

MOL 26062

Interaction of the μ -Opioid Receptor with Synaptophysin Influences Receptor Trafficking and Signaling

Ying-Jian Liang¹, Dai-Fei Wu¹, Li-Quan Yang, Volker Höllt, and Thomas Koch

Department of Pharmacology and Toxicology, Otto-von-Guericke University,
39120 Magdeburg, Germany

¹Both authors contributed equally to this publication

MOL 26062

Running Title: Synaptophysin Attenuates the Development of Opiate Tolerance

Corresponding author: Thomas Koch, Department of Pharmacology and Toxicology,

Otto-von-Guericke University, Leipziger Str. 44, 39120 Magdeburg, Germany.

Phone: +49-391-6715372, FAX: +49-391-6715869,

E-mail: thomas.koch@Medizin.Uni-Magdeburg.de

The number of text pages: 38

The number of tables: 1

The number of figures: 5

The number of references: 42

The number of words in the Abstract: 167

The number of words in the introduction: 352

The number of words in the discussion: 1477

Abbreviations: MOPr, the μ -opioid receptor; Syp, synaptophysin; GPCRs, G protein-coupled receptors. HA, influenza hemagglutinin-HA-epitope (YPYDVPDYA); HA-MOPr, HA epitope-tagged MOPr; Myc, epitope tag (MASMQKLISEEDL); DAMGO, [D-Ala², Me-Phe⁴, Glyol⁵]enkephalin; HEK293, human embryonic kidney 293; ABTS, 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)]; BRET, bioluminescence resonance energy transfer; ELISA, enzyme-linked immunosorbent assay; AP, adaptor protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; ANOVA, analysis of variance.

ABSTRACT

There is increasing evidence that the signal transduction of opioid receptors is modulated by receptor-associated proteins. In search of proteins regulating μ -opioid receptor (MOPr) endocytosis, synaptophysin was found to bind to the rat μ -opioid receptor in yeast two-hybrid assay. Co-immunoprecipitation experiment and bioluminescence resonance energy transfer (BRET) assay confirmed that the μ -opioid receptor constitutively interacts with synaptophysin in human embryonic kidney (HEK) 293 cells over-expressing MOPr and synaptophysin. Here we show that over-expression of synaptophysin enhances the μ -opioid receptor endocytosis. One explanation for the observed effects is that synaptophysin recruits dynamin to the plasma membrane facilitating fission of clathrin-coated vesicles. This suggestion is supported by our finding that over-expression of a synaptophysin truncation mutant, which breaks the interaction between synaptophysin and dynamin, prevents agonist-mediated μ -opioid receptor endocytosis. In addition, the synaptophysin-augmented μ -opioid receptor trafficking leads to an attenuated agonist-induced receptor desensitization and a faster receptor resensitization. Taken together, our findings strongly suggest that synaptophysin play an important role in the regulation of μ -opioid receptor trafficking and signaling.

Introduction

The action of most clinically important opiate drugs, e.g. morphine, as well as drugs of abuse such as heroin is mediated by the μ -opioid receptor which belongs to the G-protein coupled receptor (GPCR) family (Kieffer and Gavériaux-Ruff, 2002; Matthes et al., 1996). Similar to other GPCRs, agonist binding to opioid receptors is followed by receptor coupling to G-proteins and subsequent phosphorylation. The binding of β -arrestins to phosphorylated receptors leads to receptor uncoupling from G-proteins, which in turn causes receptor desensitization. In addition, β -arrestins bind to both clathrin and AP-2 adaptor complex. The coordinated interaction of both clathrin and AP-2 with β -arrestins is necessary for heptahelical receptor internalization via clathrin-coated vesicles. Once the plasma membrane is invaginated, GTPase dynamin wraps around and constricts the necks upon GTP hydrolysis, thus vesiculation occurs. These vesicles soon shed their clathrin coats and become early endosomes. The ligands and receptors are separated in an acidified perinuclear compartment. Dissociation of β -arrestins also occurs. Cytosolic phosphatases may dephosphorylate the receptors. The ligands are degraded while the receptors are either recycled to the plasma membrane or degraded in lysosomes (see Claing et al., 2002; Mousavi et al., 2004 for reviews).

The trafficking and signaling of G-protein coupled receptors are affected by the interaction with various proteins (see Bockaert et al., 2004 for a review). For example, the interaction of rat μ -opioid receptors with phospholipase D2 accelerates receptor internalization (Koch et al., 2003). Periplakin, a cytolinker protein, interacts with the human μ -opioid receptor and reduces the coupling of G proteins (Feng et al., 2003). Using the yeast two-hybrid system we identified

MOL 26062

another μ -opioid receptor interacting protein namely the synaptophysin. Synaptophysin is a major integral membrane glycoprotein of synaptic vesicles, and is also found in a subset of brain coated vesicles, presumably as a result of vesicle trafficking (Sudhof et al., 1987). The exact role of synaptophysin is still controversially discussed. Here, we show for the first time that synaptophysin is constitutively associated with the μ -opioid receptor, and involved in receptor trafficking. Moreover, we provide evidence that the synaptophysin-augmented μ -opioid receptor trafficking plays an important role in agonist-induced receptor desensitization and resensitization.

Materials and Methods

Yeast Two-hybrid Screen. The MATCHMAKER GAL4 Yeast Two-Hybrid System 3 (Clontech, Heidelberg, Germany) was used to investigate interacting proteins of rat μ -opioid receptor. The cDNA fragments encoding the full length, a truncation (amino acids 258-398, from the 3rd intracellular loop to the carboxyl tail) and the carboxyl tail alone (amino acids 340-398) of rat μ -opioid receptor (GI: 6981309) were amplified by PCR and subcloned into vector pGBKT7, containing the Gal4 DNA binding domain. These fusion genes were used as baits to screen a rat brain cDNA library (Clontech), constructed in the vector pACT2 containing the Gal4 DNA activation domain. The bait and library plasmids were sequentially transformed into yeast strain AH109, using the lithium acetate method. The yeast two-hybrid screen was carried out according to the protocol handbook of the manufacturer.

Plasmid Construction. For cloning of the full length cDNA of rat synaptophysin (Syp, GI: 6981621), PCR primers were designed based on the sequence of Syp cDNA segment from the rat cDNA library obtained from yeast two-hybrid assay. The sequence of forward primer introducing a Sal I restriction site is 5'-TAG CTT GTC GAC TAT GGA CGT GGT GAA TCA GCT GGT GGC TGG GGG TCA GTT CCG GGT GGT CAA GGA GCC CCT TGG CTT CGT GAA G-3', and sequence of reverse primer introducing a Kpn I restriction site is 5'-ACT TCA GGT ACC AGA TTA CAT CTG ATT GGA-3'. Using the pACT2-Syp plasmid containing part length of Syp (nucleotides 49-924) as template, the full length coding region of rat Syp was amplified and subcloned into the Sal I/Kpn I sites of pCMV-Myc expression vector (Clontech) to produce amino-terminally Myc-tagged Syp. Similarly, the Syp truncations (nSyp: amino

MOL 26062

acids 1-223 and cSyp: amino acids 219-307 of Syp) were amplified by PCR, and subcloned into pCMV-Myc. For transient transfection, the coding region of MOPr was amplified using PCR, and then subcloned into pCMV-HA expression vector, which generates amino-terminally HA-tagged MOPr.

Immunoprecipitation and Western Blot Analysis. In 100-mm dish, HEK293 cells (American Type Culture Collection, Rockville, MD) or HEK293 cells stably expressing HA-MOPr as described later were transiently transfected with pCMV-Myc-Syp plasmid using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. 48 h after transfection, cells were completely lysed in a radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and proteinase inhibitors: 0.2 mM phenylmethyl-sulfonyl fluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, and 10 µg/ml bacitracin), and the resulting lysate was subjected to Western blot analysis or immunoprecipitation as described (Pfeiffer et al., 2003). Briefly, receptor proteins were immunoprecipitated with 100 µl of protein A agarose beads preloaded with 10 µg of anti-HA antibody. After SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting, membranes were incubated with 0.5 µg/ml anti-HA or anti-Myc antibody (Clontech) followed by detection using an enhanced chemiluminescence detection system (Amersham, Braunschweig, Germany).

GST-Affinity Chromatography. cDNA fragment encoding the third intracellular loop of MOPr (3ILµ, amino acids 253-283 of MOPr) was subcloned into bacterial expression vector pGEX-2KT (Amersham) to generate the glutathione S-transferase (GST) fusion proteins by

MOL 26062

PCR using the following primers. Forward primer 5'-ACT GTG GGA TCC GGC CTG ATG ATC TTA-3' introduced a BamH I restriction site, and reverse primer 5'-AGC CAC GAA TTC CAG CAC CAT CCG GGT GAT-3' introduced a EcoR I restriction site. Prior to produce GST and GST-3IL μ affinity beads, glutathione agarose beads (Amersham) were preincubated with a buffer (20 mM HEPES, pH7.6, 100 mM NaCl, 0.2 mM EDTA, 20% Glycerol, 1 mM DTT and proteinase inhibitors). Following overnight incubation (4°C), the beads were incubated with XL1 bacterial lysate containing GST or GST fusion proteins for an additional 3 h at 4°C and followed complete wash. HEK cells transiently transfected with pCMV-Myc-Syp using Lipofectamine 2000, were completely lysed in the RIPA buffer. Debris was removed by pelleting and expressed Myc-Syp proteins were purified using 100 μ l of protein A agarose beads (Amersham) preloaded with 10 μ g anti-Myc antibody. Eluted Myc-Syp proteins from the beads were incubated with GST-3IL μ or GST beads at 4°C for 3 h. Following complete wash, bound proteins were eluted and run on SDS-PAGE, and immunoblots were visualized as described above.

