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Distinct Effects of Individual Opioids on the Morphology of Spines Depend Upon the Internalization of Mu Opioid Receptors

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Abstract

This study has examined the relationship between the effects of opioids on the internalization of mu opioid receptors (MORs) and the morphology of dendritic spines. Several opioids (morphine, etorphine, DAMGO or methadone) were applied to cultured hippocampal neurons. Live imaging and biochemical techniques were used to examine the dynamic changes in MOR internalization and spine morphology. This study reveals that MOR internalization can regulate opioid-induced morphological changes in dendritic spines: **(1)** Chronic treatment with morphine, which induced minimal receptor internalization, caused collapse of dendritic spines. In contrast, “internalizing” opioids such as DAMGO and etorphine induced the emergence of new spines. It reveals that opioid-induced changes in spines vary greatly depending on how the applied opioid agonist affects MOR internalization. **(2)** The blockade of receptor internalization by dominant negative mutant of dynamin, K44E, reversed the effects of DAMGO and etorphine. It indicates that receptor internalization is necessary for the distinct effects of DAMGO and etorphine on spines. **(3)** In neurons that were cultured from MOR knock-out mice and had been co-transfected with DsRed and MOR-GFP, morphine caused collapse of spines whereas DAMGO induced emergence of new spines, indicating that opioids can alter the structure of spines via postsynaptic MORs. **(4)** Methadone at a low concentration induced minimal internalization and had effects that were similar to morphine. At a high concentration, methadone induced robust internalization and had effects that are opposite to morphine. The concentration-dependent opioid-induced changes in dendritic spines might also contribute to the variation in the effects of individual opioids.

Keywords

Opioid Receptor; Dendritic Spines; Receptor Internalization; Synaptic Plasticity; Addiction; Cognition Deficit

INTRODUCTION

Both *in vivo* and *in vitro* studies reveal that chronic treatment with morphine profoundly alters the function and morphology of dendritic spines (Robinson et al., 2002 and 2004; Liao et al., 2005). Plasticity of dendritic spines has been proposed to be the cellular basis for experience-dependent learning and memory (Malinow et al., 2000; Matsuzaki et al., 2004). Drug addiction

has been hypothesized to present a pathological form of learning and memory (Kelley, 2004; Nestler, 2002; Williams et al., 2001). AMPA receptors, particularly GluR1 subunits, are important for morphine tolerance and dependence (Vekovischeva et al., 2001; Stephens and Mead, 2003). Most excitatory glutamatergic synaptic transmission occurs in dendritic spines in adult brains (Harris and Kater, 1994; Nimchinsky et al., 2002). Therefore, the morphine-induced changes in dendritic spines might play some roles in the addiction of opioids. Many studies demonstrate that cancer patients that have been chronically treated with opioids often have deficits in learning and memory (Bodnar and Hadjimarkou, 2005; Skaer 2004). Synaptic plasticity in the hippocampus is widely believed to be important for learning and memory (Martin et al., 2000). The density of dendritic spines in the CA1 region of the hippocampus is decreased by 50% after chronic morphine exposure (Robinson et al., 2002). This profound loss of dendritic spines is likely to contribute to the opioid-induced cognition deficits.

Although morphine causes collapse of dendritic spines in hippocampal neurons (Robinson et al., 2002; Liao et al., 2005), the effects of other opioids on the morphology of these spines are not clear. Morphine induces little receptor internalization in most cell types, including cultured hippocampal neurons, whereas other opioids, such as DAMGO, etorphine and methadone cause obvious receptor internalization (Alvarez et al., 2002; Bailey et al., 2003; Minnis et al., 2003; Sternini et al., 1996; von Zastrow, 2001; Whistler and von Zastrow, 1998; Yu et al., 1997; Kovoor et al., 1998). This property of morphine has been proposed to be responsible for the continued signaling by morphine, causing downstream adaptations that mediate addiction and tolerance (Finn and Whistler, 2001; He et al., 2002; Whistler et al., 1999). If DAMGO, etorphine and methadone can induce the internalization of MORs, these drugs might interrupt MOR-mediated signal transduction by removing MORs from cell surface. Logically, the “internalizing” opioids might have effects on dendritic spines that are different from those of morphine. Therefore, we seek to delineate the relationship between the internalization of MORs and the plasticity of dendritic spines in this current study. Live imaging and biochemical data in this current study reveal that “internalizing” opioids such as etorphine, DAMGO and methadone can induce effects that are opposite to those of morphine. The blockade of receptor internalization by the over-expression of dominant negative mutant of dynamin, K44E, reverses the effects of DAMGO and etorphine. These results together indicate that receptor internalization can regulate opioid-induced changes in dendritic spines.

EXPERIMENTAL PROCEDURES

Neuronal cultures and transfection

A 25-mm glass coverslip (thickness, 0.08 mm) was glued to the bottom of a 35-mm culture dish with a 22-mm hole using silicone sealant as previously described (Lin et al., 2004). Dissociated neuronal cultures from rat hippocampus at postnatal day 1–2 were prepared as previously described (Lin et al., 2004; Liao et al., 2001). The same method was used to make dissociated neurons from mouse hippocampus. All rat and mouse studies were approved by University of Minnesota IACUC and were performed in accordance with institutional and federal guidelines. Neurons were plated onto prepared 35-mm culture dishes at a density of 1×10^6 cells per dish. The age of cultured neurons was counted from the day of plating, one day in vitro (DIV). To label dendrites, neurons 5–7 DIV were transfected with plasmids encoding appropriate molecules as described in the text. The culture dishes fit tightly in a homemade holding chamber on a fixed platform above an inverted microscope sitting on an X-Y translation stage (Burleigh, Inc). The location of any neuron of interest was determined by the reading of the X-Y translation stage. Neurons could be found again in the next observation using the X-Y translation stage (accuracy, 4 μ m). In live imaging experiments that lasted less than 2 hours, the holding chamber was heated to 35°C and was gassed with 5% CO₂ and 95% air. Time-lapse images of the same neurons were taken every 30 minutes.

Surface Staining of HA-tag MOR-GFP

Neurons were transfected with plasmids encoding MORs with an HA tag at the N-terminus and a GFP tag at the C-terminus. These neurons at 21 DIV were treated with morphine (10 μ M), DAMGO (1 μ M), etorphine (1 μ M), or methadone (1 or 10 μ M) at 37°C for 1 hour, 1 day or 3 days. The surface staining of the HA-tag has been previously described (Liao et al., 1999). A mouse monoclonal anti-HA antibody (Covance, Richmond, CA) was added to the culture media at 37°C for 30 minutes. After the antibody incubation, neurons were fixed with 4% paraformaldehyde without permeabilization, and finally a Rhodamine-linked secondary anti-mouse antibody was used to label the HA epitope. The same area of a neuron expressing HA-MOR-GFP was photographed at both green (left) and red fluorescence channels (middle, Figure 2A–F). The intensity of green fluorescence in the soma and individual dendrites was measured using the MetaMorph program (auto-threshold for bright objects). The intensity of red fluorescence in the same region was calculated, and the ratio of red versus green fluorescence was used to estimate the ratio of surface MOR/total MOR (note that this is a relative number and the actual number of receptors is unknown; Figure 2G).

