Agonist-Dependent Postsynaptic Effects of Opioids on Miniature Excitatory Postsynaptic Currents in Cultured Hippocampal Neurons

Dezhi Liao, Olga O. Grigoriants, Horace H. Loh, and Ping-Yee Law

Departments of Neuroscience and Pharmacology, Basic Research Center on Molecular and Cell Biology of Drug Addiction, The University of Minnesota, Minneapolis, Minnesota

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Liao D, Grigoriants OO, Loh HH, Law P-Y. Agonist-dependent postsynaptic effects of opioids on miniature excitatory postsynaptic currents in cultured hippocampal neurons. J Neurophysiol 97: 1485–1494, 2007. First published November 22, 2006; doi:10.1152/jn.00790.2006. Although chronic treatment with morphine is known to alter the function and morphology of excitatory synapses, the effects of other opioids on these synapses are not clear. Here we report distinct effects of several opioids (morphine, [D-ala2,me-phe4,gly5-ol]enkephalin (DAMGO), and etorphine) on miniature excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons: 1) chronic treatment with morphine for ≥3 days decreased the amplitude, frequency, rise time and decay time of mEPSCs. In contrast, “internalizing” opioids such as etorphine and DAMGO increased the frequency of mEPSCs and had no significant effect on the amplitude and kinetics of mEPSCs. These results demonstrate that different opioids can have distinct effects on the function of excitatory synapses.

INTRODUCTION

Many previous studies show that opioids can increase the excitability of neuronal circuits by inhibiting the release of presynaptic vesicles in GABAergic neurons (Jin and Chavkin 1999; Madison and Nicoll 1988; Williams et al. 2001; Wimpney and Chavkin 1991). AMPA receptors, particularly GluR1 subunits, are reported to be important for morphine tolerance and dependence (Stephens and Mead 2003; Vekovischeva et al. 2001), suggesting that morphine might also modulate the function and morphology of excitatory synapses. Most excitatory glutamatergic synaptic transmission occurs in dendritic spines (Harris and Kater 1994; Hering and Sheng 2001; Hering and Sheng 2001; Kennedy 1997, 2000; Nimchinski et al. 2002). A recent study showed that self-administration of morphine, which maintained a high plasma concentration of morphine for a prolonged time, decreased the density of dendritic spines by 50% in the hippocampus of adult rats (Robinson et al. 2002). A previous publication by our group also showed that chronic treatment with morphine could alter both the function and morphology of excitatory synapses (Liao et al. 2005). However, chronic postsynaptic effects of opioids other than morphine on mature excitatory synaptic transmission have not yet been published. Here we report the differential effects of three opioids, including morphine, etorphine, and DAMGO, on miniature excitatory postsynaptic currents (mEPSCs) in mature cultured hippocampal neurons (≥3 wk in vitro).

Different opioids are known to have differential effects on the internalization of mu opioid receptors (MORs). Agonist-induced internalization of opioid receptors has been suggested to be one of the alterations in opiate addiction and tolerance (Dang and Williams 2004, 2005; von Zastrow et al. 2003). Morphine induces little receptor internalization in most cell types, including cultured hippocampal neurons, whereas other opioids, such as DAMGO and etorphine, cause obvious receptor internalization (Alvarez et al. 2002; Bailey et al. 2003; Kovoor et al. 1998; Minnis et al. 2003; Sternini et al. 1996; von Zastrow 2001; Whistler and von Zastrow 1998; Yu et al. 1997). This property of morphine has been proposed to be responsible for the continued signaling by morphine, which may cause downstream adaptations that mediate addiction and tolerance (Finn and Whistler 2001; He et al. 2002; Whistler et al. 1999).

The distinct difference between the abilities of morphine and other opioids in inducing receptor internalization raises an important question: Do “noninternalizing” opioids such as morphine have the same effect on excitatory synaptic transmission as “internalizing” opioids? Primary hippocampal cultures were used in the current study because the hippocampus is one of the regions that contain the highest levels of MOR (Arvidsson et al. 1995) and is a part of the “learning and memory circuits,” which have been implicated in drug addiction in recent models and experiments (Biala et al. 2005; Fan et al. 1999; Kelley 2004; Nestler 2002; Vorel et al. 2001). In this current study, cultured hippocampal neurons were chronically treated with several opioids including morphine, DAMGO, and etorphine for ≥3 days. Our results revealed that “internalizing” opioids such as etorphine and DAMGO increased the frequency of mEPSCs, an effect that was opposite to morphine’s effect. These and other results together indicate...
that receptor internalization can modulate opioid-induced postsynaptic plasticity of excitatory synaptic transmission.