Bioluminescence Resonance Energy Transfer (BRET) Assay. The coding sequence without a stop codon of MOPr was amplified by PCR, and ligated into humanized pRluc-N₃ expression vector (BioSignal Packard, Montreal, Canada). The full length Syp and its truncations (nSyp and cSyp) were amplified by PCR, and subcloned into humanized pGFP-C₃ expression vectors (BioSignal Packard). For BRET measurements, HEK293 cells were transiently co-transfected with Rluc fusion and GFP fusion plasmids using Lipofectamine 2000. 45 h post-transfection, cells were detached with PBS/EDTA and resuspended in PBS buffer containing 0.1% glucose (w/v) and 2 μ g/ml aprotinin. Approximately 1×10^5 cells per well

MOL 26062

were transferred into a white bottom 96-well microplate (BioSignal Packard). Deep Blue C (a modified form of coelenterazine, BioSignal Packard) was added at a final concentration of 5 μ M, and readings were collected using a Fusion Universal Microplate Analyzer (BioSignal Packard) that allows the sequential integration of the signals detected in the 330-490 nm and 485-545 nm windows using filters with the appropriate band pass. The BRET signal is determined by calculating the ratio of the light emitted by the GFP (485-545 nm) over the light emitted by the Rluc (330-490 nm) after subtracting background signal detected in non-transfected cells. Using the Fusion Universal Microplate Analyzer, the expression level of GFP-Syp, GFP-cSyp and GFP-nSyp can be examined by fluorescence assay (light source, halogen CW; excitation, 485 nm; emission, 530 nm), and the expression level of MOPr-Rluc or Rluc can be evaluated (emission, 410 nm) after the addition of Deep Blue C.

Confocal Microscopy. Cells were grown onto poly-L-lysine-coated coverslip overnight. After treatment as indicated in text, cell fixation, permeabilization, and antibody incubation with anti-HA and/or anti-Myc antibodies were carried out as previously described (Koch et al., 2003). Bound primary antibodies were detected with cyanine 2.18- and/or cyanine 3.18-conjugated secondary antibodies (1: 400, Jackson ImmunoResearch, West Grove, PA). Cells were then permanently mounted in DPX (Fluka, Neu-Ulm, Germany) and examined using a Leica TCS-NT confocal microscope (Heidelberg, Germany).

Primary Neuronal Cell Culture, Transfection and Immunostaining. Neuronal cultures were prepared from the rat cortex of embryonic day 17 embryos (E17). All animal procedures were approved by the Otto-von-Guericke-University Magdeburg. In 12-well plate with poly-D-lysine-coated coverslips, primary cortical cells were seeded at 4×10^5 cells/well in 1.5

MOL 26062

ml Neurobasal medium (Invitrogen) supplemented with 2% B-27, 0.5 mM Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured at 37°C and 5% CO₂ in a humidified incubator. Half of the volume was removed on day 3 after seeding and replaced with fresh culture medium. Primary neuronal cultures on day 4 were transfected with pCMV-HA-MOPr and pCMV-Myc-Syp plasmids using Lipofectamine 2000. About 45 h after transfection, cells were treated and subsequently fixed. The distribution of expressed proteins of interest was detected by confocal microscopy as described above.

Generation of Stable Cell Lines. MOPr or Syp was tagged at the amino terminus with the HA or Myc epitope tag using PCR, and subcloned into the pEAK10 (Edge Bio Systems, Gaithersburg, MD) or pcDNA3.1 expression vector (Invitrogen), respectively. HEK293 cells were first transfected with pEAK10-HA-MOPr plasmid using Lipofectamine 2000. Stable transfectants were selected in the presence of 1.25 µg/ml puromycin (Sigma, Deisenhofen, Germany). To generate cell lines co-expressing HA-MOPr and Myc-Syp, GFP-nSyp (amino acids 1-223 of Syp) or GFP-cSyp (amino acids 219-307 of Syp), the cells were subjected to a second round of transfection with pcDNA3.1-Myc-Syp, pGFP-C₃-nSyp or pGFP-C₃-cSyp plasmid as described above, and selected in the presence of 1.25 µg/ml puromycin plus 1000 µg/ml G418 or 100 µg/ml Zeocin (Invitrogen), respectively. Multi-clones were used for further study. Receptor and/or synaptophysin expression were monitored using Western blot and/or confocal microscopy.

Radioligand Binding Assay. Binding studies were performed on membranes prepared from stably transfected cells. The dissociation constant (K_D) and number of [³H]DAMGO binding sites (B_{max}) were calculated by Scatchard analysis using at least seven concentrations of

MOL 26062

[³H]DAMGO in a range from 0.25 to 10 nM as previously described (Koch et al., 2006).

Quantitative Analysis of Receptor Trafficking. Cells were seeded at a density of 4.0×10^4 per well and grown onto poly-L-lysine-treated 96-well plates overnight. To estimate endocytosis, cells were specifically surface-labeled with anti-HA antibody (1 µg/ml) in UltraMEM (Cambrex, Verviers, Belgium) at 4°C for 1.5 h. Following the treatment as indicated in text, cells were fixed and incubated with peroxidase-conjugated antibody (1:1000, Amersham) for 1.5 h at room temperature. Subsequently, plates were developed with 50 µl ABTS solution (Roche, Mannheim, Germany). The reaction was analyzed at 405 nm using an Expert Plus Microplate Reader (ASYS, Eugendorf, Austria) during 10-20 min in real time. Receptor endocytosis was evaluated as the loss of surface-labeled receptors. To measure recycling, cells were first exposed to 10 µM etorphine (Gampian Pharamceutical Limited, Dundee Scotland, UK) for 2 h to drive agonist-induced endocytosis to a steady-state level. Following wash, cells were incubated at 37°C for 30 and 60 min in the presence of 10 µM naloxone to block residual agonist-stimulated internalization of MOPr. Then cells were chilled with 4°C PBS to stop receptor trafficking. Afterward receptors were surface-labeled at 4°C for 1.5 h. After wash of unbound antibody, cells were fixed and surface receptors were detected as described above. The recycling rate was estimated as a percentage of recovered surface receptors to endocytosed receptors.

Determination of Receptor Desensitization and Resensitization by Measurement of cAMP Accumulation. Approximately 1.0×10^5 cells per well were plated onto poly-L-lysine coated 24-well plate overnight. For desensitization assay, cells were exposed to 5 µM DAMGO (Bachem, Heidelberg, Germany) with and without 50 µM monensin (Sigma) for either 0, 1, 2, 4,

MOL 26062

or 6 h. For resensitization assay, cells were washed after 5-h exposure of 5 μ M DAMGO plus 50 μ M monensin followed by an additional incubation period of either 0, 5, 10, 30 or 50 min in the absence of agonist. For the measurement of cAMP accumulation, cells were washed one time with 0.5 ml serum-free RPMI medium (Cambrex). Immediately, medium was removed and replaced by 0.25 ml serum-free RPMI medium containing 25 μ M forskolin (Biotrend, Köln, Germany) in the presence or absence of 5 μ M DAMGO. The cells were incubated at 37°C for 15 min. After one time wash with cold PBS, the intracellular cAMP was extracted immediately with 0.5 ml of cold HCl/ethanol (1 volume of 1 N HCl/100 volumes of ethanol, stored at -20°C). The supernatant was transferred into a 1.5-ml tube, and evaporated by vacuum at 30°C. The extracted cAMP content was determined using a commercially available cyclic AMP (3 H) assay system (Amersham).

Results

Identification of Synaptophysin as a μ -Opioid Receptor Interacting Protein by Yeast

Two-hybrid Screen. A yeast two-hybrid screening of a rat cDNA library using both full length μ -opioid receptor and its truncation mutant (amino acids 258-398, from the 3rd intracellular loop to the carboxyl tail) as baits led to the finding of two cDNA fragments encoding amino acids 17-307 and 81-307 of synaptophysin (GI: 6981621), respectively. The interaction was verified in yeast mating and β -galactosidase assay. Synaptophysin interacts strongly with full length μ -opioid receptor and its truncation mutant (amino acids 258-398), but not with the carboxyl cytoplasmic tail (amino acids 340-398 of MOPr) alone, suggesting the 3rd intracellular loop of the receptor is the potential site for the interaction. As negative controls, empty Gal4-BD with Gal4-AD-Syp and empty Gal4-AD with Gal4-BD-MOPr, as well as a fusion of Gal4-BD with human lamin C, which neither forms complexes nor interacts with most other proteins, were used.

