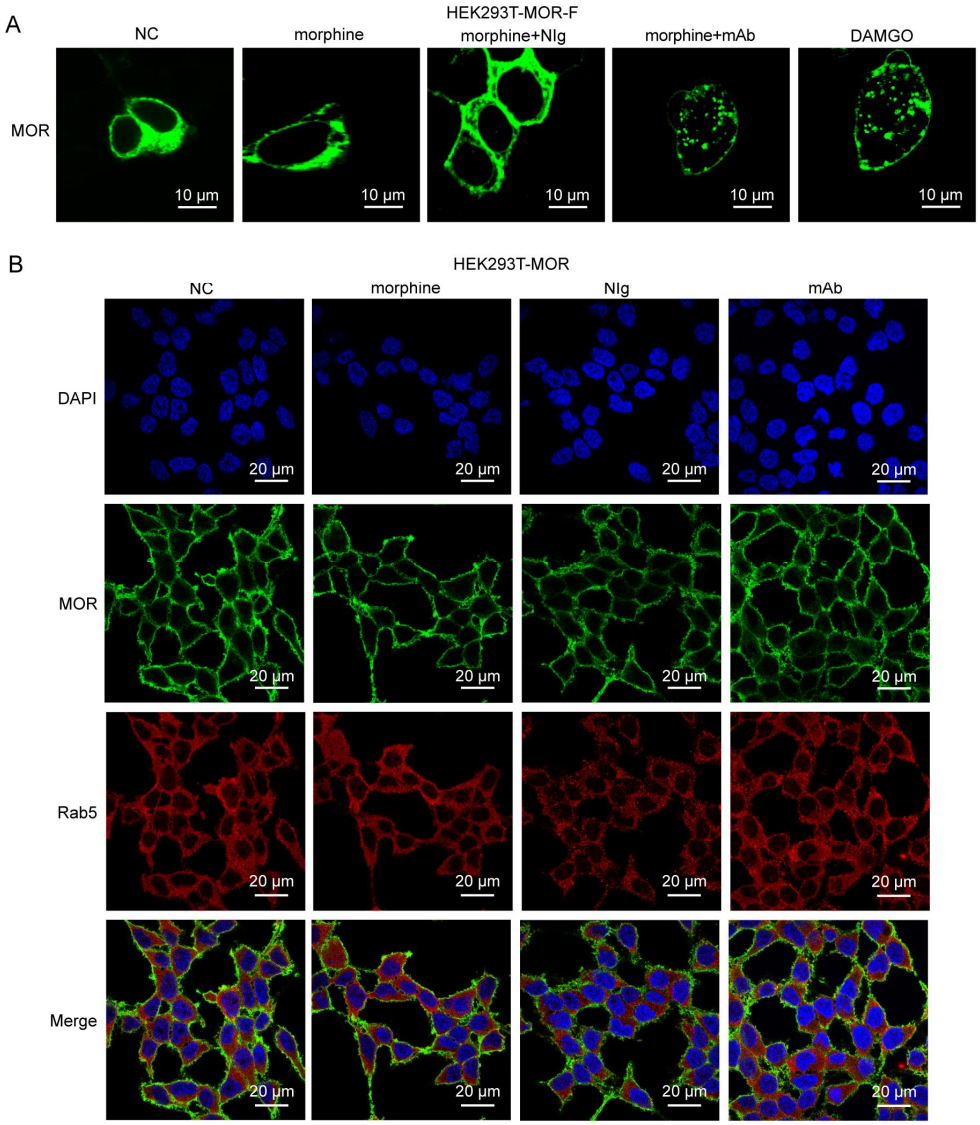

Fig. S5. Effects of 3A5C7 monoclonal antibody (mAb) on morphine-induced mu-opioid receptor (MOR) endocytosis. (A) Immunofluorescence staining showing the endocytosis of MOR in HEK293T-MOR-F cells treated with morphine and 3A5C7 mAb. HEK293T-MOR-F cells are HEK293T cell lines transfected with pEGFP-N1-MOR vector (a fluorescence infusion plasmid) using lipofectamine 2000, and the transfected cells were selected with puromycin after 48 h for about 30 days. D-ala2-nmephe4-gly-ol-enkephalin (DAMGO) was used as positive control. (B) Effect of 3A5C7 mAb alone on the endocytosis of MOR in HEK293T-MOR cells. *n* = 3 independent experiments. NIg: normal IgG; DAPI: 4′,6-Diamidino-2-phenylindole dihydrochloride.