**METHODS**

**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 and striatum at postnatal days 1–2 were prepared as previously described (Brustovetsky et al. 2001; Liao et al. 1999, 2001). The same method was used to make dissociated neurons from mouse hippocampus. To prepare cortico- striatal cultures, neurons were dissociated from the cortex (frontal and parietal) and the striatum, respectively. Equal number of dissociated cortical and striatal neurons were mixed together and plated at a density of $1 \times 10^6$ per dish (each type contained $0.5 \times 10^6$). 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 culture dishes at a density of $1 \times 10^6$ cells/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 (Lin et al. 2004).

**Electrophysiology**

mEPSCs were recorded from cultured hippocampal neurons as previously described (Liao et al. 2005). Neurons at 21 DIV were treated with various opioid drugs for 3 days before recording. No opioid drug was present during the recording. Visual fields were randomly moved to approximate the center of the culture dish. Attempts were made to patch the first encountered “spiny” transfected neuron expressing fluorescence proteins or fluorescence protein-tagged proteins. If the establishment of whole cell configuration was not successful, the second encountered transfected neuron was attempted. No more than two neurons were attempted from the same culture dish. mEPSCs were recorded while holding potentials of $-55$ to $-60$ mV and filtered at 1 kHz. Input and series resistances were checked before and after the recording of mEPSCs, which lasted $\sim 10–30$ min. There were no significant difference in the series resistances and input resistance among various groups of experiments. One recording sweep lasting 200 ms was sampled for every 1 s. mEPSCs were recorded in cultured dissociated neurons in standard Earle’s balanced salts solution (EBSS) at room temperature with 200 µM APV [N-methyl-D-aspartate (NMDA) receptor blocker], TTX (sodium current blocker), and 100 µM picrotoxin (GABA receptor antagonist), gassed with 95% O$_2$, 5% CO$_2$. To increase the number of mEPSCs, an extra 2 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ were added to the bath solution. The internal solution in the patch pipette contained (in mM) 100 cesium gluconate, 0.2 EGTA, 0.5 MgCl$_2$, 2 ATP, 0.3 GTP, and 40 HEPES (pH 7.2 with CsOH).

All mEPSCs were analyzed with the MiniAnalysis program designed by Synaptosoft Inc. Detection criteria for mEPSCs was set as the peak amplitude $>3$ pA. Each mEPSC event was visually inspected and only events with a distinctly fast-rising phase and a slow-decaying phase were accepted. The frequency and amplitude of all accepted mEPSCs were directly read out by using the analysis function in the MiniAnalysis program. The amplitudes of all events in all neurons in each experimental group were pooled together and plotted as a cumulative frequency curve. The Kolmogorov-Smirnov test was used to test the difference between two cumulative frequency distributions. In further statistical analyses, the averaged amplitude of mEPSCs from each neuron was treated as a single sample. These averaged amplitudes were further averaged to calculate the mean amplitude in each experimental group and plotted as histograms. The MiniAnalysis program often erroneously locates the beginning of the rising phase of a small mEPSC event, which leads to a substantial underestimate of rise time. To correct this potential error, each previously accepted event was visually inspected again. An event would further be accepted to another group for time-course analysis only when the yellow spot was at the beginning of the rising phase and the red spot was at the peak of the response (see the tutorial in MiniAnalysis). The rise and decay times of all events in the new group would be estimated and averaged for neuron. The rise time was defined as the interval between the very beginning of the rising phase and the peak, and the decay time was the interval between the peak and 90% of the decaying phase. The averaged parameters from each neuron were treated as single samples in any further statistical analyses. Student’s t-tests were used to test the difference between two experimental groups, and ANOVA was used to examine the difference among multiple groups.