Co-immunoprecipitation of the μ -Opioid Receptor and Synaptophysin. To confirm the interaction between MOPr and synaptophysin, we first carried out co-immunoprecipitation studies. The μ -opioid receptor was tagged with the HA epitope tag (HA-MOPr), and synaptophysin was tagged with the Myc epitope tag (Myc-Syp) both at the amino terminus. HEK293 cells with and without stable expression of HA-MOPr were transiently transfected with Myc-Syp expressing plasmid. Expression of HA-MOPr and/or Myc-Syp was examined by directly immunoblotting lysates from these cells with antibodies against HA and/or Myc epitope tag. A MOPr band migrating at about 75 kD and a synaptophysin band migrating at

MOL 26062

about 38 kD were detected (Fig. 1A, lane 1 and 5, lysate). For co-immunoprecipitation, HA-MOPr receptors were precipitated from the lysates of HA-MOPr stably expressing cells and cells co-expressing HA-MOPr and Myc-Syp using anti-HA antibody. The resulting precipitates were immunoblotted with antibody directed against Myc epitope tag. As shown in Fig. 1A (lane 2, IP HA), Myc tagged synaptophysin migrating at about 38 kD was detected in immunoprecipitates from the cells co-expressing HA-MOPr and Myc-Syp, suggesting that MOPr is physically associated with synaptophysin *in vivo*. In contrast, no band was detectable in immunoprecipitates prepared under identical conditions from cells expressing only HA-MOPr or Myc-Syp, or from a mixture of cells expressing HA-MOPr or Myc-Syp individually (Fig. 1A, lane 3, 4 and 6, IP HA, respectively). This finding indicated that the MOPr-synaptophysin complex preexisted in cells prior to cell lysis and was not artificially formed during sample preparation.

Synaptophysin Binds Directly to the Third Intracellular Loop of MOPr. As suggested from the yeast two-hybrid screen, the third intracellular loop of the MOPr seems to be the important site for the interaction with synaptophysin. To confirm this suggestion, GST-affinity chromatography was carried out. The third intracellular loop of MOPr (3IL μ) was expressed as glutathione S-transferase fusion protein (GST-3IL μ). The purified GST and GST-3IL μ were then tested in GST pull-down experiments performed on purified synaptophysin from transfected HEK293 cells expressing full-length synaptophysin. To ensure that approximately equal amounts of the eluted GST and GST fusion protein were loaded on the gel, nitrocellulose membranes were probed with anti-GST antibody (data not shown). As depicted in Fig. 1B, synaptophysin was precipitated by GST-3IL μ . Control experiments performed with GST alone

MOL 26062

yielded no precipitation. These results demonstrate that synaptophysin binds specifically to the third intracellular loop of the MOPr.

Analysis of the Interaction between the μ -Opioid Receptor and Synaptophysin by BRET Assay. Detergent solubilization of cells during co-immunoprecipitation studies could promote artificial aggregation of hydrophobic proteins such as transmembrane proteins. Bioluminescence resonance energy transfer (BRET) is a non-destructive, living cell-based proximity assay, which overcomes the drawback of co-immunoprecipitation (Pfleger and Eidne, 2003). We therefore additionally analyzed the interaction of MOPr with synaptophysin in living cells using BRET assay.

To assess the interaction of MOPr with synaptophysin and their interacting domain, fusion constructs linking Rluc to the receptor carboxyl terminus and linking GFP to the amino terminus of full length synaptophysin (amino acids 1-307) or its truncation mutant (nSyp: amino acids 1-223 or cSyp: amino acids 219-307 of synaptophysin) were co-transfected in HEK293 cells. The transfer of energy between the two partners was assessed following the addition of Deep Blue C. The BRET signal is determined by calculating the ratio of the light emitted by GFP fusion protein over the light emitted by Rluc or MOPr-Rluc. Expression level of GFP fusion proteins, MOPr-Rluc or Rluc alone was evaluated as described in materials and methods. There was no significant difference in the ratio of acceptor protein (GFP fusion protein) and donor protein (MOPr-Rluc or Rluc alone) (data not shown), indicating similar expression level for GFP-Syp, GFP-nSyp and GFP-cSyp. As shown in Fig. 1C, a small BRET signal was obtained when GFP-Syp was co-expressed with Rluc alone (control). A significant BRET signal was detected in the combination of MOPr-Rluc with GFP-Syp or GFP-nSyp. And

MOL 26062

there was no significant BRET signal between MOPr-Rluc with GFP-cSyp. This indicates that the constitutive interaction between MOPr and synaptophysin also occurs in living mammalian cells.

Agonist Binding or Inhibition of Adenylate Cyclase by the μ -Opioid Receptor Is Not Affected by the Co-expression of Synaptophysin. To elucidate the potential function of the interaction between MOPr and synaptophysin, we stably co-expressed both amino-terminally HA-tagged MOPr and Myc-tagged synaptophysin in HEK293 cells. Co-expression of HA-MOPr and Myc-Syp was monitored by immunocytochemical analyses and Western blot revealing that more than 90% of the cells coexpressed both proteins. Saturation binding experiments revealed no substantial differences between MOPr and MOPr-Syp expressing cells with respect to their affinities (K_D) to [3 H]DAMGO and their numbers of binding sites (B_{max}) (Table 1). For the functional property of μ -opioid receptors to inhibit adenylate cyclase, there was no significant difference on the maximal inhibition of forskolin-induced cAMP accumulation by the μ -agonist (Table 1). These indicate that synaptophysin has no substantial effect on the μ -opioid receptor both agonist binding and functional inhibition of adenylate cyclase by the receptor.

Co-expression of Synaptophysin Augments μ -Opioid Receptor Trafficking. In HEK293 cells, μ -opioid receptor endocytosis was thought to be mediated via dynamin dependent clathrin-coated vesicle pathway. Over-expression of a dominant-negative mutant of dynamin I greatly inhibits μ -opioid receptor endocytosis (Zhang et al., 1998). Previous studies showed that synaptophysin interacts with dynamin I, which regulates the fission of endocytic vesicles (Daly et al., 2000; Daly and Ziff, 2002). To investigate whether synaptophysin

MOL 26062

influences μ -opioid receptor trafficking, Myc-tagged synaptophysin and HA-tagged MOPr were co-expressed in HEK293 cells and their subcellular distribution was examined using confocal microscopy. In MOPr transfected cells, the receptor was strictly localized in the cell membrane (Fig. 2A) (Finn and Whistler, 2001; Koch et al., 2003). However, as depicted in Fig. 2C, MOPr was both cytosolically and membraneously localized and co-localized with synaptophysin in cells co-expressing HA-MOPr and Myc-Syp. To test the possibility that there is an augmented constitutive trafficking of MOPr in cells with co-expression of synaptophysin, cells were exposed to 50 μ M monensin (a blocker of receptor recycling) for 1 h. As shown in Fig. 2D, MOPr was distributed mainly in cytosolic compartments in cells with high expression of synaptophysin whereas MOPr was still mainly localized at the plasma membrane in adjacent cells with low expression of synaptophysin. In cells without over-expression of synaptophysin, monensin treatment induced the distribution of MOPr in the cytoplasm, but MOPr was still mainly localized at the plasma membrane (Fig. 2B). This observation indicates that the synaptophysin-mediated μ -opioid receptor accumulation in cytoplasm occurs in recycling vesicles.

The μ -opioid receptor and synaptophysin are generally transported to the processes in native neurons. In transfected cortical neurons co-expressing HA-MOPr and Myc-Syp, the μ -opioid receptor and synaptophysin were co-localized at the plasma membrane and in the cytoplasm in both of the cell body and processes (Fig. 2E). Consistent with this observation, electron microscopy in brain slice of nucleus accumbens revealed that μ -opioid receptors are distributed at the plasma membrane and in the cytoplasm of both dendrites and axons, and also at the membrane of synaptic vesicles (Svingos et al., 1996).

Quantitative Analysis of μ -Opioid Receptor Trafficking. Confocal observation in Fig. 2D indicated that synaptophysin enhances the constitutive trafficking of μ -opioid receptors. To further confirm this finding, we quantified μ -opioid receptor trafficking by ELISA. As expected, there was an enhanced constitutive internalization of μ -opioid receptors in cells co-expressing synaptophysin (11.15 ± 1.99 % versus 28.00 ± 2.36 % for MOPr and MOPr-Syp, respectively, Fig. 3A). Moreover, we found that synaptophysin also enhances the agonist-induced μ -opioid receptor internalization. In the presence of $1\mu\text{M}$ DAMGO for 30 min, an augmented rate of μ -opioid receptor endocytosis by synaptophysin was detected (40.43 ± 1.62 % versus 61.48 ± 1.95 % for MOPr and MOPr-Syp, respectively, Fig. 3A). As shown in Figure 3B, we found that synaptophysin significantly increased the recycling of endocytosed receptors. These results provide evidences that synaptophysin enhances μ -opioid receptor trafficking in HEK293 cells.