Biotinylation

The biotinylation technique was used to estimate the internalization of both MOR-GFP (Figure 2H–I) and endogenous MORs (Figure 2J–K). In Figure 2H–I, primary neuronal cell cultures in 60 mm dishes were transiently transfected with MOR-GFP. After 14 days, cells were washed twice with ice-cold PBS, and cell surfaces were biotinylated by incubating with 300 μ g/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in PBS for 30 min at 4 °C. Un-reacted biotin was quenched and washed twice with ice-cold TBS (10 mM Tris, pH 7.4, 154 mM NaCl). Biotinylated cells were then incubated with serum free, pre-warmed DMEM medium (37°C) and treated with 1 μ M etorphine, morphine, or DAMGO (NIDA, Bethesda, MD) for 2 hours. After washing the drug, remaining cell-surface biotinylated receptors in designated plates were stripped in 50 mM glutathione, 0.3 M NaCl, 75 mM NaOH, and 1% fetal bovine serum at 4 °C for 30 min. Cells were then lysed in cell lysis buffer (0.2% Triton X-100, 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, complete protease inhibitor cocktail tablet (1 tablet/50 ml, Roche Applied Science)). Cellular debris was removed by centrifuging at $12,000 \times g$ for 10 min at 4°C. Clarified cell lysates were immunoprecipitated with immobilized streptavidin (Pierce, Rockford, IL) at 4°C overnight. After washing five times with cell lysis buffer, the washed beads were extracted with SDS sample buffer, and eluted proteins were fractionated by SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred to Immobilon™ PVDF Transfer membrane (Millipore, Bedford, MA) in transfer buffer (48 mM Tris-HCl, 39 mM Glycine, 20% methanol) and blocked for 60 min in 10% nonfat milk in 0.1% Tween-20 in TBS. Receptors were detected by sequential incubation with mouse anti-GFP (1:2000; Covance, Richmond, CA) and goat anti-mouse antibody conjugated to AP (1:5000; Bio-Rad, Hercules, CA) for 60 min each. Protein bands were detected by ECF substrate (Amersham, Piscataway, New Jersey) and membrane scanned with Storm 840 (Molecular Dynamics, Sunnyvale, CA). Band intensities were quantified and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

In Figure 2J–K, the internalization of endogenous MORs was examined by using a rabbit polyclonal antibody against the C-terminus of MORs (Arvidsson et al., 1995). In order to collect a large amount of proteins, neurons that were dissociated from the hippocampus of rats were plated on 100 mm dishes at the density of 8–12 million cells per dish. The surface proteins in cultured neurons at 21 DIV were biotinylated and the internalized MORs would be detected using the above anti-MOR antibodies. The ratio of internalized MORs (+)/total MORs (–) was quantified and was compared between untreated control and opioid-treated neurons (see the left solid bars in Figure 2K). To rule out potential non-specific changes induced by opioids, western-blot membranes that had been used to estimate this ratio were further stained with

streptavidin-HRP conjugates. As expected, multiple bands of proteins were detected by this staining because the streptavidin-HRP conjugates could bind to any biotinylated proteins. The amount of proteins in all visible bands in the same loading lane were added together to estimate the amount of biotinylated proteins in each lane. The ratios of internalized biotinylated proteins (+, with stripping)/total biotinylated proteins (–, with no stripping) were quantified in all experimental groups (see the right open bars in Figure 2K).

Image analysis

Time-lapse live images from the same neuron were taken before and at various time points after drug treatments as previously described (Liao et al., 2005). All digital images were analyzed with MetaMorph Imaging System (Universal Imaging Co.). Unless stated otherwise, all images of live neurons were taken as stacks and were averaged into one image before further analysis. In addition to simple averaging, stacks of images were also processed by deconvolution analyses using the MetaMorph software with the nearest planes. A stack of deconvoluted images was further averaged into one single image. A dendritic protrusion with an expanded head that was 50% wider than its neck was defined as a spine. The number of spines or non-spine protrusions from one neuron was manually counted and normalized per 100 μm dendritic length. One-way ANOVA was used for comparison between multiple groups of data and two-way ANOVA was used for comparison between parameters that were collected at different time points from the same neurons (n - number of neurons; $p < 0.05$, significant). If the ANOVA test indicated that there were significant changes, t-test was used to further test the significance. If a difference passed the ANOVA test and t-test ($p < 0.05$), we considered this change to be statistically significant. To measure the fluorescence intensity in dendrites and the soma, these regions were highlighted and selected in the MetaMorph program with the “autothreshold bright objects” function, and the averaged fluorescence intensity in each region was calculated. In order to highlight dendritic protrusions and spines, detection threshold was set at 75% of the fluorescent intensity in the center of the dendritic protrusion or spine to be measured. The spine was manually separated from the dendrite using the line tool, and the area and fluorescence intensity of highlighted dendritic spines were measured by the MetaMorph program. In neurons expressing both MOR-GFP and DsRed, all regions were first highlighted and selected in the DsRed image and then transferred to the MOR-GFP image for further analyses. Neurons were fixed and stained with antibodies as previously described (Liao et al., 1999). In neurons expressing HA-MOR-GFP that were stained with Anti-HA antibody (red), the HA-MOR-GFP images (green) were used to select the regions. All data are reported as Mean $\pm$ Standard Error. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

In order to examine the temporal dynamics of receptor internalization, variance of MOR-GFP aggregates was used as an index to reflect the degree of internalization. Images of neurons expressing MOR-GFP were taken before and at various times after treatment with various opioids (Figure 3A–F). A line with the width of 5 pixels was drawn in the middle of a dendrite and the fluorescence intensity of these 5 pixels was averaged into 1 single value for each 1 pixel-long forward step. The variance of all values along the line was calculated as a measurement to estimate the degree of aggregation in dendrites. The same line was used for all images collected before and at various time after the application of opioids, and all estimated variances were normalized by the variance before the application (Figure 3G–H).

RESULTS

DAMGO and etorphine cause effects on dendritic spines that are opposite to morphine

A live imaging system was used to examine how various opioids affect the morphology of dendritic spines (Liao et al., 2005). Images of GFP-labeled neurons were taken before and after treatment with known “non-internalizing” (morphine) and “internalizing” opioids (DAMGO

and etorphine). Chronic treatment with morphine caused collapse of dendritic spines (Figure 1A) and significantly decreased the density of dendritic protrusions and spines (Figure 1D–E; at 1 and 10 μM ; $n=8$ in each group). In contrast, DAMGO (1 μM) and etorphine (1 μM) induced the emergence of new spines (Figure 1B–C) and significantly increased the density of dendritic protrusions and spines (Figure 1D–E). These results led us to hypothesize that MOR internalization can modulate opioid-induced structural plasticity of dendritic spines.