**Image analysis**

Neurons that had been transfected with GFP alone (Figs. 1 and 5) or MOR-GFP and DsRed (Fig. 3) were photographed immediately after the electrophysiological recording (see Electrophysiology for the selection of neurons). In addition, images of MOR-GFP-expressing neurons cultured from the hippocampus and striatum were also...
photographed to compare the distribution of MORs between these two types of cultures (Fig. 2). For a further comparison, the distribution of MORs in cortico-striatal neurons was also examined (Fig. 2). All digital images were analyzed with the MetaMorph Imaging System (Universal Imaging). Unless stated otherwise, all images of live neurons were taken as stacks (series of optical sections) 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 a single composite image. A dendritic protrusion with an expanded head 50% wider than its neck was defined as a spine. The number of spines or nonspine protrusions from one neuron was manually counted and normalized as number per 100 μm of dendritic length. One-way ANOVA was used for comparison among multiple groups of data (n, number of neurons; P < 0.05, significant). If the ANOVA test indicated significant changes, a 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 soma, regions of interest were highlighted and selected in the MetaMorph program with the “autothreshold bright objects” function, and the averaged fluorescence intensity in each region was calculated. To highlight dendritic protrusions and spines, the 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. All data are reported as means ± SE, *P < 0.05, **P < 0.01, ***P < 0.001.

**RESULTS**

**DAMGO and etorphine cause effects on mEPSCs that are different from morphine**

It is well known that etorphine and DAMGO can rapidly induce the internalization of MORs, whereas morphine induces little or no internalization in hippocampal neurons (Cielcer et al. 2004; Cox 2005). This fact led us to hypothesize that various opioid agonists might have differential effects on excitatory synaptic transmission depending on their abilities to internalize the opioid receptors. mEPSCs were recorded in 3-wk-old GFP-labeled neurons with clearly visible dendritic spines (Fig. 1A). Chronic treatment with morphine (10 μM) caused mEPSCs to become smaller and faster than untreated control (Fig. 1B) and significantly decreased the amplitude, frequency, rise time, and decay time of mEPSCs (Fig. 1, C—F, n = 10 in each group; ■ and ●). In contrast, DAMGO (1 μM) and etorphine (1 μM) had no significant effect on the amplitude and time kinetics of mEPSCs (Fig. 1, C, D, and F) but significantly increased their frequency (Fig. 1E), indicating that internalizing opioids such as DAMGO and etorphine can induce effects that are distinctly different from those of morphine. These results support our hypothesis that MOR internalization modulates opioid-induced plasticity of dendritic spines.

**Distribution of MORs in cultured hippocampal neurons is markedly different from cultured striatal and cortico-striatal neurons**

It is still controversial whether morphine’s effects are due to pre- or postsynaptic changes (Liao et al. 2005; Williams et al. 2001). One potential reason for this controversy is that the distribution of MORs might be different in various types of neurons. To address this controversy, neurons were cultured from either the hippocampus (Fig. 2A) or the striatum of rats (Fig. 2, B and C) and were transfected with MOR-GFP. In the hippocampal neurons, MOR-GFP was predominantly expressed in dendrites and was clustered and concentrated in dendritic spines after 21 DIV (Fig. 2A). This distribution is very similar to that of endogenous MORs as described in our previous studies (Liao et al. 2005). In these cultured hippocampal neurons, 91% of MOR-GFP-expressing neurons were spiny neurons, which were likely pyramidal neurons (Fig. 2F; n = 10 dishes). This preferential expression of MOR-GFP is not surprising because previous immunohistochemical studies show that MORs are exceptionally abundant in the dendrites of hippocampal pyramidal neurons (Arvidsson et al. 1995). In contrast, MOR-GFP molecules were predominantly expressed in the axon-like processes of nonspiny neurons in primary...
neurons (Fig. 3). MOR-GFP was used to rescue the MOR deficit in cultured neurons that were cultured from MOR knock-out mice (MOR\(^{-/-}\)) (Loh et al. 1998; Fig. 3). mEPSCs were recorded in an untreated neuron cultured from the hippocampus of MOR knock-out mice (MOR\(^{-/-}\)). This neuron was double-transfected with DsRed and MOR-GFP. Left, both illumination and red fluorescent lights were turned on. Middle, only red fluorescent light was turned on. Right, green fluorescent light was turned on. →, location of the patch electrode. B: zoom-in images from A (denoted by △ in A) including DsRed (left), MOR-GFP (middle), and overlay (right). Clustering of MOR-GFP in dendritic spines was estimated in untreated neurons. △, a ratio calculated from images taken in red fluorescence channel. ■, a similar ratio was calculated from images in green fluorescence channel. Therefore MOR-GFP molecules (green) are clustered in dendritic spines as this ratio is larger than 100%. E: The density of dendritic protrusions and spines in untreated, morphine-treated, and DAMGO-treated neurons in DsRed images.