Co-expression of Synaptophysin Truncation Mutants Blocks Agonist-Induced μ -Opioid Receptor Endocytosis. As shown above (Fig. 1C), the μ -opioid receptor interacted with the truncation mutant nSyp (amino acids 1-223 of synaptophysin), but not with cSyp (amino acids 219-307, carboxyl cytoplasmic tail of synaptophysin). Previous report showed that the carboxyl cytoplasmic tail of synaptophysin interacts with dynamin I and mediates the formation of a protein complex containing GTPase dynamin (Daly and Ziff, 2002). We assumed that the observed effect of synaptophysin on μ -opioid receptor endocytosis might be due to the synaptophysin-enhanced recruitment of dynamin to the plasma membrane during the budding of clathrin-coated vesicles. If this is true, agonist-induced μ -opioid receptor endocytosis should be decreased when the connection between receptor and dynamin via

MOL 26062

synaptophysin is disrupted. Indeed, after transient over-expression of both GFP-tagged truncation mutants (amino acids 1-223 and 219-307) of synaptophysin, the DAMGO-induced μ -opioid receptor endocytosis was almost completely blocked whereas receptors in adjacent cells that did not express these mutants internalized efficiently in response to μ -agonist (Fig. 4, B-C). However, both mutants do not cause a general impairment of dynamin-mediated endocytosis because the endocytosis of transferrin receptors (marked by Alexa Fluor 488-conjugated transferrin) was not affected by the expression of both mutants (Fig. 4, D-E). In addition, in cells transiently co-expressing GFP-tagged full length synaptophysin, μ -opioid receptors were internalized efficiently under the same stimulation (Fig. 4A), suggesting GFP tag did not block agonist-induced receptor endocytosis. The negative effect of these mutants on μ -opioid receptor endocytosis was further confirmed by quantitative ELISA analysis in stable multi-clones with the co-expression of MOPr and synaptophysin truncation mutant (Fig. 3F). These findings indicate an essential role of synaptophysin in μ -opioid receptor internalization. In addition, the full length of synaptophysin is required to enhance the internalization.

Synaptophysin Decreases Agonist-Induced Desensitization of the μ -Opioid Receptor.

We have demonstrated that synaptophysin influences μ -opioid receptor internalization. Agonist-induced endocytosis of G protein-coupled receptors was reported to modulate receptor desensitization (Koch et al., 2004, 2005; Pak et al., 1996). For investigating the effect of synaptophysin on receptor desensitization, we exposed the cells expressing μ -opioid receptors with and without co-expression of synaptophysin to μ -agonist for various time periods, and measured the receptor activity by determining the agonist-induced inhibition of forskolin-stimulated cAMP accumulation. As shown in Fig. 5A, prolonged exposure of

MOL 26062

μ -opioid receptors to the selective μ -agonist DAMGO led to a time-dependent decrease in the inhibition of cAMP accumulation. After 6 h of μ -agonist exposure, the μ -opioid receptor alone exhibited the desensitization with about 58% loss in DAMGO-induced inhibition of cAMP accumulation whereas over-expression of synaptophysin significantly decreased receptor desensitization, with only about 36% loss in the inhibition.

It is generally assumed that rapid receptor internalization contributes to rapid receptor desensitization. For investigating whether the relative slow desensitization of μ -opioid receptors with co-expression of synaptophysin is due to the augmented receptor recycling, we tested the effect of monensin, a blocker of receptor recycling, on receptor desensitization. As depicted in Fig. 5A, co-application of monensin (mon) with DAMGO resulted in an enhanced μ -opioid receptor desensitization. The receptor was completely desensitized after 5-h DAMGO treatment in the presence of monensin. In the cells co-expressing MOPr and synaptophysin, a significantly faster desensitization was observed as compared to cells expressing receptor alone in the presence of monensin (Fig. 5A). The finding indicated that the synaptophysin-mediated slow desensitization of μ -opioid receptors resulted from the enhanced-receptor recycling, which induces receptor reactivation. Consistent with this opinion, over-expression of synaptophysin significantly increased μ -opioid receptor recycling (Fig. 3B) and reactivation (resensitization) (Fig. 5B).

Discussion

A large body of both in vitro and in vivo experimental data indicate that synaptophysin is involved in multiple, important aspects of exo-endocytosis of synaptic vesicles, including regulation of SNARE assembly into the fusion core complex, formation of the fusion pore initiating neurotransmitter release, activation of synaptic vesicle endocytosis and biogenesis. Synaptophysin has been found to interact in vitro with various nerve terminal proteins, which include dynamin I, adaptor protein 1 (AP-1), the v-SNARE vesicle-associated membrane protein 2/synaptobrevin II (VAMP2), the vesicular proton pump V-ATPase and myosin V (for review, see Valtorta et al., 2004). Synaptophysin also binds to cholesterol and this interaction has been proposed to be essential for the biogenesis of synaptic-like microvesicles (Thiele et al., 2000). In the present study, synaptophysin was identified as a direct binding partner for the μ -opioid receptor, and influences receptor trafficking and signaling.

Synaptophysin was found to interact with full length μ -opioid receptor, but not with the receptor carboxyl cytoplasmic tail in yeast two-hybrid assay. This suggests that the μ -opioid receptor carboxyl tail is not the direct binding region for synaptophysin though this terminal tail has been shown to be an important domain for other μ -opioid receptor interacting protein (Koch et al., 2003; Onoprishvili et al., 2003). However, our experiments indicate that the 3rd intracellular loop of the receptor interacts with synaptophysin since a μ -opioid receptor truncation mutant containing only this loop binds to synaptophysin in GST pull-down assay. An interaction between the μ -opioid receptor and synaptophysin was further confirmed by co-immunoprecipitation and BRET assay in HEK293 cells. Fluorescence studies using confocal microscopy provided evidences for a co-localization of the μ -opioid receptor and

MOL 26062

synaptophysin in both transfected HEK293 cells and primary cultured neurons. This is in line with the finding that a high degree of co-localization between endogenous μ -opioid receptor and synaptophysin was detected in mature synapses in both cultured cortical and hippocampal neurons (Liao et al., 2005). In addition, in the brain slice of the pallidum, the co-localization of the μ -opioid receptor and synaptophysin was also found (Olive et al., 1997). These findings provide a strong support for a direct interaction between the μ -opioid receptor and synaptophysin under physiological condition.

In transfected HEK293 cells co-expressing the μ -opioid receptor and synaptophysin, over-expression of synaptophysin has no effect on receptor properties, both agonist-binding and agonist-induced adenylate cyclase inhibition. However, the μ -opioid receptor shows co-localization with synaptophysin not only at the plasma membrane, but also in the cytosolic compartments. This result is surprising because μ -opioid receptors are found mainly at cell surface in transfected HEK293 cells without over-expression of synaptophysin (Finn and Whistler, 2001; Koch et al., 2003). In transfected CHO cells and in endocrine cell lines (PC12 cells and RINm5F cells), synaptophysin is co-localized in the constitutively recycling vesicles with transferrin receptors, and follows the same trafficking pathway as transferrin receptors (Cameron et al., 1991). In addition, previous work (Keith et al., 1998) and our current observation (data not shown) show that μ -opioid and transferrin receptors share the same trafficking pathway. Based on these findings, it seems likely that the synaptophysin-enhanced μ -opioid receptor distribution in cytosolic compartments results from the synaptophysin-augmented constitutive receptor trafficking. This view is further supported by our observation that treatment with monensin (a blocker of receptor recycling) significantly

MOL 26062

increases the distribution of μ -opioid receptors in the cytoplasm. Furthermore, we additionally quantified the receptor trafficking and provided the direct evidence that over-expression of synaptophysin enhances the constitutive μ -opioid receptor trafficking. The synaptophysin-enhanced intracellular localization of μ -opioid receptors in HEK293 cells is in line with previous report that μ -opioid receptors are constitutively localized in both of the plasma membrane and the cytoplasm of native neurons (Svingos et al., 1996), which endogenously express synaptophysin.