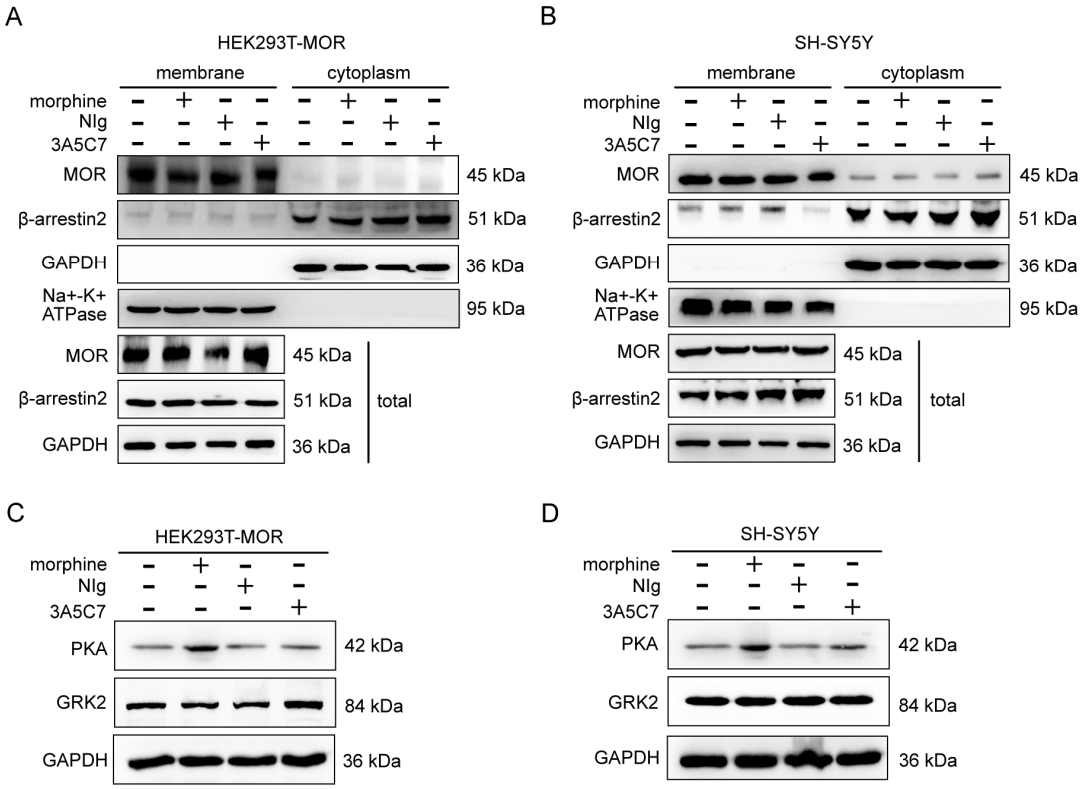

Fig. S6. Effects of 3A5C7 monoclonal antibody (mAb) itself on the endocytosis of mu-opioid receptor (MOR) and morphine tolerance-related molecules. (A) Representative western blots showing the impacts of 3A5C7 mAb alone on MOR endocytosis and translocation of β-arrestin2 in HEK293T-MOR cells. (B) Representative western blots showing the impacts of 3A5C7 mAb alone on MOR endocytosis and translocation of β-arrestin2 in SH-SY5Y cells. Cells were treated with 3A5C7 mAb (10 μg/mL), NIg (normal IgG, 10 μg/mL), or morphine (10 μM) for 1 h respectively, then membrane and plasma proteins of cells were separated for western blot assays. (C) Representative western blots manifesting the effects of 3A5C7 mAb itself on the expression of G protein-coupled receptor kinase 2 (GRK2) and the morphine tolerance-related marker, protein kinase A (PKA), in HEK293T-MOR cells. (D) Representative western blots manifesting the effects of 3A5C7 mAb itself on the expression of GRK2 and the morphine tolerance-related marker, PKA, in SH-SY5Y cells. Na^+-K^+ adenosine triphosphatase (ATPase) α1 subunit was used as internal reference of cell membrane and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference of cytoplasm. $n = 3$ independent experiments.