**Opioids postsynaptically modulate the morphology of excitatory synapses in cultured hippocampal neurons**

A distinct characteristic of molecules that can modulate postsynaptic function of excitatory synapses is that these molecules often cluster in dendritic spines (Kim and Sheng 2004; Malinow et al. 2000). MOR-GFP molecules clustered at dendritic spines in cultured rat hippocampal neurons and most MOR-GFP-expressing hippocampal neurons (91%) were spiny neurons (Fig. 2). The relative homogeneity of these hippocampal neurons gave us a useful tool to test postsynaptic effects of opioids. To address the issue of postsynaptic MOR activities, two plasmids encoding GFP-tagged \(\mu\)-opioid receptors (MOR-GFP) and DsRed were co-transfected into hippocampal neurons that were cultured from MOR knock-out mice (MOR\(^{-/-}\)) (Loh et al. 1998; Fig. 3). mEPSCs were recorded to examine the function of dendritic spines (see the recording pipette in Fig. 3A, left; mEPSC traces in Fig. 4). DsRed was used to label dendritic morphology (Fig. 3A, middle), and MOR-GFP was used to rescue the MOR deficit in cultured neurons (Fig. 3A, right). Similar to cultured rat neurons (Fig. 2), MOR-GFP molecules also clustered in dendritic spines in mouse cultures (Fig. 3, B and E). Chronic treatment with morphine for 3–6 days significantly decreased the density of dendritic protrusions and dendritic spines (\(n = 10\) in each group, Fig. 3, C and F). In contrast, DAMGO, another MOR agonist, significantly increased the density of dendritic protrusions and spines after 3–6 days of treatment (Fig. 3, D and F). Because MORs were absent in all untransfected cells, including nearby neurons and glial cells, these results provide evidence that morphine can directly act on postsynaptic excitatory neurons, causing the collapse of dendritic spines. In contrast, DAMGO, which is a known “internalizing” opioid, causes effects that are opposite to morphine via postsynaptic MORs.

**Opioids postsynaptically modulate the function of excitatory synapses in cultured hippocampal neurons**

To further test whether morphine and DAMGO can postsynaptically modulate the function of dendritic spines, mEPSCs were recorded in neurons co-transfected with DsRed and
MOR-GFP (see Figs. 3A, left for configuration, and 4A for sample traces). The transfected neurons (left 3 averaged traces in Fig. 4B) contain exogenous MORs, whereas the untransfected neurons contain neither endogenous nor exogenous MORs (Fig. 4B, right trace). Morphine decreased the frequency, amplitude, rise time, and decay time in neurons that expressed exogenously introduced MORs (Fig. 4, A—F; n = 10 in each group) but did not suppress excitatory synaptic transmission in neurons that did not express MORs (untransfected neurons; Fig. 4, B—F). These results indicate that morphine can directly act on postsynaptic excitatory neurons and postsynaptically suppress excitatory synaptic transmission. In contrast, the frequency of mEPSCs in DAMGO-treated MOR-GFP-expressing neurons was significantly higher than that in untreated MOR-GFP-expressing neurons (Fig. 4E). This effect is clearly opposite to that of morphine. This result further confirms that "internalizing" opioid may cause effects that are opposite to morphine.

As controls, neurons were cultured from the hippocampus of wild-type mice with similar genetic background (for breeding, see Loh et al. 1998). Morphine decreased the density of spines and DAMGO had the opposite effect in these neurons with endogenous MORs (Fig. 5, A and B; n = 10 in each group). Morphine significantly decreased the amplitude, frequency, rise time, and decay time of mEPSCs (Fig. 5, C—F; n = 10 in each group). DAMGO had no effect on the amplitude and time-kinetics of mEPSCs but significantly increased the frequency of mEPSCs, an effect that was opposite to morphine (Fig. 5, C—F). Data in Figs. 3—5 together indicate that exogenously introduced MOR-GFP and endogenous MORs can mediate similar chronic effects on excitatory synapses probably through the same signaling pathway.