Receptor endocytosis is thought to be initiated by the formation of receptor/ β -arrestin/AP-2/clathrin complexes. Subsequently, the rapid fission of budding vesicles depends essentially on dynamin, a GTPase that acts as a molecular scissor in sequestering the newly formed clathrin-coated vesicles away from the plasma membrane (Claing et al., 2002; Hinshaw, 2000; Slepnev et al., 2000). Synaptophysin has been shown to interact with dynamin I, and plays a role in the endocytosis of synaptic vesicles by recruiting dynamin to the vesicle membrane (Daly and Ziff, 2002). In squid giant terminal, disruption of the synaptophysin-dynamin interaction by microinjection of the carboxyl cytoplasmic tail of synaptophysin results in a block of vesicle endocytosis (Daly et al., 2000). In transfected 3T3 cells, deletion of this carboxyl tail has been found to impede internalization of the truncation mutant while full length synaptophysin is internalized efficiently (Linstedt and Kelly, 1991). These findings are consistent with a model in which the interaction between synaptophysin and dynamin facilitates the formation of endocytotic vesicles. Indeed, over-expression of synaptophysin significantly increases μ -opioid receptor internalization. It is thus reasonable to assume that synaptophysin augments μ -opioid receptor internalization by targeting dynamin to

MOL 26062

the plasma membrane during the formation of endocytotic vesicles. Consistent with this idea, over-expression of synaptophysin carboxyl tail, which is thought to disrupt the normal targeting of dynamin to the plasma membrane, is shown to almost completely block agonist-induced μ -opioid receptor internalization. Since the N-terminal fragment of synaptophysin (nSyp) also blocks MOPr internalization without affecting dynamin function, it is reasonable to suggest that binding of nSyp to MOPr prevents binding of endogenously expressed full-length synaptophysin to MOPr and subsequently impedes the receptor mediated targeting of dynamin to the plasma membrane. Transferrin receptor endocytosis was not affected by over-expression of both the mutants, indicating that nSyp and cSyp do not lead to a general impairment of dynamin function.

Agonist-induced endocytosis of G protein-coupled receptors is a process that can contribute to functional desensitization of signal transduction by reducing the number of receptors present at cell surface (Pak et al., 1996). However, in the present studies, the synaptophysin-augmented μ -opioid receptor endocytosis significantly decreases agonist-induced receptor desensitization. Though endocytosis enhances receptor desensitization, it also contributes to functional resensitization of signal transduction by promoting recycling of internalized receptors to cell surface (Ferguson et al., 1998; Koch et al., 2004; Law et al., 2000; Lefkowitz et al., 1998). After internalization, distinct G protein-coupled receptors are differentially sorted between the degradative and recycling pathways (Tsao and von Zastrow, 2000). Internalization of receptors is a rapid process and for the μ -opioid receptor 30 min of DAMGO treatment resulted in a 40% loss of membrane receptors without markedly affecting functional activity at this time (Koch et al., 1998, 2005). The simplest explanation for

MOL 26062

this finding is that only 60% of intact MOPr are necessary for maximum inhibition of intracellular cAMP formation. The decreasing pool of receptors in the membrane after chronic agonist treatment may then be balanced by receptor recycling after internalization, counteracting fast receptor desensitization. For the μ -opioid receptor, it has been shown that agonist-induced receptor endocytosis is followed by a rapid receptor recycling and reactivation, thus reducing receptor desensitization (Finn and Whistler, 2001; He et al., 2002; Koch et al., 2005; Qiu et al., 2003). We therefore speculate that the observed slower rate of μ -opioid receptor desensitization after over-expression of synaptophysin is predominantly due to the synaptophysin-augmented receptor recycling and reactivation. Consistent with this speculation is that synaptophysin mediates a faster DAMGO-induced receptor desensitization when the recycling pathway is blocked by monensin. This is also in line with the finding that the internalizing μ -agonist DAMGO leads to a faster resensitization of μ -opioid receptors with the co-expression of synaptophysin.

After receptor internalization, the receptor might be recycled to cell surface, degraded in lysosomes, or transported for axonal targeting in neurons (Leterrier et al., 2006). Though it is clear that μ -opioid receptors are localized in both dendrites and axons, the mechanisms whereby this transportation occurs are still far from understanding. The AP-1 adaptor complex recruits clathrin to membranes in the trans-Golgi network (TGN) to form transport vesicles (Robinson and Bonifacino, 2001; Seaman et al., 1996). Horikawa et al. (2002) reported that synaptophysin interacts with the AP-1 adaptor complex via γ -adaptin, and provides adaptor sites for microtubule-based vesicle transport to axons and nerve terminals. Thus, it appears to be possible that synaptophysin may have multiple functional roles in μ -opioid receptor

MOL 26062

regulation. The transportation of newly synthesized μ -opioid receptors to neuronal processes might be involved in the complex formation of MOPr/Syp/AP-1. Regulation of specific synaptophysin function might be provided by protein kinases, as synaptophysin is known to be efficiently phosphorylated on both serine/threonine and in particular tyrosine residues (Evans and Cousin, 2005; Horikawa et al., 2002).

In conclusion, we have identified synaptophysin as a new μ -opioid receptor interacting protein, and demonstrated its role in receptor trafficking. Furthermore, the synaptophysin-augmented μ -opioid receptor trafficking attenuates agonist-induced receptor desensitization. It has been demonstrated in numerous studies that μ -opioid receptor desensitization contributes to the development of opiate tolerance (Bohn et al., 2000; Finn and Whistler, 2001; He et al., 2002; Koch et al., 2004, 2005). We therefore suggest that the synaptophysin-augmented μ -opioid receptor trafficking decreases the development of opiate tolerance.

MOL 26062

Acknowledgements

We thank Sandra Großeheimann, Evelyn Kahl, Stefanie Holze and Michaela Böx for excellent technical assistance.

References

- Bockaert J, Fagni L, Dumuis A and Marin P (2004) GPCR interacting proteins (GIP). *Pharmacol Ther* **103**:203-221.
- Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ and Caron MG (2000) Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* **408**:720-723.
- Cameron PL, Sudhof TC, Jahn R and De Camilli P (1991) Colocalization of synaptophysin with transferrin receptors: implications for synaptic vesicle biogenesis. *J Cell Biol* **115**:151-164.
- Claing A, Laporte SA, Caron MG and Lefkowitz RJ (2002) Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog Neurobiol* **66**:61-79.
- Daly C, Sugimori M, Moreira JE, Ziff EB and Llinas R (2000) Synaptophysin regulates clathrin-independent endocytosis of synaptic vesicles. *Proc Natl Acad Sci U S A* **97**:6120-6125.
- Daly C and Ziff EB (2002) Ca²⁺-dependent formation of a dynamin-synaptophysin complex: potential role in synaptic vesicle endocytosis. *J Biol Chem* **277**:9010-9015.
- Evans GJ and Cousin MA (2005) Tyrosine phosphorylation of synaptophysin in synaptic vesicle recycling. *Biochem Soc Trans* **33**:1350-1353.
- Feng GJ, Kellett E, Scorer CA, Wilde J, White JH and Milligan G (2003) Selective interactions between helix VIII of the human mu-opioid receptors and the C terminus of periplakin disrupt G protein activation. *J Biol Chem* **278**:33400-33407.

MOL 26062

- Ferguson SS, Zhang J, Barak LS and Caron MG (1998) Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. *Life Sci* **62**:1561-1565.
- Finn AK and Whistler JL (2001) Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* **32**:829-839.
- He L, Fong J, von Zastrow M and Whistler JL (2002) Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* **108**:271-282.
- Hinshaw JE (2000) Dynamin and its role in membrane fission. *Annu Rev Cell Dev Biol* **16**:483-519.
- Horikawa HP, Kneussel M, El Far O and Betz H (2002) Interaction of synaptophysin with the AP-1 adaptor protein gamma-adaptin. *Mol Cell Neurosci* **21**:454-462.
- Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ and von Zastrow M (1998) mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Mol Pharmacol* **53**:377-384.
- Kieffer BL and Gaveriaux-Ruff C (2002) Exploring the opioid system by gene knockout. *Prog Neurobiol* **66**:285-306.
- Koch T, Brandenburg LO, Liang Y, Schulz S, Beyer A, Schröder H and Höllt V (2004) Phospholipase D2 modulates agonist-induced mu-opioid receptor desensitization and resensitization. *J Neurochem* **88**:680-688.
- Koch T, Brandenburg LO, Schulz S, Liang Y, Klein J and Höllt V (2003) ADP-ribosylation factor-dependent phospholipase D2 activation is required for agonist-induced mu-opioid receptor endocytosis. *J Biol Chem* **278**:9979-9985.