DAMGO, etorphine and methadone induced robust internalization of MORs whereas morphine had little effect

To test our hypothesis, we have examined how various opioids affect the internalization of MORs in rat primary hippocampal cultures. Neurons expressed HA-MOR-GFP were treated with opioids for 1 hour, 1 day or 3 days (Figure 2A–F, also see Surface Staining of HA-tag MOR-GFP in Experimental Procedures for details), and the ratio of surface and total MORs were calculated (Figure 2G). MORs clustered on the surface of dendritic spines (Figure 2A), indicating that MORs are likely to play some functional roles in these spines. Morphine did not significantly reduce the surface MORs in comparison to untreated control (Figure 2G; $n=8$, in each group) within 1 hour as MORs were abundant on cell surface in both morphine-treated neurons and untreated control (arrows in Figure 2A–B). In contrast, DAMGO, etorphine and methadone all significantly decreased the amount of surface MORs (Figure 2C–G) within 1 hour. Interestingly, there were still visible amounts of surface MORs in neurons that were treated with 1 μM of methadone whereas most surface MORs disappeared in neurons that were treated with 10 μM of methadone (Figure 2E–G) within 1 hour. Chronic treatment with morphine for 1–3 days induced little MOR internalization whereas treatment with etorphine for 1–3 days induced robust internalization (Figure 2G). Interestingly, neurons with green fluorescence were rarely found after treatment with DAMGO for > 1 day, indicating that DAMGO induced the degradation of MORs (also see Figure 3 for DAMGO-induced degradation).

The biotinylation technique was used to further test whether individual opioids have different effects on the internalization of either exogenous MOR-GFP (Figure 2H–I) or endogenous MORs (Figure 2J–K). All surface proteins were biotinylated first and neurons were later on treated with morphine, etorphine, or DAMGO (1 μM each) for two hours. All biotinylated surface proteins that remained after drug treatments were stripped away by glutathione, therefore, the biotinylated receptors after stripping should reflect the amount of internalized MORs (Figure 2H–K, +). The un-stripped fraction should reflect the total surface MORs before drug treatment (Figure 2H–K, –). Morphine caused little internalization of MOR-GFP whereas etorphine and DAMGO caused robust internalization (Figure 2H–I). In order to detect endogenous MORs with high resolution in western blots (Figure 2J–K), high-density hippocampal neurons (8–12 million cells per dish) were plated onto large culture dishes (100 mm). A typical internalizing opioid, etorphine, and a typical non-internalizing opioid, morphine, were tested in the above high-density neuronal cultures. Figure 2J–K demonstrates that endogenous MORs existed in primary hippocampal cultures, and morphine induced little internalization of endogenous MORs whereas etorphine induced robust internalization. To test the specificity of these effects, biotinylated proteins were non-discriminately stained with streptavidin-HRP conjugates. As shown in Figure 2K (right open bars), the proportion of internalized surface proteins versus total surface proteins after 2 hour incubation with morphine (40%) or etorphine (42%) was not significantly different from that in the untreated control (36%; $n=4$ western-blot membranes). It indicates that the differential effects of opioids were not due to non-specific effects on all surface proteins (Figure 2K, right open bars).

Temporal dynamics of agonist-induced internalization

As shown above in Figure 2, MORs often forms aggregates in the soma and dendrites after agonist-induced internalization. This phenomenon provided a tool to examine the temporal dynamics of internalization in live neurons expressing MOR-GFP (Figure 3). Dendritic variance of green fluorescence was used to reflect the degree of receptor internalization (see last paragraph of Image Analysis in Experimental Procedures). In acute experiments (Figure 3A–C), neurons were put on a heated chamber (at 35°C, gassed with 95% air and 5% CO₂) that was mounted on a microscope. Both etorphine and DAMGO significantly increased MOR aggregation within 30 minutes to 1 hour after application (Figure 3B–C) whereas morphine caused no significant change (Figure 3A; n=8 in each group; quantification in Figure 3G). For chronic treatment with opioids (Figure 3D–F), the same neuron was photographed repeatedly and was put back in the tissue culture incubator (37°C, 95% air and 5% CO₂) after each observation (Liao et al., 2005). Etorphine significantly increased the variance after 1 hour and 1 day of treatment whereas morphine had no significant effect during the same period of incubation (n=8 for each group; Figure 3H). DAMGO also significantly increased the variance after 1 hour (n=8, Figure 3F and 3H). However, the variance was decreased back to the previous level after 1 day of DAMGO treatment, which is likely due to a significant decrease in the overall fluorescence of the dendrites (Figure 3I, $p<0.001$, n=8, paired t-test). This result indicates that DAMGO-internalized MORs are more likely to be degraded than etorphine-internalized MORs.

DAMGO and etorphine remove opioid receptors from dendritic spines whereas morphine continuously acts on opioid receptors existing in spines during chronic treatment

MORs cluster in dendritic spines, indicating that opioids might act locally on MOR clusters in dendritic spines (Figure 2). To test this possibility, 3 week old neurons expressing both DsRed and MOR-GFP were treated with morphine, DAMGO or etorphine. Images in both red and green fluorescence channels were taken before and at various times (1 hour and 1 day) after the treatment (Figure 4). As shown in our previous studies (Liao et al., 2005), it takes 1–3 days for morphine to have obvious effects on dendritic spines in neurons expressing GFP alone. In contrast, morphine caused collapse of dendritic spines within only 1 hour in DsRed and MOR-GFP double-transfected neurons and had a profound effect after 1 day (Figure 4A–C and 4H), showing that morphine has a strong effect on neurons over-expressing exogenous MORs. The density of dendritic protrusions and spines in neurons expressing GFP alone (n=40) is significantly higher than neurons expressing both DsRed and MOR-GFP (n=32), indicating that the constitutive activity of MOR-GFP or endogenous opioids acting through MOR-GFP can postsynaptically modulate dendritic spines (Figure 4I).

DAMGO and etorphine significantly increased the density of dendritic spines after 1 day of treatment, which is obviously opposite to morphine's effect (Figure 4H, n=8 in each group). Does this difference result from DAMGO's and etorphine's ability to cause the internalization of MORs, specifically, the loss of MORs in dendritic spines? Indeed, DAMGO and etorphine mobilized MORs from dendritic spines to dendritic shafts to form numerous aggregates in the middle of the dendrites within 1 hour, removing MORs from spines (images in Figure 4D–G; quantification in Figure 4J). Interestingly, MOR-GFP and DsRed shrunk together during morphine treatment (Figure 4A–C) and morphine treatment had no significant effect on the ratio of MORs versus DsRed (Figure 4J), indicating that continuous activation of MORs in dendritic spines during morphine treatment caused the collapse of spines.