**Acute effects of morphine on mEPSCs in neurons expressing GFP or MOR-GFP**

An important but unanswered question is: can morphine induce acute functional plasticity in excitatory synaptic transmission? To examine the acute effects of morphine, three groups of experiments were performed (methodological details are provided in the legend of Fig. 6A). In the control group, no morphine was applied to the GFP-labeled neurons during the recording of mEPSCs (Fig. 6A, left). In the other two groups, mEPSCs were recorded in neurons expressing GFP or MOR-GFP before and after treatment with 10 μM morphine (Fig. 6A, middle and right). In comparison to the baseline before treatment, morphine significantly decreased the amplitude of mEPSCs in neurons expressing MOR-GFP after 25 min of treatment (Fig. 6, B—D and F). Morphine had no significant

![Fig. 4. Chronic treatment with opioids postsynaptically modulates the function of excitatory synapses. A: sample mEPSC traces recorded from neurons cultured from MOR knock-out mice (MOR−/−) that had been transfected with DsRed and MOR-GFP. Left: untreated neurons; right: morphine-treated neurons. B: averaged mEPSCs from 4 groups of neurons (from left to right): group 1, untreated neurons expressing DsRed and MOR-GFP; groups 2 and 3, neurons expressing DsRed and MOR-GFP were treated with morphine or DAMGO for 3—6 days; group 4, nearby untransfected neurons that had been treated with morphine for 3—6 days. C: amplitudes of mEPSCs from the preceding 4 groups of neurons plotted as histograms. D: cumulative frequency curves of mEPSC amplitudes were estimated in the same 4 groups of neurons: untreated control (□), morphine-treated (●), DAMGO treated (▲) and untransfected (■).](http://jn.physiology.org/doi/abs/10.1152/jn.00783.2006)

![Fig. 5. Chronic effects of opioids mediated via endogenous MORs are similar to those mediated via exogenous MORs. A: neurons were cultured from the hippocampus of wild-type mice, transfected with GFP and treated with morphine (middle) or DAMGO (right) for >3 days. Untreated neurons were used as control (left). B: density of dendritic spines in the 3 groups of neurons plotted as histograms (□, untreated; ●, morphine-treated; ▲, DAMGO-treated). C: amplitudes of mEPSCs plotted as histograms. D: cumulative frequency curves of mEPSC amplitudes plotted. Bin size = 0.5 pA. E and F: frequency, rise time, and decay time of mEPSCs plotted as histograms.](http://jn.physiology.org/doi/abs/10.1152/jn.00783.2006)
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MOR-GFP but had no effect on GFP-labeled cultured rat hippocampal neurons expressing neuron with no morphine application; cation (gray trace).

recorded in GFP-expressing neurons for 5 min before morphine application (black trace) and were continuously recorded for another 25 min after morphine application (gray trace).

Middle: mEPSCs were recorded in GFP-expressing neurons for 5 min before morphine application (black trace) and were continuously recorded for another 25 min after morphine application (gray trace).

Right: mEPSCs were recorded in MOR-GFP-expressing neurons before and after morphine application. B, top: traces from a GFP-expressing neuron with no morphine application; bottom: traces from a MOR-GFP-expressing neuron before and after morphine application. C, cumulative frequency curves of mEPSCs recorded from the 3 groups of neurons as described in A before (black) and after (gray) the application of morphine (bin size, 0.5 pA). Note that the Kolmogorov-Smirnov (K-S) tests are significant ($P < 0.001$) in both left and middle panels even though paired t-test show that the mean mEPSC amplitudes are not significantly changed during the period of recording (see D). The gray curves were slightly shifted to the left almost at every percentage level, which leads to significant K-S test results. It suggests that there might be a tiny "run-down" in mEPSCs even in control neurons. D and E, amplitude (D) and frequency (E) of mEPSCs were compared before (b) and after (a) morphine application. F: to rule out potential artifacts from "run-down" (see K-S test results in C), the amplitude (left 3 bars) and frequency (right 3 bars) after morphine application were normalized to the baseline value before morphine application and were further compared among experimental groups using ANOVA tests. G: no significant difference was detected in the rise and decay times after morphine application (bin size, 0.5 pA). Note that the Kolmogorov-Smirnov (K-S) tests are significant ($P < 0.001$) in both left and middle panels even though paired t-test show that the mean mEPSC amplitudes are not significantly changed during the period of recording (see D). The gray curves were slightly shifted to the left almost at every percentage level, which leads to significant K-S test results. It suggests that there might be a tiny "run-down" in mEPSCs even in control neurons.