MOL 26062

- Koch T, Schulz S, Schröder H, Wolf R, Raulf E and Höllt V (1998) Carboxyl-terminal splicing of the rat mu opioid receptor modulates agonist-mediated internalization and receptor resensitization. *J Biol Chem* **273**:13652-13657.
- Koch T, Widera A, Bartsch K, Schulz S, Brandenburg LO, Wundrack N, Beyer A, Grecksch G and Höllt V (2005) Receptor endocytosis counteracts the development of opioid tolerance. *Mol Pharmacol* **67**:280-287.
- Koch T, Wu DF, Yang LQ, Brandenburg LO and Höllt V (2006) Role of phospholipase D2 in the agonist-induced and constitutive endocytosis of G-protein couple receptors. *J Neurochem* **97**:365-372.
- Law PY, Erickson LJ, El-Kouhen R, Dicker L, Solberg J, Wang W, Miller E, Burd AL and Loh HH (2000) Receptor density and recycling affect the rate of agonist-induced desensitization of mu-opioid receptor. *Mol Pharmacol* **58**:388-398.
- Lefkowitz RJ, Pitcher J, Krueger K and Daaka Y (1998) Mechanisms of beta-adrenergic receptor desensitization and resensitization. *Adv Pharmacol* **42**:416-420.
- Leterrier C, Laine J, Darmon M, Boudin H, Rossier J and Lenkei Z (2006) Constitutive activation drives compartment-selective endocytosis and axonal targeting of type 1 cannabinoid receptors. *J Neurosci* **26**:3141-3153.
- Liao D, Lin H, Law PY and Loh HH (2005) Mu-opioid receptors modulate the stability of dendritic spines. *Proc Natl Acad Sci U S A* **102**:1725-1730.
- Linstedt AD and Kelly RB (1991) Endocytosis of the synaptic vesicle protein, synaptophysin, requires the COOH-terminal tail. *J Physiol (Paris)* **85**:90-96.
- Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A,

MOL 26062

- Le Meur M, Dolle P, Tzavara E, Hanoune J, Roques BP and Kieffer BL (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* **383**:819-823.
- Mousavi SA, Malerod L, Berg T and Kjekshus R (2004) Clathrin-dependent endocytosis. *Biochem J* **377**:1-16.
- Olive MF, Anton B, Micevych P, Evans CJ and Maidment NT (1997) Presynaptic versus postsynaptic localization of mu and delta opioid receptors in dorsal and ventral striatopallidal pathways. *J Neurosci* **17**:7471-7479.
- Onopriashvili I, Andria ML, Kramer HK, Ancevska-Taneva N, Hiller JM and Simon EJ (2003) Interaction between the mu opioid receptor and filamin A is involved in receptor regulation and trafficking. *Mol Pharmacol* **64**:1092-1100.
- Pak Y, Kouvelas A, Scheideler MA, Rasmussen J, O'Dowd BF and George SR (1996) Agonist-induced functional desensitization of the mu-opioid receptor is mediated by loss of membrane receptors rather than uncoupling from G protein. *Mol Pharmacol* **50**:1214-1222.
- Pfeiffer M, Kirscht S, Stumm R, Koch T, Wu D, Laugsch M, Schröder H, Höllt V and Schulz S (2003) Heterodimerization of substance P and mu-opioid receptors regulates receptor trafficking and resensitization. *J Biol Chem* **278**:51630-51637.
- Pfleger KD and Eidne KA (2003) New technologies: bioluminescence resonance energy transfer (BRET) for the detection of real time interactions involving G-protein coupled receptors. *Pituitary* **6**:141-151.
- Qiu Y, Law PY and Loh HH (2003) Mu-opioid receptor desensitization: role of receptor phosphorylation, internalization, and representation. *J Biol Chem* **278**:36733-36739.

MOL 26062

- Robinson MS and Bonifacino JS (2001) Adaptor-related proteins. *Curr Opin Cell Biol* **13**:444-453.
- Seaman MN, Sowerby PJ and Robinson MS (1996) Cytosolic and membrane-associated proteins involved in the recruitment of AP-1 adaptors onto the trans-Golgi network. *J Biol Chem* **271**:25446-25451.
- Slepnev VI, Ochoa GC, Butler MH and De Camilli P (2000) Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of clathrin coat function by amphiphysin fragments comprising these sites. *J Biol Chem* **275**:17583-17589.
- Sudhof TC, Lottspeich F, Greengard P, Mehl E and Jahn R (1987) A synaptic vesicle protein with a novel cytoplasmic domain and four transmembrane regions. *Science* **238**:1142-1144.
- Svingos AL, Moriwaki A, Wang JB, Uhl GR and Pickel VM (1996) Ultrastructural immunocytochemical localization of mu-opioid receptors in rat nucleus accumbens: extrasynaptic plasmalemmal distribution and association with Leu5-enkephalin. *J Neurosci* **16**:4162-4173.
- Thiele C, Hannah MJ, Fahrenholz F and Huttner WB (2000) Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. *Nat Cell Biol* **2**:42-49.
- Tsao PI and von Zastrow M (2000) Type-specific sorting of G protein-coupled receptors after endocytosis. *J Biol Chem* **275**:11130-11140.
- Valtorta F, Pennuto M, Bonanomi D and Benfenati F (2004) Synaptophysin: leading actor or walk-on role in synaptic vesicle exocytosis? *Bioessays* **26**:445-453.
- Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY and Caron MG (1998) Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid

MOL 26062

receptor responsiveness. *Proc Natl Acad Sci U S A* **95**:7157-7162.

MOL 26062

Footnotes

This work was supported by grants HO 1027/10-1 and SFB 426/A2 from the Deutsche Forschungsgemeinschaft (to VH) and BMBF-grant 01ZZ0407 (to TK).

MOL 26062

Legends for Figures

Fig. 1. Interaction of the μ -opioid receptor and synaptophysin. A, co-immunoprecipitation. Lysates from cells co-expressing HA-MOPr and Myc-Syp were extracted and either immunoblotted directly (lane 1, lysate) or immunoprecipitated using anti-HA antibody (lane 2, IP HA). In controls, lysates from cells expressing HA-MOPr (lane 3) or Myc-Syp (lane 4 and 5) alone, or their mixture (lane 6) were extracted and either immunoprecipitated using anti-HA antibody or immunoblotted directly. Arrows point to Syp and MOPr, respectively. B, in vitro binding between the third intracellular loop of MOPr (3IL μ) and synaptophysin. Purified Myc-tagged synaptophysin proteins were incubated with GST or GST-3IL μ beads in GST pull-down assay as described under *Materials and Methods*. Following complete wash, precipitated protein was analysed by Western blotting with anti-Myc antibody. C, BRET assay. Cells were transiently co-transfected with MOPr-Rluc in combination with GFP-Syp (full length), GFP-nSyp (amino acids 1-223 of Syp) or GFP-cSyp (amino acids 219-307, carboxyl cytoplasmic tail of Syp). A combination of Rluc and GFP-Syp was used as negative control (control). Cells were harvested 45 h post-transfection. The energy transfer was initiated by addition of 5 μ M Deep Blue C, and BRET signal was assessed as described under *Materials and Methods*. Data are presented as mean \pm SEM of 3 independent experiments performed in triplicate. $^{**}p < 0.01$ compared with negative control (ANOVA followed by Bonferroni's test).

Fig. 2. A-D, role of synaptophysin in the subcellular distribution of μ -opioid receptors. HA-MOPr expressing cells with and without co-expression of Myc-Syp were either not treated

(A and C) or treated with 50 μ M monensin for 1 h (B and D). Cells were subsequently fixed, and subjected to immunofluorescent staining as described under *Materials and Methods*. Note that over-expression of synaptophysin augmented the constitutive trafficking of μ -opioid receptors. E, co-localization of MOPr and Syp in co-transfected primary cultured neurons. Newly prepared cortical cells (E17) were grown on poly-D-lysine-coated glass coverslips for 4 days, and then subjected to co-transfection with pCMV-HA-MOPr and pCMV-Myc-Syp plasmids. The receptor and synaptophysin were detected as described above. The overlay image was shown in large magnification (4 folds in comparison with single image). Shown are representative results from one of three independent experiments.

Fig. 3. Quantitative analysis of μ -opioid receptor trafficking. A, receptor endocytosis. HEK293 cells expressing HA-MOPr alone or co-expressing with Myc-Syp were surface-labeled with anti-HA antibody at 4°C for 1.5 h. Subsequently, cells were either not exposed or exposed to 1 μ M DAMGO at 37°C for 30 min, and then fixed. Receptor internalization, quantified as the percent loss of cell surface receptors, was measured by ELISA as described under *Materials and Methods*. B, receptor recycling. After agonist-induced MOPr internalization to a stable level, the cells were exposed to 10 μ M naloxone for 30 and 60 min in agonist free medium to allow receptor recycling. The recycling rate was estimated as a percentage of recovered surface receptors to internalized receptors as described under *Materials and Methods*. Data in A and B are presented as means \pm SEM of 3-5 independent experiments performed in triplicate. * p <0.05 and *** p <0.001 compared with MOPr alone in relative group (ANOVA followed by Bonferroni's test).

Fig. 4. Effect of synaptophysin truncation mutants on agonist-induced μ -opioid receptor internalization. A-E, cells with and without stable expression of HA-MOPr were transiently transfected with GFP-Syp, GFP-nSyp, GFP-cSyp, Myc-nSyp or Myc-cSyp plasmid using calcium phosphate precipitation as described (Koch et al., 2003). About 60 h after transfection, following the preincubation of 50 μ M monensin for 30 min, cells were exposed to 1 μ M DAMGO (A-C) or 5 μ g/ml Alexa Fluor 488-conjugated transferrin (Molecular Probes)(D-E) in the presence of 50 μ M monensin for another 30 min at 37°C. Cells were subsequently fixed and subjected to immunofluorescent staining as described under *Materials and Methods*. The images were taken by confocal microscopy. Representative images from two independent experiments are shown. Scale bar, 8 μ m. F, in cells stably expressing HA-MOPr alone or co-expressing with GFP-nSyp or GFP-cSyp, receptor endocytosis was detected as described in Fig.3. Data are presented as means \pm SEM of 3 independent experiments performed in triplicate. * p <0.05 and *** p <0.001 compared with MOPr alone, respectively (ANOVA followed by Bonferroni's test). Note that over-expression of synaptophysin truncation mutants significantly decreased μ -opioid receptor endocytosis.