The blockade of receptor internalization by dominant negative dynamin reverses the effect of DAMGO and etorphine

It has been previously reported that dynamin-dependent cellular mechanisms are necessary for agonist-induced internalization of MORs (Chu et al., 1997). In this study, we have tested

whether dynamin-dependent mechanisms are involved in the internalization of MORs and subsequent MOR-mediated changes in dendritic spines in our culture system. Dominant negative dynamin (the K44E mutant, referred to as Dyn-) was co-transfected with either GFP or MOR-GFP to block the internalization of MORs (Figure 5). If the effects of DAMGO and etorphine are mainly due to agonist-induced receptor internalization, the inhibition of internalization should reverse their effects. Treatments with DAMGO and etorphine caused a significant loss of dendritic protrusions and spines (Figure 5A and 5C) and significantly decreased the density of protrusions and spines after 3 days (Figure 5F–G, $n=10$ in each group) in neurons expressing both GFP and Dyn-. Obviously, these effects were opposite to neurons that contained no Dyn- (see Figure 1 for comparison). Experiments using neurons expressing MOR-GFP and Dyn- revealed that DAMGO- and etorphine-induced internalization of MORs was indeed greatly diminished (Figure 5D–E). DAMGO and etorphine caused significantly less aggregations of MOR-GFP in neurons expressing both MOR-GFP and Dyn- than control neurons expressing only MOR-GFP ($n=10$ in each group, Figure 5H). The reversal of DAMGO's and etorphine's effects by the blockade of receptor internalization further supports that continuous activation of MORs on the neuronal surface causes collapse of dendritic spines. To test whether the suppression of dynamin activity itself could alter dendritic spines, the density of spines was compared between neurons expressed GFP alone and neurons expressed both GFP and Dyn- (Figure 5J). There was no significant difference between these two groups of neurons ($n=8$ in each group; Figure 5J), suggesting that redundant signaling molecules could have compensated for the lack of dynamin activity in other MOR-independent signaling pathways.

Morphine-induced collapse of dendritic spines is due to direct activation of MORs in postsynaptic neurons

To test whether opioid-induced structural plasticity of dendritic spines is mediated via postsynaptic activation of MORs, neurons were cultured from MOR knock-out mice (MOR^{-/-}) and co-transfected with DsRed and MOR-GFP (Figure 6A–B). DsRed (a soluble protein with red fluorescence) was used to label the morphology of spines and MOR-GFP was used to rescue the deficits of MOR^{-/-} neurons. Morphine (10 μ M) caused collapse of spines (Figure 6A) and significantly decreased the density of dendritic protrusions and spines after 1 hour and 1 day of treatment (Figure 6C; $n=10$ in each group). In contrast, DAMGO induced the emergence of new spines (Figure 6B) and significantly increased the density of dendritic protrusions and spines after 1 day of treatment (Figure 6C). Morphine caused no obvious aggregation of MOR-GFP (Figure 6A, lower panel) while DAMGO induced the formation of many MOR aggregates in dendrites (Figure 6B, lower panel). DAMGO caused loss of MORs in spines after 1 hour and 1 day of treatment (Figure 6D). In our previous studies, morphine does not cause collapse of dendritic spines in neurons cultured from MOR knock-out mice (Liao et al., 2005), and thus the morphine-induced collapse of spines in Figure 6 is likely to be mediated via the exogenous MOR-GFP molecules. Neither endogenous MORs nor MOR-GFP should exist in untransfected cells, including either pre-synaptic neurons or nearby glial cells. Therefore, these results demonstrate that opioids can directly act on postsynaptic neurons, causing collapse of dendritic spines.

The concentration-dependent effect of methadone on dendritic spines is related to its ability to induce the internalization of MORs

As previously shown in Figure 2E–G, methadone-induced internalization of MORs is concentration-dependent as there was a fair amount of surface MORs present on the cell surface after 1 hour exposure of 1 μ M methadone, whereas most surface MORs disappeared after 1 hour exposure of 10 μ M methadone. To further test our hypothesis that MOR internalization regulates opioid-induced changes of dendritic spines, these two concentrations of methadone were applied to GFP-labeled neurons (Figure 7A–B) and neurons expressing MOR-GFP

(Figure 7C–D). The 1 μ M concentration of methadone caused an effect that was similar to morphine, significantly decreasing the density of dendritic protrusions and spines after 3 days ($n=8$, Figure 7A and 7E) whereas the 10 μ M concentration had an opposite effect, increasing the density of protrusions and spines ($n=8$, Figure 7B and 7E). Interestingly, both concentrations significantly caused MORs to aggregate in dendrites after 1 hour exposure (Figure 7C–D, middle panels; Figure 7G; $n=8$ in each group). Nevertheless, aggregates still persisted in neurons after 1 day of exposure to 10 μ M methadone, whereas most of the aggregates had disappeared after 1 day in neurons treated with 1 μ M of methadone (Figure 7C–D, right panels). Methadone did not significantly change fluorescence intensity after 1 day (Figure 7F, $n=8$ in each group), suggesting that the internalized MORs were not degraded. Therefore, most of the internalized and aggregated MORs might have been re-inserted into the cell surface in the neurons that had been treated with 1 μ M methadone. These results further support our hypothesis that the opioid-induced collapse of dendritic spines requires the continuous presence of MORs in the cell surface.

DISCUSSION

MOR internalization and Variation in the Effects of Opioids

This study demonstrates that the effects of “internalizing” opioids such as DAMGO and etorphine on the density of dendritic spines are opposite to that of morphine. The internalization of opioid receptors has initially been proposed to mediate desensitization and tolerance of opiate addiction (von Zastrow et al., 2003; Dang and Williams 2004 and 2005; Kieffer and Evans 2002). However, morphine causes little receptor internalization in most cell types but can strongly lead to drug addiction and tolerance (Alvarez et al., 2002; Bailey et al., 2003; Minnis et al., 2003; Sternini et al., 1996; von Zastrow, 2001; Whistler and von Zastrow, 1998; Yu et al., 1997; Kovoov et al., 1998). To resolve this “morphine paradox”, a “rave” hypothesis has subsequently been proposed. In this hypothesis, morphine’s inability to induce receptor internalization contributes to the prolonged activation of MORs, which mediates the addictive liability and tolerance development (He et al., 2002; Whistler et al., 1999). If this is the case, then there should be a correlation between the agonist’s ability to induce receptor internalization and to modulate dendritic spines. In this study, morphine induced little internalization of MORs, and caused collapse of dendritic spines. Morphine-induced collapse of dendritic spines requires the presence of either endogenous MORs or exogenous MORs, indicating that morphine can directly act on postsynaptic neurons to induce plasticity of dendritic spines. In contrast, DAMGO and etorphine induced the emergence of new spines, an effect that was obviously opposite to morphine. Furthermore, the effects of DAMGO and etorphine are shown to result from the ability of these agonists to induce MOR endocytosis, since these effects were reversed when receptor internalization was blocked by the expression of dominant negative dynamin. These results indicate that DAMGO and etorphine can also cause loss of dendritic spines if MORs are continuously present in the spines. This study demonstrates that the internalization of MORs might contribute to the variation in the effects of individual opioids on dendritic spines.