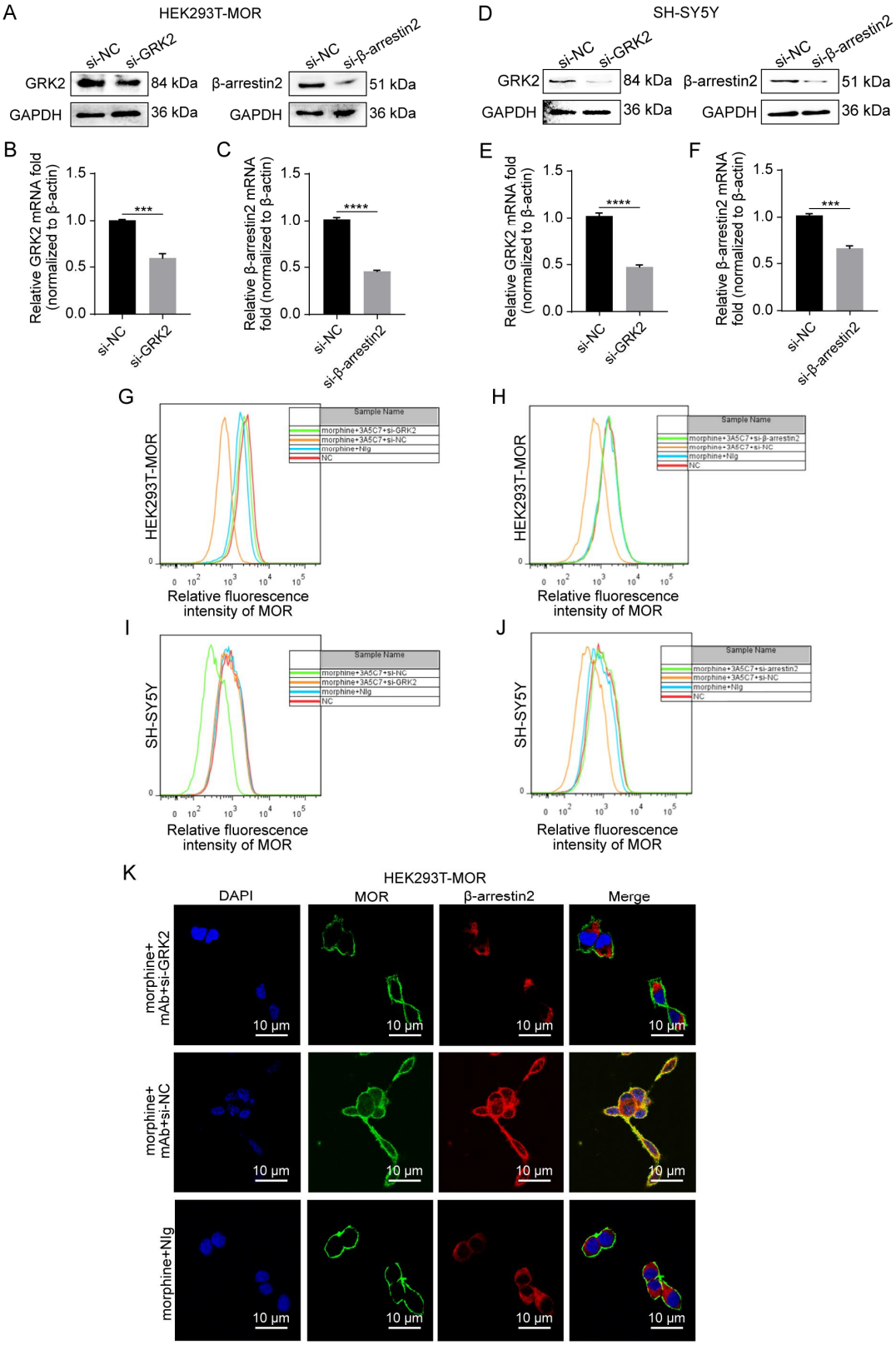

Fig. S7. Identification and the influence of G protein-coupled receptor kinase 2 (GRK2)/β-arrestin2 knockdown. (A) Western blot showing the protein level of GRK2 and β-arrestin2 in HEK293T-MOR cells with GRK2 and β-arrestin2 being knocked down by small interfering ribonucleic acid (siRNA) for GRK2 (si-GRK2) and β-arrestin2 (si-β-arrestin2). (B) Relative messenger ribonucleic acid (mRNA) level of GRK2 in HEK293T-MOR cells with GRK2 being knocked down by si-GRK2. (C) Relative