Fig. 5. Effect of synaptophysin on agonist-induced μ -opioid receptor desensitization and resensitization. A, DAMGO-induced receptor desensitization. Cells expressing HA-MOPr with and without co-expression of Myc-Syp were exposed to 5 μ M DAMGO in the presence or absence of 50 μ M monensin (mon) for the indicated time periods. After washing, cells were treated with forskolin or forskolin plus DAMGO for 15 min, and cAMP levels were determined

MOL 26062

as described under *Materials and Methods*. B, μ -opioid receptor resensitization. Cells were exposed to 5 μ M DAMGO plus 50 μ M monensin for 5 h followed by a drug-free interval of 0, 5, 10, 30 and 50 min. Then, the effect of DAMGO on forskolin-stimulated cAMP accumulation was measured as described above. DAMGO-induced inhibition of cAMP accumulation without a drug-free interval (0 min) was defined as 0%, whereas maximum inhibition of intracellular cAMP accumulation in cells that were not pretreated with DAMGO was defined as 100%. Values represent mean \pm SEM of 3-4 independent measurements performed in duplicate. * p <0.05 and ** p <0.01 compared with MOPr alone (ANOVA followed by Bonferroni's test).

MOL 26062

TABLE 1

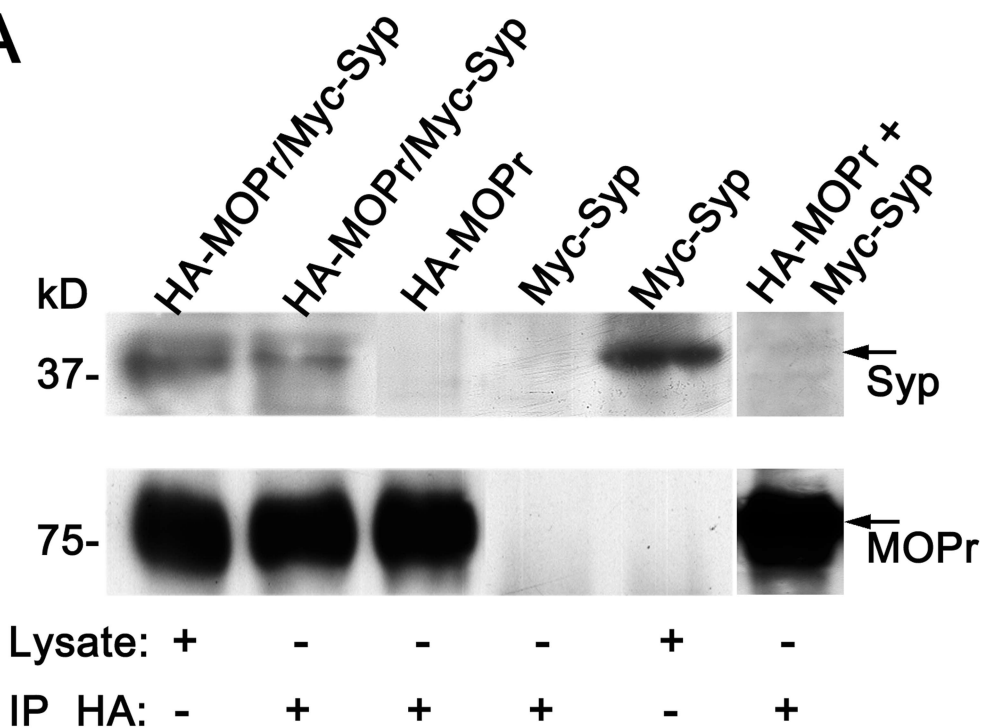
Binding characteristics of the μ -opioid receptor with and without co-expression of synaptophysin

K_D and B_{max} for the binding of [3H] DAMGO to μ -opioid receptors in stable cell line expressing HA epitope-tagged MOPr with or without co-expression of Myc epitope-tagged Syp in HEK293 cells were determined by Scatchard analyses. The effect of 5 μ M DAMGO on forskolin-stimulated cAMP accumulation was determined as described under *Materials and Methods*. Values shown are the mean \pm SEM of three independent experiments performed in duplicate.

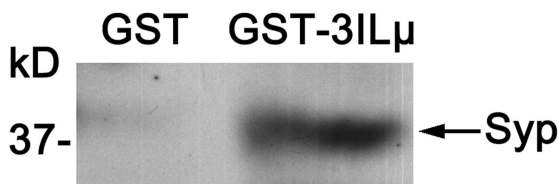
Stable cell lines	K_D (nM)	B_{max} (pmol/mg)	Maximal inhibition of cAMP accumulation
MOPr	2.15 ± 0.21	2.21 ± 0.35	97.2 ± 0.8 %
MOPr - Syp	2.08 ± 0.35	1.95 ± 0.21	96.0 ± 2.1 %

Fig. 1.

A



B



C

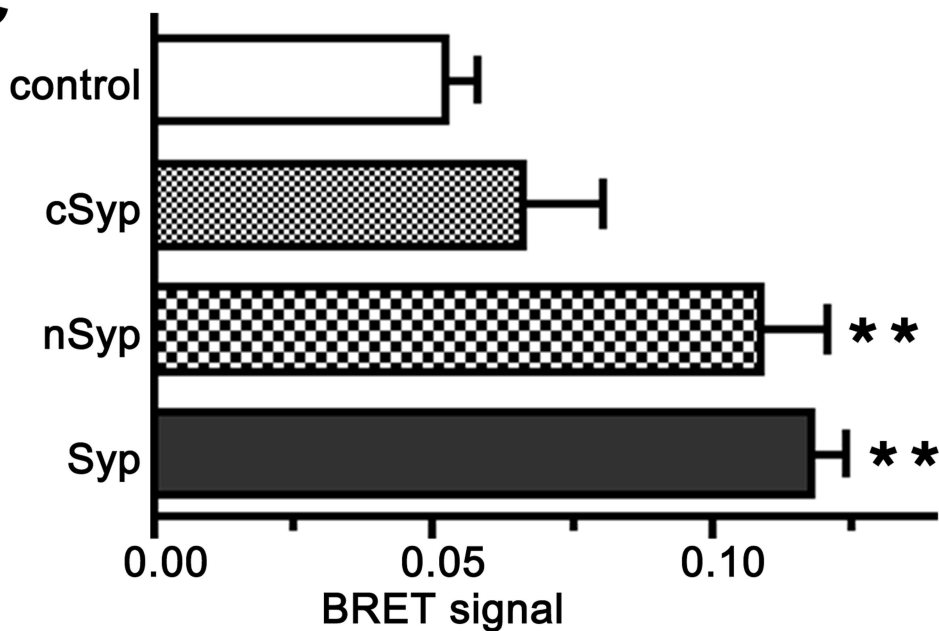


Fig. 2.