Function of postsynaptic modulation of dendritic spines: Cognition or Addiction?

This study reveals a post-synaptic mechanism that can mediate opioid-induced morphological changes in dendritic spines: (1) MORs were clustered and concentrated on the surface of dendritic spines (Figure 2A). As previously reported, molecules that cluster in dendritic spines often play either an essential or a regulatory role in these spines (Shirao and Sekino, 2001; Hering and Sheng, 2001). (2) Postsynaptic over-expression of dominant negative dynamin, which blocks endocytosis, reverses the effects of DAMGO and etorphine (Figure 5). (3) Morphine does not cause the collapse of spines in neurons that were cultured from MOR knockout mice (Liao et al., 2005). However, when these neurons were transfected with MOR-

GFP, the postsynaptically introduced MOR-GFP can rescue the deficit in these neurons, allowing morphine to cause collapse of spines (Figure 6). (4) DAMGO and etorphine postsynaptically remove MORs from dendritic spines whereas morphine does not remove MORs from dendritic spines (Figure 4). Interestingly, only morphine can cause collapse of spines in neurons expressing both DsRed and MOR-GFP. It suggests that morphine can continuously act on postsynaptic MORs in spines, leading to the eventual collapse of these spines. The postsynaptic signaling pathway that mediates opioid-induced changes in spine morphology is unknown. It has recently been reported that Rho GTPases including Rac1, Cdc42 and Rho A are key regulators of spine morphology and spinogenesis (Nakayama et al., 2000; Luo 2000; Wiens et al., 2005; Tashiro and Yuste 2004). It is possible that MORs might regulate the stability of dendritic spines via these Rho GTPases.

Many previous studies demonstrated that morphine could acutely inhibit pre-synaptic release of GABA (the “disinhibition” theory; Wimpey and Chavkin, 1991; Madison and Nicoll, 1988; Williams et al., 2001). Consistent with these previous pre-synaptic findings, our recent experiments also revealed that the MORs were often concentrated in axon-like process in “non-spiny” neurons, which are likely to be GABAergic neurons (Liao et al., 2007). In contrast, MORs were clustered on the surface of dendritic spines in “spiny” neurons, which were probably glutamatergic neurons (Figure 2A). Therefore, it is not surprising that different research groups might obtain either pre- or post-synaptic effects if different cell types were examined.

What is the functional role of the opioid-induced postsynaptic changes in dendritic spines? Addiction has traditionally been proposed to be mediated through the dopaminergic reward pathway, which includes the prefrontal cortex, nucleus accumbens and ventral tegmental area (Rang et al., 1995). One unsolved puzzle in opiate addiction is that the hippocampus contains a very high level of MOR proteins (Arvidsson, et al., 1995) and MOR mRNAs (see Figure 1 in Stumm et al, 2005). Self-administered morphine, which often maintains high blood concentrations of morphine for a long time, strongly decreases the density of dendritic spines in the hippocampus in adult rats (Robinson et al., 2002). More and more evidence supports the involvement of the hippocampus in drug addiction (Biala et al., 2005; Vorel et al., 2001; Fan et al., 1999; Kelley 2004). The notion of distinct “addiction circuits” and “learning and memory circuits” has recently been replaced with a much more integrative view of many brain regions including the hippocampus (Nestler 2002). Based on these previous studies, we believe that opioid-induced changes in dendritic spines are likely to play some roles in opiate addiction. However, further *in vivo* experiments are clearly needed to prove the link between opiate addiction and spine plasticity.

Many previous studies reveal that cancer patients who have chronically received opioid analgesics often manifest severe deficits in cognition (Bodnar and Hadjimarkou, 2005; Skaer 2004). Synaptic plasticity in the hippocampus is known to be critical for learning and memory (Martin et al., 2000). Therefore, the profound decrease in the density of dendritic spines in the CA1 region of the hippocampus after chronic morphine treatment (by ~50% in Robinson et al., 2002) is very likely to impair learning and memory, causing cognition deficits.

Concentration-Dependent Effects of Methadone on Dendritic Spines

Another puzzle in opioid pharmacology is “methadone paradox”. Morphine, oxycodone, methadone and fentanyl are among the most commonly prescribed opioids for pain management (Hanks and Reid, 2005). Individual opioids may manifest variability in clinical responses and tolerance (Hanks and Reid, 2005). It is clinically well known that methadone and fentanyl, which can also induce receptor internalization, are highly addictive opiate agonists (Kreek 2000; Compton and Volkow, 2005). Our results provide a probable explanation for the “methadone paradox”, as methadone-induced plasticity of dendritic spines is

bidirectional and concentration-dependent (Figure 7). Methadone induced the collapse of dendritic spines at a low concentration (1 μ M) and this effect was lost only when the concentration of methadone reached as high as 10 μ M. Perhaps, methadone might not reach such a high concentration in some clinical usages, and methadone at lower concentrations leads to opioid-induced plasticity of spines, causing opiate addiction and tolerance. Tail flick experiments in mice indicated that some clinical opioid drugs, such as fentanyl, produced less tolerance than morphine (Duttaroy and Yoburn, 1995). Because methadone and fentanyl can clearly be addictive in clinical usages, it is still controversial whether an “internalizing” opioid is less addictive than a “non-internalizing” opioid (Kreek 2000; Compton and Volkow, 2005). It would be interesting to examine the effects of other clinical opioid drugs such as oxycodone and fentanyl on dendritic spines at different concentrations. If these drugs can also cause concentration-dependent bidirectional effects on dendritic spines, and then the concentration-dependent agonist-induced MOR internalization might provide a probable explanation for the variability in clinical responses and addictive liabilities of individual opioids.

Conclusions

This study has provided a mechanistic link between two key cellular alterations after opiate exposure, the internalization of opioid receptors and the plasticity of dendritic spines. This study demonstrates that non-internalizing opioids such as morphine induce collapse of spines whereas internalizing opioids such as DAMGO and etorphine have the opposite effect. Importantly, the linkage of these two cellular events is dependent on agonist concentrations as shown in our methadone experiments. In this respect, certain concentrations of an opiate drug might cause variation in clinical responses and drug addiction by preventing MOR-mediated collapse of spines via receptor internalization.