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DISCUSSION

Differential effects of internalizing and noninternalizing opioids

This study demonstrates that different MOR agonists can have differential postsynaptic effects on excitatory synaptic transmission. Previous studies showed that chronic treatment with morphine can cause collapse of dendritic spines and suppression of excitatory synaptic transmission (Liao et al. 2005; Robinson and Kolb 2004; Robinson et al. 2002). In a proposed "RAVE" (receptor activity vs. endocytosis) hypothesis, morphine's inability to induce receptor internalization allows the continuous signaling of MORs, which contributes to additive liability and tolerance development (He et al. 2002; Whistler et al. 1999). 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; Kovoor et al. 1998; Minnis et al. 2003; Sternini et al. 1996; von Zastrow 2001; Whistler and von Zastrow 1998; Yu et al. 1997).

In contrast, DAMGO and etorphine cause robust internalization and desensitization of opioid receptors (Dang and Williams 2004; Kieffer and Evans 2002; von Zastrow et al. 2003). Naloxone increased the density of spines and the frequency of mEPSCs, effects that are opposite to those of morphine (Liao et al. 2005). If DAMGO and etorphine can cause robust internalization of MORs, they should induce effects that are similar to naloxone because the signaling of MORs might be interrupted when MORs are removed from cell surface. This is in fact what we saw. Therefore the observed correlation between the agonist’s ability to induce receptor internalization and to alter excitatory synaptic transmission shown in this study suggests that receptor internalization can modulate opioid-induced synaptic plasticity. Activity-dependent alterations in excitatory synaptic transmission is widely believed to be the cellular model of learning and memory (Martin et al. 2000) and addiction is proposed to be a pathological form of learning and memory (Nestler 2002). Therefore opioid-induced plasticity of excitatory synaptic transmission might contribute to opiate addiction. In support of the RAVE hypothesis, this study demonstrates that opioids with different abilities in inducing receptor internalization can modulate opioid-induced synaptic plasticity. Activity-dependent alterations in excitatory synaptic transmission is widely believed to be the cellular model of learning and memory (Martin et al. 2000) and addiction is proposed to be a pathological form of learning and memory (Nestler 2002). Therefore opioid-induced plasticity of excitatory synaptic transmission might contribute to opiate addiction. In support of the RAVE hypothesis, this study demonstrates that opioids with different abilities in inducing receptor internalization can modulate opioid-induced synaptic plasticity. Activity-dependent alterations in excitatory synaptic transmission is widely believed to be the cellular model of learning and memory (Martin et al. 2000) and addiction is proposed to be a pathological form of learning and memory (Nestler 2002). Therefore opioid-induced plasticity of excitatory synaptic transmission might contribute to opiate addiction. In support of the RAVE hypothesis, this study demonstrates that opioids with different abilities in inducing receptor internalization can modulate opioid-induced synaptic plasticity. Activity-dependent alterations in excitatory synaptic transmission is widely believed to be the cellular model of learning and memory (Martin et al. 2000) and addiction is proposed to be a pathological form of learning and memory (Nestler 2002). Therefore opioid-induced plasticity of excitatory synaptic transmission might contribute to opiate addiction. In support of the RAVE hypothesis, this study demonstrates that opioids with different abilities in inducing receptor internalization can modulate opioid-induced synaptic plasticity. Activity-dependent alterations in excitatory synaptic transmission is widely believed to be the cellular model of learning and memory (Martin et al. 2000) and addiction is proposed to be a pathological form of learning and memory (Nestler 2002). Therefore opioid-induced plasticity of excitatory synaptic transmission might contribute to opiate addiction. In support of the RAVE hypothesis, this study demonstrates that opioids with different abilities in inducing receptor internalization can modulate opioid-induced synaptic plasticity.

Morphological changes and mEPSCs

AMPLITUDE AND FREQUENCY OF MEPPS. In this study, chronic treatment with morphine for >3 days decreased both the frequency of mEPSCs and the density of dendritic spines, whereas chronic treatment with DAMGO or etorphine had opposite effects. Clearly, our current results cannot prove the
causal relationship between the morphological and functional changes. Nonetheless, a decrease in the frequency of mEPSCs is consistent with a decrease in the density of dendritic spines. In the classical mathematical model (del Castillo and Katz 1954), the strength of evoked excitatory postsynaptic potentials (EPSPs) in neuromuscular junction (NMJ) is determined by quantal content \( m \) and quantal size \( q \). Quantal content is the average number of released vesicles per stimulus, \( m = n^p \), where \( p \) is the probability of release and \( n \) is the number of releasing sites. Similarly, the frequency of mEPSPs in NMJ or mEPSCs in the CNS, \( F_m = n^{p'} \), where \( p' \) is the probability of spontaneous release and \( n \) is the number of releasing sites. In the CNS, mEPSCs are often recorded by a patch electrode in the soma of a neuron so that the electrode receives signals from all synapses in that neuron. In this case, an increase in the density of dendritic spines would also increase the number of apposing presynaptic termi, leading to an increase in the total number of releasing sites \( n \). Therefore an increase in mEPSC frequency is consistent with an increase in the density of dendritic spines. The amplitude of mEPSPs or mEPSCs is equivalent to the quantal size \( q \) in evoked synaptic transmission. Quantal size is the average postsynaptic response per one released vesicle. As the amount of neurotransmitters per vesicle is assumed to be constant, a decrease in the amplitude of mEPSCs would indicate a loss of AMPA receptors in the postsynaptic membrane (Liao et al. 2001).