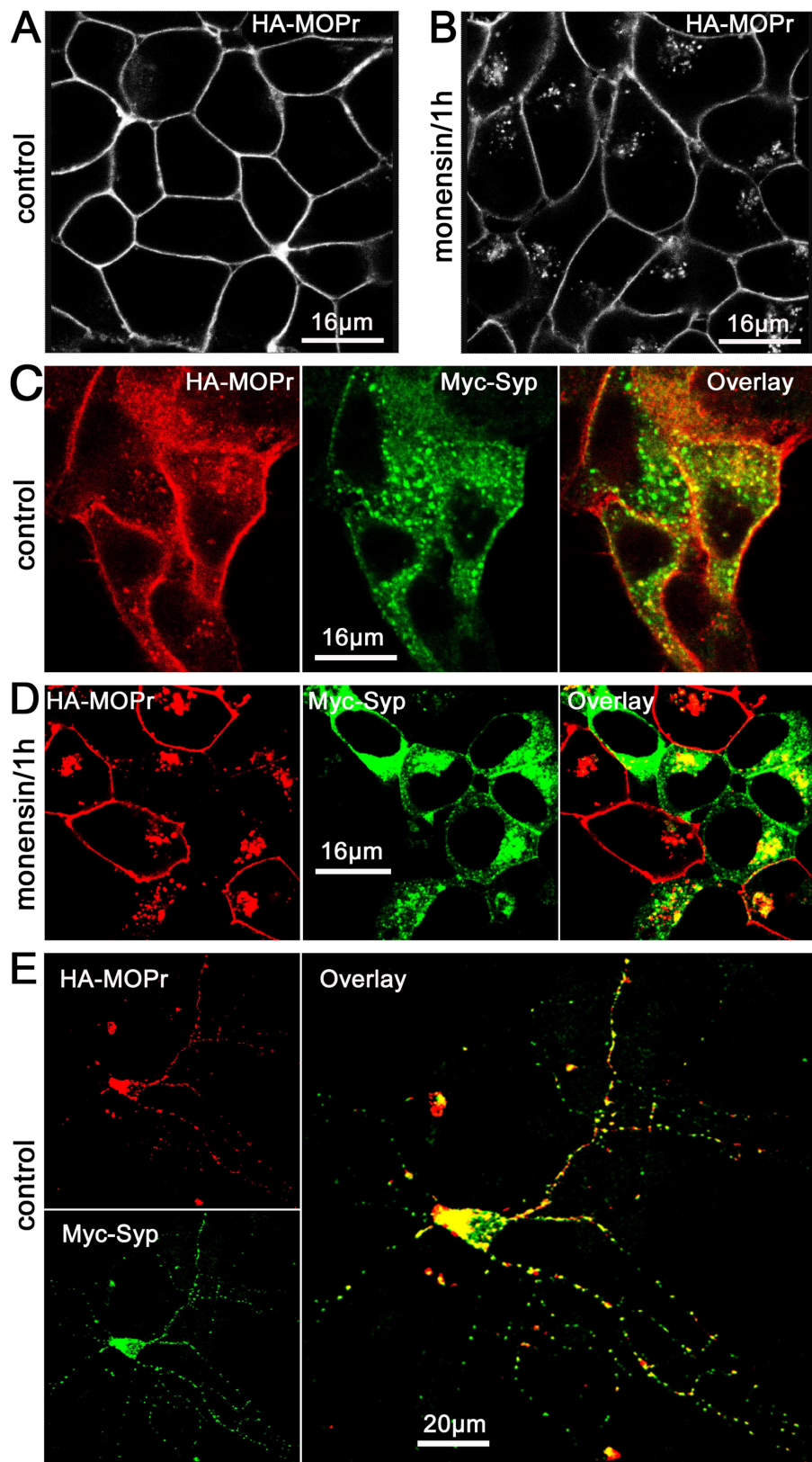
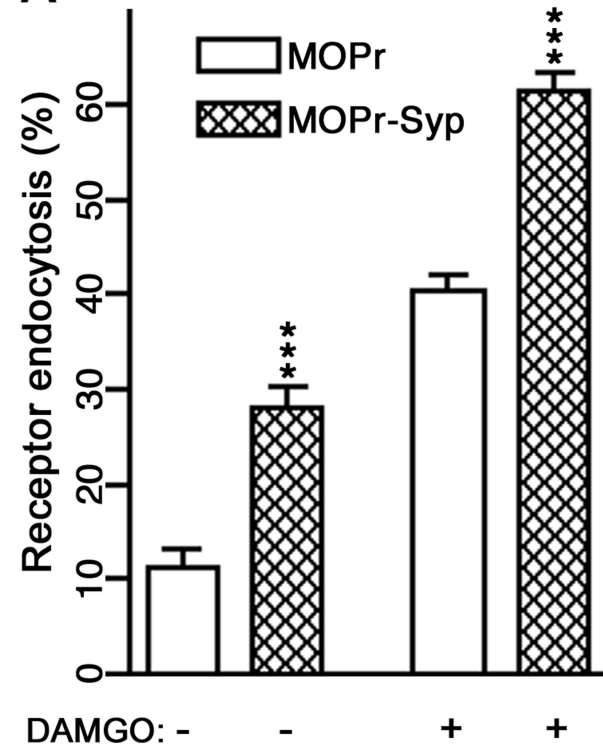


Fig. 3.

A



B

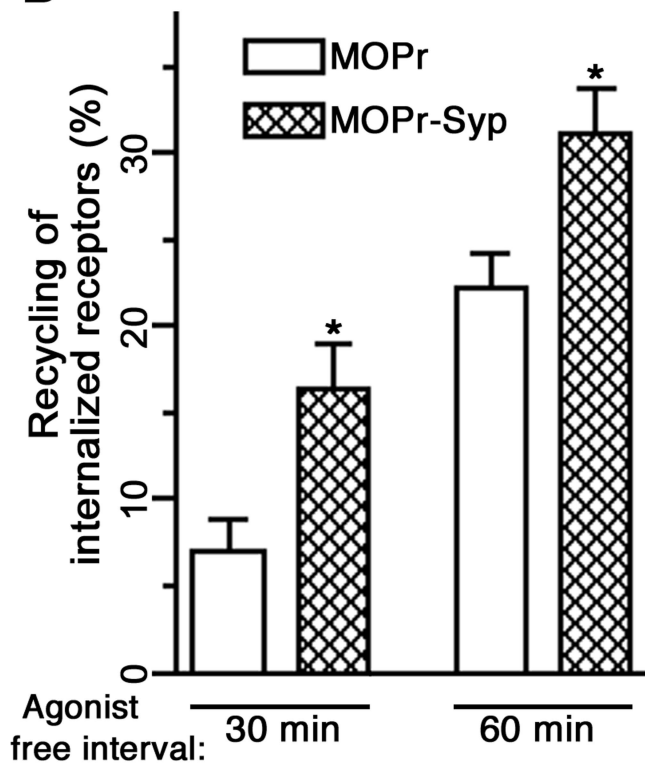


Fig. 4.

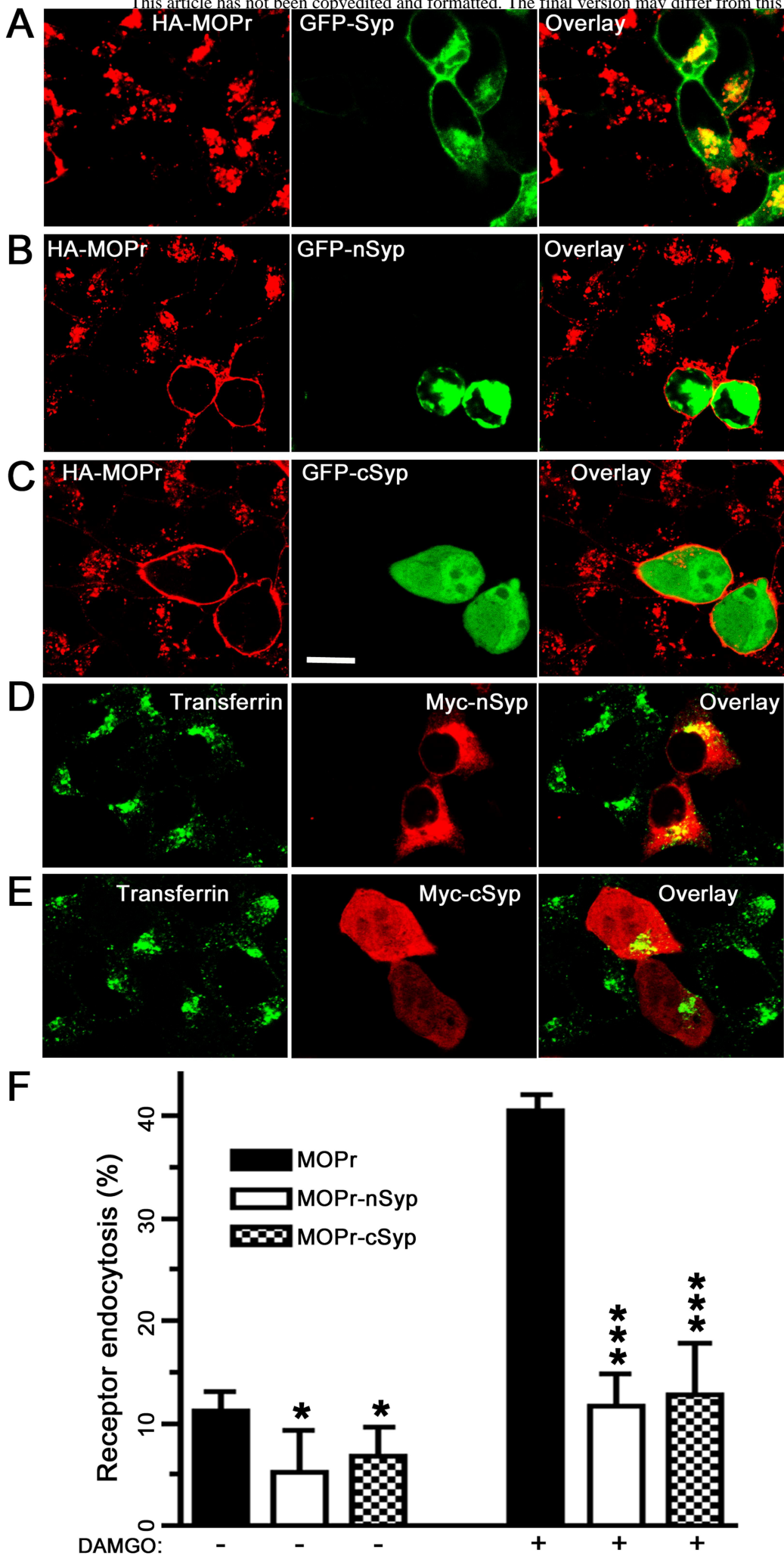


Fig. 5.