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References

- Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, von Zastrow M, Grandy DK, Williams JT. μ -Opioid receptors: ligand-dependent activation of potassium conductance, desensitization, and internalization. *J Neurosci* 2002;22:5769–5776. [PubMed: 12097530]
- Arvidsson U, Riedl M, Chakrabarti S, Lee JH, Nakano AH, Dado RJ, Loh HH, Law PY, Wessendorf MW, Elde R. Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. *J Neurosci* 1995;15:3328–3341. [PubMed: 7751913]
- Bailey CP, Couch D, Johnson E, Griffiths K, Kelly E, Henderson G. Mu-opioid receptor desensitization in mature rat neurons: lack of interaction between DAMGO and morphine. *J Neurosci* 2003;23:10515–10520. [PubMed: 14627635]
- Bodnar, Hadjimarkou. Endogenous opiates and behavior: 2004. *Peptides* 2005;26:2629–2711. [PubMed: 16039752]
- Celver J, Xu M, Jin W, Lowe J, Chavkin C. Distinct domains of the mu-opioid receptor control uncoupling and internalization. *Mol Pharmacol* 2004;65:528–537. [PubMed: 14978231]
- Chu P, Murray S, Lissin D, von Zastrow M. Delta and kappa opioid receptors are differentially regulated by dynamin-dependent endocytosis when activated by the same alkaloid agonist. *J Biol Chem* 1997;272:27124–27130. [PubMed: 9341153]
- Compton WM, Volkow ND. Major increases in opioid analgesic abuse in the United States: concerns and strategies. *Drug Alcohol Depend* 2005;81:103–107. [PubMed: 16023304]

- Dang VC, Williams JT. Chronic morphine treatment reduces recovery from opioid desensitization. *J Neurosci* 2004;24:7699–7706. [PubMed: 15342737]
- Dang VC, Williams JT. Morphine-induced μ -opioid receptor desensitization. *Mol Pharmacol* 2005;68:1127–1132. [PubMed: 16020743]
- Fan GH, Wang LZ, Qiu HC, Ma L, Pei G. Inhibition of calcium/calmodulin-dependent protein kinase II in rat hippocampus attenuates morphine tolerance and dependence. *Mol Pharmacol* 1999;56:39–45. [PubMed: 10385682]
- Finn AK, Whistler JL. Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* 2001;32:829–839. [PubMed: 11738029]
- Haberstock-Debic H, Kim KA, Yu YJ, von Zastrow M. Morphine promotes rapid, arrestin-dependent endocytosis of μ -opioid receptors in striatal neurons. *J Neurosci* 2005;25:7847–7857. [PubMed: 16120787]
- Haberstock-Debic H, Wein M, Barrot M, Colago EE, Rahman Z, Neve RL, Pickel VM, Nestler EJ, von Zastrow M, Svingos AL. Morphine acutely regulates opioid receptor trafficking selectively in dendrites of nucleus accumbens neurons. *J Neurosci* 2003;23:4324–4332. [PubMed: 12764121]
- Hanks GW, Reid C. Contribution to variability in response to opioids. *Support Care Cancer* 2005;13:145–152. [PubMed: 15761703]
- Harris KM, Kater SB. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Annu Rev Neurosci* 1994;17:341–371. [PubMed: 8210179]
- He L, Fong J, von Zastrow M, Whistler JL. Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* 2002;108:271–282. [PubMed: 11832216]
- Hering H, Sheng M. Dendritic spines: structure, dynamics and regulation. *Nat Rev Neurosci* 2001;2:880–888. [PubMed: 11733795]
- Kelley AE. Memory and Addiction: Shared Neural Circuitry and Molecular Mechanisms. *Neuron* 2004;44:161–179. [PubMed: 15450168]
- Kieffer BL, Evans CJ. Opioid tolerance-in search of the holy grail. *Cell* 2002;108:587–590. [PubMed: 11893329]
- Kovoor A, Celver JP, Wu A, Chavkin C. Agonist induces homologous desensitization of mu-opioid receptors mediated by G protein-coupled receptor kinases is dependent on agonist efficacy. *Mol Pharmacol* 1998;54:704–711. [PubMed: 9765514]
- Kreek MJ. Methadone-related opioid agonist pharmacotherapy for heroin addiction. History, recent molecular and neurochemical research and future in mainstream medicine. *Ann NY Acad Sci* 2000;909:186–216. [PubMed: 10911931]
- Liao D, Zhang X, O'Brien R, Ehlers MD, Huganir RL. Regulation of morphological postsynaptic silent synapses in developing hippocampal neurons. *Nat Neurosci* 1999;2:37–43. [PubMed: 10195178]
- Liao D, Scannevin RH, Huganir R. Activation of silent synapses by rapid activity-dependent synaptic recruitment of AMPA receptors. *J Neurosci* 2001;21:6008–6017. [PubMed: 11487624]
- Liao D, Lin H, Law PY, Loh HH. Mu-opioid receptors modulate the stability of dendritic spines. *Proc Natl Acad Sci USA* 2005;102:1725–1730. [PubMed: 15659552]
- Liao D, Grigoriants OO, Loh HH, Law PY. Agonist-dependent postsynaptic effects of opioids on miniature excitatory postsynaptic currents in cultured hippocampal neurons. *J Neurophysiol* 2007;97:1485–1494. [PubMed: 17122315]
- Lin H, Huganir R, Liao D. Temporal dynamics of NMDA receptor-induced changes in spine morphology and AMPA receptor recruitment to spines. *Biochem Biophys Res Commun* 2004;316:501–511. [PubMed: 15020245]
- Luo L. Rho GTPases in neuronal morphogenesis. *Nature Reviews Neuroscience* 2000;1:73–180.
- Madison DV, Nicoll RA. Enkephalin hyperpolarizes interneurons in the rat hippocampus. *J Physiol (Lond)* 1988;398:123–130. [PubMed: 3392667]
- Malinow R, Mainen ZF, Hayashi Y. LTP mechanisms: from silence to four-lane traffic. *Curr Opin Neurobiol* 2000;10:352–357. [PubMed: 10851179]
- Martin SJ, Grimwood PD, Morris RGM. Synaptic plasticity and memory: An evaluation of the hypothesis. *Annu Rev Neurosci* 2000;23:649–711. [PubMed: 10845078]