**KINETICS OF MEPCS.** The mechanism underlying the morphine-induced decrease in rise and decay times of mEPSCs is unknown. It is likely that this alteration is largely attributed to morphine-induced shortening of dendritic spines (see the theoretical model in Fig. 7). Assuming that a dendrite is a passive cable, dendritic membrane is never perfectly voltage-clamped when a voltage clamp is applied using a patch electrode on the soma (Rall and Segev 1985; Spruston et al. 1993). The clamping voltage along a dendrite progressively decreases as described by the equation: \( V(X)/V_o = \cosh(L - X)/\cosh L \) (continuous cable model) (Carnevale and Johnston 1982; Rall and Segev 1985). Due to this attenuation of clamping voltage, the rise time of an EPSC from a remote synapse would be slower than that from a proximate synapse (Spruston et al. 1993). The electrical resistance of a spine stem (or neck) is probably the most important parameter that determines the electrical behavior of a dendritic spine (Johnston and Wu 1995; Segev and Rall 1988; Tsay and Yuste 2004). The high electrical resistance of the spine stem is expected to dramatically increase the electrotonic distance from the head of dendritic spines to the dendritic shaft, and consequently the total electrotonic distance from the spine head to the soma (Fig. 7A). When the length of the spine stem is decreased by morphine (Fig. 7B–D), the electrotonic distance would be decreased, space-clamp errors would be reduced, and the attenuation of the kinetics of mEPSCs would also be decreased. According to our estimate, the shortening of the spine stem alone would decrease the rise time of mEPSCs by \( \sim 20\% \) and a decrease in Ri (resistivity of the spine neck) might account for another \( \sim 10\% \) reduction (Fig. 7D). The most critical assumption in our theoretical model is that the resistivity of spine neck (Ri) is much higher than that in the dendrite (Ri). This difference in resistivity cannot be explained by geometry alone and requires that the neck of spine must be able to obstruct the free movement of ions. The neck of a dendritic spine is now known to pose a barrier to the diffusion of molecules (Bloodgood and Sabatini 2005). In addition, morphine might decrease the membrane capacitance by decreasing the size of the spine head and consequently increase the rise time of mEPSCs by decreasing the charging of the capacitance. Morphine-induced change in glutamate uptake might also contribute to the altered kinetics of mEPSCs (Xu et al. 2003). Perhaps the major contributor to the decrease in mEPSC kinetics is the morphine-induced reduction in the electrical resistance at the spine neck (Fig. 7; due to decreases in bc and Ri), although other factors such as spine size and glutamate uptake may also play minor roles.

Compared with previous studies (Stellwagen et al. 2005; Thiagarajan et al. 2005), the kinetics of mEPSCs in this current study is very slow. This is mainly due to the advanced age of...
our cultured neurons (>24 DIV: 21 DIV +3 days of treatment). As we previously reported, the time course of mEPSCs from 3-wk-old neurons is much slower than that from 2-wk-old neurons (Liao et al. 2005). Dendritic spines start to form at 14 DIV and become elongated and stable after 21 DIV (Liao et al. 1999). According to our theoretical model in Fig. 7, the kinetics of mEPSCs from a dendritic spine should be slower than that from a nonspiny synapse. Therefore it is not surprising that mEPSCs in more mature neurons are slower than those in immature neurons. In addition, mEPSCs were recorded only from spiny neurons in this study, whereas mixed types of neurons were patched in previous studies from other groups.

Which comes first—morphology or function?