mRNA level of β-arrestin2 in HEK293T-MOR cells with β-arrestin2 being knocked down by si-β-arrestin2. (D) Western blot showing the protein level of GRK2 and β-arrestin2 in SH-SY5Y cells with GRK2 and β-arrestin2 being knocked down by si-GRK2 and si-β-arrestin2. (E) Relative mRNA level of GRK2 in SH-SY5Y cells with GRK2 being knocked down by si-GRK2. (F) Relative mRNA level of β-arrestin2 in SH-SY5Y cells with β-arrestin2 being knocked down by si-β-arrestin2. Student's *t* tests were used to analyze data. Data were presented as mean ± standard error of mean. ***$P < 0.001$, ****$P < 0.0001$. (G, H) Flow cytometry indicated that MOR endocytosis induced by morphine and 3A5C7 was reduced in HEK293T-MOR cells with GRK2 or β-arrestin2 knockdown. (I, J) Flow cytometry indicated that MOR endocytosis induced by morphine and 3A5C7 was reduced in SH-SY5Y cells with GRK2 or β-arrestin2 knockdown. (K) Immunofluorescence staining showed that GRK2 knockdown reduced translocation of β-arrestin2 from plasma to membrane in HEK293T-MOR cells. *n* = 3 independent experiments. si-NC: control siRNA; NIg: normal IgG; DAPI: 4′,6-Diamidino-2-phenylindole dihydrochloride.