- Matsuzaki M, Honkura N, Ellis-Davies GC, Kasai H. Structural basis of long-term potentiation in single dendritic spines. *Nature* 2004;429:761–766. [PubMed: 15190253]
- Minnis JG, Patierno S, Kohlmeier SE, Brecha NC, Tonini M, Sternini C. Ligand-induced mu opioid receptor endocytosis and recycling in enteric neurons. *Neuroscience* 2003;119:33–42. [PubMed: 12763066]
- Nakayama AY, Harms MB, Luo L. Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *J Neurosci* 2000;20:5329–5338. [PubMed: 10884317]
- Nestler EJ. Common molecular and cellular substrates of addiction and memory. *Neurobiol Learning Memory* 2002;78:637–647.
- Nimchinsky EA, Sabatini BL, Svoboda K. Structure and function of dendritic spines. *Annu Rev Physiol* 2002;64:313–353. [PubMed: 11826272]
- Rang, HP.; Dale, MM.; Ritter, JM.; Gardner, P. *Pharmacology*. 3. Chapter 24. Churchill Livingstone Inc.; 1995. p. 495-499.
- Robinson TE, Gorny G, Savage VR, Kolb B. Widespread but regionally specific effects of experimenter- versus self-administered morphine on dendritic spines in the nucleus accumbens, hippocampus, and neocortex of adult rats. *Synapse* 2002;46:271–279. [PubMed: 12373743]
- Robinson TE, Kolb B. Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology* 2004;47(Suppl 1):33–46. [PubMed: 15464124]
- Skaer TL. Practice guidelines for transdermal opioids in malignant pain. *Drugs* 2004;64:2629–2638. [PubMed: 15537367]
- Shirao T, Sekino Y. Clustering and anchoring mechanisms of molecular constituents of postsynaptic scaffolds in dendritic spines. *Neurosci Res* 2001;40:1–7. [PubMed: 11311400]
- Stephens DN, Mead AN. What role do GluR1 subunits play in drug abuse? *Trends Neurosci* 2003;26:181–182. [PubMed: 12689767]
- Sternini C, Spann M, Anton B, Keith DE Jr, Bunnett NW, von Zastrow M, Evans C, Brecha NC. Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. *Proc Natl Acad Sci USA* 1996;93:9241–9246. [PubMed: 8799185]
- Stumm R, Ruthrich H, Schulz S, Zhou C, Holtt V. Expression of the mu-opioid receptor is induced in dentate gyrus granule cells after focal cerebrocortical ischaemia and stimulation of entorhinal afferents. *Eur J Neurosci* 2005;22:1032–1044. [PubMed: 16176345]
- Tashiro A, Yuste R. Regulation of dendritic spine motility and stability by Rac1 and Rho kinase: evidence for two forms of spine motility. *Mol Cell Neurosci* 2004;26:429–440.
- Vekovischeva OY, Zamanillo D, Echenko O, Seppala T, Uusi-Oukari M, Honkanen A, Seeburg PH, Sprengel R, Korpi ER. Morphine-induced dependence and sensitization are altered in mice deficient in AMPA-type glutamate receptor-A subunits. *J Neurosci* 2001;21:4451–4459. [PubMed: 11404432]
- von Zastrow M. Endocytosis and downregulation of G protein-coupled receptors. *Parkinsonism Relat Disord* 2001;7:265–271. [PubMed: 11331198]
- von Zastrow M, Svingos A, Habersack-Debic H, Evans C. Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. *Curr Opin Neurobiol* 2003;13:348–353. [PubMed: 12850220]
- Vorel SR, Liu X, Hayes RJ, Spector JA, Gardner EL. Relapse to cocaine-seeking after hippocampal theta burst stimulation. *Science* 2001;292:1175–1178. [PubMed: 11349151]
- Whister JL, von Zastrow M. Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc Natl Acad Sci USA* 1998;95:9914–9919. [PubMed: 9707575]
- Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* 1999;23:737–746. [PubMed: 10482240]
- Wiens KM, Lin H, Liao D. Rac1 induces the clustering of AMPA receptors during spinogenesis. *J Neurosci* 2005;25:10627–10635. [PubMed: 16291935]
- Williams JT, Christie MJ, Manzoni O. Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev* 2001;81:299–343. [PubMed: 11152760]

- Wimpey TL, Chaykin C. Opioids activate both an inward rectifier and a novel voltage-gated potassium conductance in the hippocampal formation. *Neuron* 1991;6:281–289. [PubMed: 1993123]
- Yu Y, Zhang L, Yin X, Sun H, Uhl GR, Wang JB. Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. *J Biol Chem* 1997;272:28869–28874. [PubMed: 9360954]

Figure 1. The effects of DAMGO and etorphine on dendritic spines are distinctly different from morphine

A. Neurons expressing GFP alone were photographed before and after chronic treatment with morphine (1 μ M). *Arrows* denote that spines gradually shrunk and eventually collapsed after 3 days of treatment. **B–C.** Neurons expressing GFP were photographed before and after treatment with DAMGO (1 μ M; B) or Etorphine (1 μ M; C). *Arrows* denote gradual emergence of new dendritic spines. **D–E.** The density of dendritic protrusions and spines in 5 groups of neurons was estimated before (open), 1 day (gray) and 3 days (black) after drug treatment.

Figure 2. The effects of individual opioids on receptor internalization are both agonist dependent and concentration dependent in cultured rat hippocampal neurons

A. An untreated neuron expressing HA-MOR-GFP (green, *left*) was stained with anti-HA antibodies (red, *middle*). *Right*, overlay. *Arrows* denote MORs clustered on the surface of dendritic spines. **B–F.** Similar neurons treated with morphine, DAMGO, etorphine and methadone for 1 hour. *Arrows* denote clustered MORs. *Triangles* denote MOR aggregates in dendrites after internalization. **G.** Neurons were treated with various opioids for 1 hour (left 6 bars), 1 day (middle 3 bars) or 3 days (right 3 bars) and were stained with a mouse anti-HA antibody. The percentage of surface (detected by anti-HA antibody) and total MORs (as GFP fluorescence) is plotted in G. Open bar, untreated control (ctl); black bar, morphine (Mor);

gray bar, DAMGO (DA); slashed bar, etorphine (Et); vertically lined bar, 1 μ M methadone (Meth); horizontally lined bar, 10 μ M methadone (Meth). *Note* that MOR-GFP molecules were often degraded after treatment with DAMGO with 1 day, and thus DAMGO-induced MOR internalization cannot be examined after this prolonged treatment. **H.** Neurons were transfected with MOR-GFP at 7 DIV and neurons at 14 DIV were treated with morphine (Mor), etorphine (Et) or DAMGO (DA) for 2 hours. The internalization of the exogenous GFP-labeled MORs was examined using an anti-GFP antibody with the biotinylation technique (see Experimental Procedures). (–) denotes total biotinylated MORs with no stripping. (+) denotes internalized MORs as MORs remain in the surface after drug treatment were removed by glutathione stripping. **I.** The ratio of internalized MORs (+)/total MORs (–) from H was quantified. **J.** The internalization of endogenous MORs in neurons at 21 DIV was examined using a rabbit polyclonal antibody against the C-terminus of MORs (Arvidsson et al., 1995) with the biotinylation technique. **K.** The ratio of internalized MORs (+)/total MORs (–) from J was quantified as the left solid bars. As additional control, streptavidin-HRP conjugates were used to non-discriminately detect surface biotinylated proteins (see Experiment Procedures for details) and the ratio of internalized surface proteins (+)/total surface proteins (–) was quantified (the right open bars).