REMOVAL OF AMPA RECEPTORS. According to the cable theory, the shortening of a spine stem alone should increase the amplitude of mEPSCs as there is less attenuation of synaptic inputs (Tsay and Yuste 2004). Therefore the morphine-induced decrease in mEPSC amplitude cannot be directly caused by the morphological change. It is instead probably due to the removal of synaptic AMPA receptors (Liao et al. 2005). Interestingly, acute perfusion of morphine altered only the amplitude of mEPSCs in neurons expressing MOR-GFP, not affecting the frequency, rise time and decay time of mEPSCs (Fig. 6). This result suggests that the activation of MORs might be able to remove AMPA receptors from dendritic spines before altering spine size or density. As GluR2 subunits of AMPA receptors are important for the growth and/or stability of dendritic spines (Passafaro et al. 2003), it is possible that the morphine-induced collapse of dendritic spines might be a secondary effect due to the loss of AMPA receptors. It remains to be determined whether the removal of AMPA receptors and the collapse of spines are regulated through the same or a different independent signaling pathway.

INTRACELLULAR SIGNALING PATHWAYS. Morphine treatment requires 1–3 days to cause collapse of dendritic spines in neurons with only endogenous opioid receptors being expressed (Liao et al. 2005). Logically, if morphine requires such a long time to alter dendritic spines, the downstream signaling pathway must also remain available for a prolonged period of time. Even though morphine causes little internalization, most known signaling pathways are rapidly desensitized on the application of morphine (Law et al. 2000). Therefore morphine-induced collapse of dendritic spines is likely to be caused by an unconventional signaling pathway that regulates the actin-cytoskeleton. Both filamin A (Onoprishvili et al. 2003) and Rho GTPases are potential signaling candidates in this pathway (Lippman and Dunaevsky 2005; Luo 2000). A recent previous study by us shows that Rac1, a Rho GTPase, can mediate both the clustering of AMPA receptors and the formation and maintenance of dendritic spines (Wiens et al. 2005). Therefore it is possible that opioid treatment might simultaneously affect the trafficking of AMPA receptors and the morphology of spines by altering Rac1 activity.

PRE- AND POSTSYNAPTIC MECHANISMS. This current study demonstrates that chronic morphine treatment can postsynaptically modulate the function of dendritic spines. In contrast, numerous previous studies demonstrate that morphine can acutely inhibit presynaptic release of GABA (the “disinhibition” theory) (Madison and Nicoll 1988; Williams et al. 2001; Wimpey and Chavkin 1991). As shown in Fig. 2, the distribution of MORs in hippocampal neurons is distinctly different from striatal and cortico-striatal neurons. Therefore it is not surprising that different research groups might obtain either pre- or postsynaptic effects if different cell types were examined. However, we believe that MOR-mediated effects on hippocampal pyramidal neurons are mainly postsynaptic for the following reasons. 1) The pyramidal layer in the hippocampus is one of the regions that contain the highest levels of MORs and the dendritic trees of pyramidal neurons clearly contain abundant MORs (Arvidsson et al. 1995). 2) When adult animals were chronically exposed to self-administered morphine (the plasma concentration is high for a long time in this group), the strongest effect on dendritic spines was observed in the CA1 region of the hippocampus (Robinson et al. 2002). 3) Endogenous MORs are clustered in dendritic spines in cultured hippocampal neurons (Liao et al. 2005). 4) MOR-GFP molecules are clustered in dendritic spines and are preferentially expressed in spiny neurons in primary hippocampal cultures (Fig. 2). 5) In neurons cultured from MOR knock-out mice, chronic treatment with morphine only suppressed mEPSCs in neurons expressing exogenous MOR-GFP (Figs. 3 and 4).

Conclusions

This study has contributed two key pieces of knowledge explaining normal opioid neurophysiology and pathology. First it provides evidence that opioids can postsynaptically modulate the structure and function of dendritic spines in cultured hippocampal neurons. Endogenous opioids may participate in maintaining normal morphology and function of spines via this postsynaptic plasticity, whereas abnormal alteration of spines may occur when MORs in dendritic spines are overactivated. The normal level of endogenous MORs allows only slow opioid-induced plasticity of dendritic spines. Second, this study demonstrates a correlation between receptor internalization and opioid-induced plasticity of excitatory synaptic transmission by showing that noninternalizing opioids such as morphine suppresses synaptic transmission, whereas internalizing opioids such as DAMGO and etorphine have the opposite effect. Therefore this study suggests that the receptor internalization might modulate opioid-induced plasticity of excitatory synapses.

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References

DISTINCT EFFECTS OF OPIOIDS ON mEPSCs