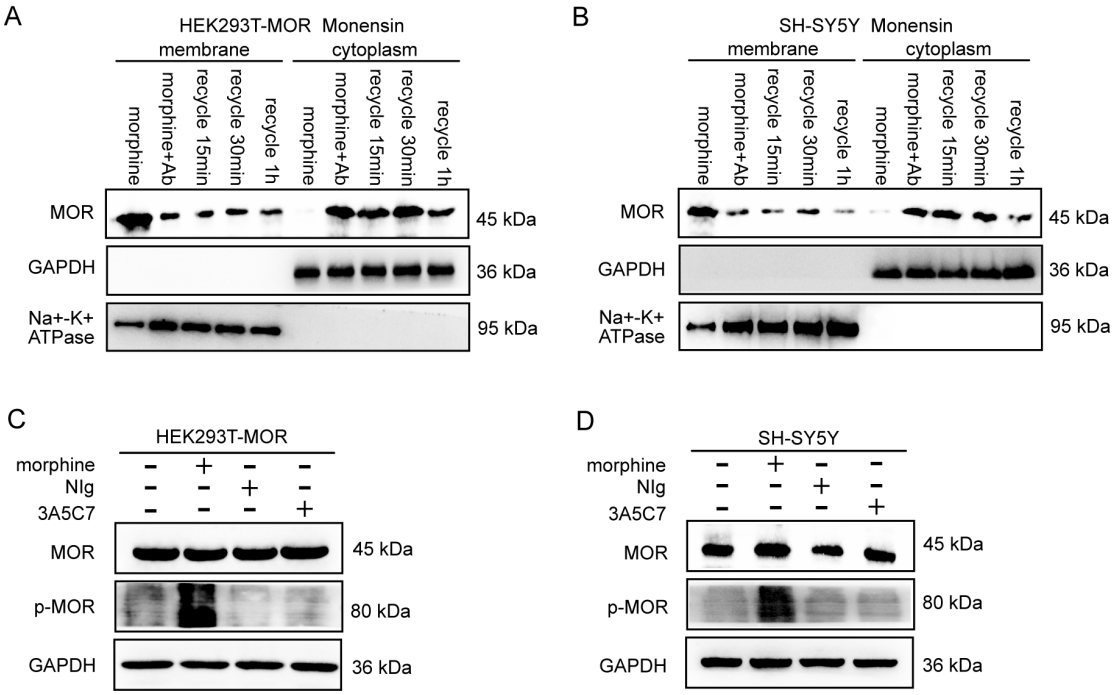

Fig. S8. The influence of monensin on mu-opioid receptor (MOR) endocytosis and the effects of 3A5C7 monoclonal antibody (mAb) itself on phospho-MOR (p-MOR). (A) Representative western blots showing the effects of monensin on MOR recycling in HEK293T-MOR cells. (B) Representative western blots showing the effects of monensin on MOR recycling in SH-SY5Y cells. HEK293T-MOR cells and SH-SY5Y cells were treated with morphine (10 μM) or morphine + 3A5C7 mAb (10 μg/mL) for 1 h, then morphine or mAb were washed out and mononsin (50 μM) was added to the media and incubated for 60 min. At 15 min, 30 min and 60 min after the addition of mononsin, cell membrane and plasma proteins were extracted for western blot assays. (C) Representative western blots manifesting the effects of 3A5C7 mAb itself on the expression of p-MOR in HEK293T-MOR cells. (D) Representative western blots manifesting the effects of 3A5C7 mAb itself on the expression of p-MOR in SH-SY5Y cells. Na^+-K^+ adenosine triphosphatase (ATPase) α1 subunit was used as internal reference of cell membrane and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference of cytoplasm. *n* = 3 independent experiments. NIg: normal IgG.

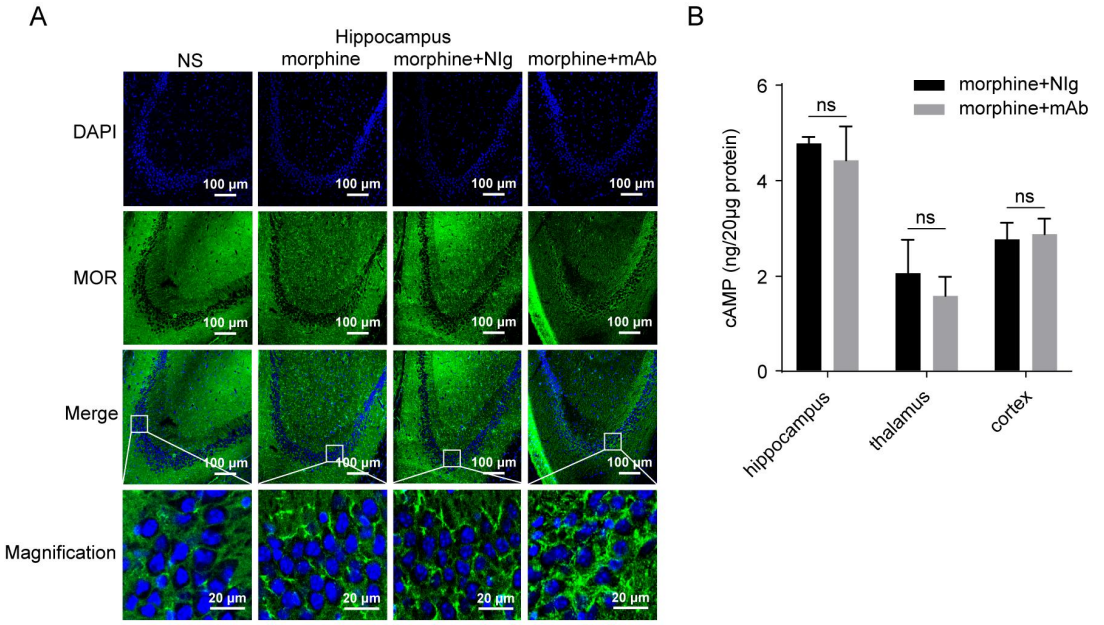

Fig. S9. Effects of 3A5C7 monoclonal antibody (mAb) on morphine-induced mu-opioid receptor (MOR) endocytosis and adenylate cyclase activation in mice. (A) Representative immunofluorescence staining images of MOR for each treatment in mouse hippocampus. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was used as nuclear marker. n = 3 mice in each group. (B) Cyclic adenosine monophosphate (cAMP) concentrations of different brain regions in mice from each treatment. n = 3 mice in each group. Student's t tests were used for statistical analysis. Data were presented as mean ± standard error of mean.