Figure 3. Temporal dynamics of opioid-induced internalization of MOR-GFP in cultured rat hippocampal neurons

A–C. Acute time-lapse images (0–1 hour) were taken in neurons expressing MOR-GFP in a heated recording chamber before and after treatment with morphine (A), etorphine (B) and DAMGO (C). **D–F.** Chronic time-lapse images were taken on neurons expressing MOR-GFP before, 1 hour and 1 day after treatment with morphine (D), etorphine (E) and DAMGO (F). **G.** Variance was used to estimate the aggregation of MOR-GFP molecules in dendrites before (open bars), 0.5 hour (gray bars) and 1 hour (black bars) after treatment with morphine, etorphine and DAMGO. The variance was used to measure receptor internalization by assuming that the formation of aggregates reflects the internalization of MORs (see Haberstock-Debic et al., 2003, 2005). **H.** Changes in variance before (open bars), 1 hour (gray bars) and 1 day (black bars) after treatment with morphine, etorphine and DAMGO. **I.** DAMGO significantly decreased the fluorescence intensity after 1 day, indicating degradation of MORs.

The fluorescence intensity in the same dendrites before treatment was used to normalize the changes after 1 day of drug treatment.

Figure 4. Agonist-induced receptor internalization removed MORs from spines and altered opioid-induced plasticity of dendritic spines in cultured rat hippocampal neurons

A–B. Images of a neuron expressing MOR-GFP (A) and DsRed (B) were taken before, 1 hour and 1 day after treatment with morphine. *Arrows* denote that MOR-GFP and DsRed moved together when the spines collapsed. **C.** Overlay images of A and B. **D–F.** Images similar to A, B and C were taken in a DAMGO treated neuron. *Arrows* denote that MOR-GFP was removed from spines even when DsRed-labeled spines still existed after treatment with DAMGO. *Triangles* denote aggregates of MOR-GFP. **G.** Overlay of DsRed and MOR-GFP images in an etorphine-treated neuron.

H. The density of dendritic spines was calculated in neurons double-transfected with DsRed and MOR-GFP before (b), 1 hour (1h) and 1 day (1d) after treatment with morphine, DAMGO and etorphine. The density was calculated in the DsRed images and untreated neurons were used as control. **I.** All neurons expressing GFP alone (open

bars) and neurons expressing both DsRed and MOR-GFP (black bars) with no treatment with opioids were pooled together. The over-expression of MOR-GFP decreased the density of dendritic protrusions and spines. **J.** DAMGO and etorphine caused loss of MORs in dendritic spines. The amount of MORs in each spine was estimated by the formula in the *right inset*. In this formula, the amount of MORs in a spine was normalized by the MORs in the adjacent dendrite. *MORspine*, green fluorescence intensity in the spine; *DsRedspine*, red fluorescence intensity in the spine; *MORdendrite*, green fluorescence in the adjacent dendrite; *DsReddendrite*, red fluorescence in the dendrite.

Figure 5. The blockade of receptor internalization reverses the effects of DAMGO and etorphine on dendritic spines in cultured rat hippocampal neurons

A. Changes of dendritic spines (arrows) before and after DAMGO treatment in a neuron that was double-transfected with GFP and dominant negative dynamin (Dyn-). **B.** The expression of Dyn- was confirmed by an anti-HA antibody. *Left*, a shrunk live image from the same neuron as shown in A. *Middle 2 panels*, GFP and HA stained images of the same neuron after fixation. *Right*, Overlay of the middle 2 images. **C.** A neuron expressing GFP and Dyn- was photographed before and after treatment with etorphine. **D–E.** Redistribution of MOR-GFP in neurons expressing both MOR-GFP and Dyn- was examined before and after treatment with DAMGO (D) and etorphine (E). **F–G.** The density of dendritic protrusions and spines was calculated in the double-transfected neurons before (open bars), 1 day (gray bars) and 3 days (black bars) after treatment with DAMGO and etorphine. Untreated neurons were used as

control. **H.** Variance of dendritic fluorescence was compared between neurons with (black bars) or without the expression of Dyn-(open bars) to estimate the internalization of MOR-GFP after 1 hour of DAMGO and etorphine treatment. The values after treatment were normalized by values before treatment. **J.** The density of spines was compared between neurons expressed GFP alone (open bar) and neurons expressed both GFP and Dyn-(black bar).

Figure 6. The structural plasticity of spines is likely to be mediated through postsynaptic MORs
A. Neurons were cultured from MOR knock-out mice (MOR^{-/-} neuron) and were co-transfected with DsRed (upper 3 panels) and MOR-GFP (lower 3 panels). Images of these neurons were taken before, 1 hour and 1 day after treatment with morphine (10 μ M). *Arrows and triangles* denote that dendritic spines gradually shrunk and eventually collapsed after treatment with morphine. These results indicate that postsynaptic over-expression of MOR-GFP can rescue the deficits of MOR^{-/-} neurons because our previous studies reveal that morphine does not cause the collapse of spines in MOR^{-/-} neurons. **B.** MOR^{-/-} neurons expressing DsRed (upper) and MOR-GFP (lower) were treated with DAMGO (1 μ M). *The arrows* denote that a small dendritic protrusion became a spine and *the triangles* denote that a big spine was split into 3 spines after treatment with DAMGO. **C.** The density of dendritic protrusions (left 9 bars) and spines (right 9 bars) was calculated in neurons before (b), 1 hour

(1h) and 1 day (1d) after treatment with morphine and DAMGO. Untreated neurons were used as control. **D.** DAMGO caused loss of MORs in dendric spines. *Right inset*, the formula that was used to estimate the amount of MORs in spines. *MORspine*, green fluorescence intensity in the spine; *DsRedspine*, red fluorescence intensity in the spine; *MORDendrite*, green fluorescence in the adjacent dendrite; *DsReddendrite*, red fluorescence in the dendrite.

Figure 7. Methadone-induced bidirectional changes in dendritic spines are concentration-dependent

A and B. Changes in dendritic spines before and after treatment with methadone at 2 different concentrations. **C and D.** DAMGO at high concentration (10 μ M) induced prolonged changes in the redistribution of MORs in dendrites (D). In contrast, DAMGO at low concentration (1 μ M) induced short-lasting changes in MOR redistribution in dendrites (C). **E.** Density of dendritic protrusions and spines after treatment with methadone. The methadone modulation of dendritic protrusions and spines is concentration dependent. Methadone at a low concentration (1 μ M) causes the collapse of spines whereas methadone at a high concentration (10 μ M) induces the emergence of new spines. **F.** No change in total fluorescence intensity of MOR-GFP in dendrites 1 day after treatment with 1 or 10 μ M methadone, indicating that

methadone did not cause degradation of MORs. **G.** Variance was used to estimate the internalization of MORs before (open bars), 1 hour (gray bars) and 1 day (black bars) after treatment with methadone.