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

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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; Wimpey 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; Nimchinsky 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; Stermini et al. 1996; von Zastrow 2001; Whistler and von Zastrow 1998; Yu et al. 1997). The 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 at 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 ~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; d,l-form, the concentration is equivalent to active form at 100 μM), 1 μM TTX (sodium current blocker), and 100 μM picrotoxin (GABA receptor antagonist), gassed with 95% O2-5% CO2. To increase the number of mEPSCs, an extra 2 mM Ca2+ and 1 mM Mg2+ were added to the bath solution. The internal solution in the patch pipette contained (in mM) 100 cesium gluconate, 0.2 EGTA, 0.5 MgCl2, 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

FIG. 1. The effects of DAMGO and etorphine on miniature excitatory postsynaptic currents (mEPSCs) are distinctly different from morphine. A: mEPSCs were recorded in neurons cultured from the hippocampus of rats and transfected with GFP alone. Top: both illumination light and green fluorescent light were turned on. Middle: only green fluorescent light was turned on. Right: an enlarged image from the middle (denoted by the triangle). B: averaged mEPSCs from 4 groups of neurons (from left to right): untreated, morphine treated, DAMGO treated, and etorphine treated. C: amplitudes of mEPSCs in untreated (○), morphine-treated (●), DAMGO-treated (■), and etorphine-treated neurons (□), plotted as histograms. D: cumulative frequency curves of mEPSC amplitudes from untreated (○), morphine-treated (●), DAMGO-treated (■), and etorphine-treated neurons (□). E: frequency and time kinetics of mEPSCs in the preceding 4 groups of neurons are plotted as histograms.
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%-
wide 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 “autotreshold 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 protru-
sion 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 (Celver et al.
2004; Cox 2005). This fact led us to hypothesize that various
opioid agonists might have differential effects on excitatory syn-
aptic transmission depending on their abilities to internalize the
opioid receptors. mEPSCs were recorded in 3-wk-old GFP-la-
beled 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 den-

dritic 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 ex-
pressed 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 hippocam-
pal 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 sur-
prising 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

![Image](https://example.com/image.png)

**Fig. 2.** The distribution of μ opioid receptor (MOR)-GFP in cultured
hippocampal neurons is different from that in cultured striatal and cortico-
striatal neurons. A: MOR-GFP is clustered and concentrated in dendritic spines
in cultured hippocampal neurons ( □). B and C: MOR-GFP is highly con-
centrated in axon-like processes in nonspiny striatal neurons. →, axon in C is
continued from B at this location. D: enlarged image from the same neuron as
in B and C shows that this is a nonspiny neuron. E: cortico-striatal cultures
contain both spiny (left) and nonspiny neurons (right). The arrows denote
MOR-GFP is clustered and concentrated in dendritic spines (left). □, strong
expression of MOR-GFP in axon-like processes in nonspiny neurons (right). F:
proportion of “spiny” neurons total MOR-GFP-expressing neurons in hippo-
campal (H), striatal (S), and cortico-striatal (CS) cultures. The visual field
was moved to approximately the center of a culture dish and randomly moved
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striatal cultures after 21 DIV (only 24% of MOR-GFP-expressing neurons were spiny neurons; \( n = 10 \) dishes; Fig. 2, B–D and F). Strikingly, many dishes of these striatal cultures were often extensively covered by numerous green thin thread-like axons. These results clearly demonstrate that the cellular distribution of MORs might vary in different regions of the brain. Occasionally, MORs also expressed in nonspiny neurons in cultured hippocampal neurons. Interestingly, MORs were exceptionally abundant in axon-like processes in these nonspiny neurons, which might be GABAergic neurons. This observation reveals that the distribution of MORs might vary in different types of neurons even in the same region of the brain. Therefore morphine might induce either pre- or postsynaptic changes in specific types of neurons. The diverse MOR distribution in various types of neurons might provide reconciliation for many previously reported pre- or postsynaptic effects of opioids.

It has been previously reported that cultured striatal neurons contained almost no dendritic spines if they were grown alone probably due to lack of glutamatergic presynaptic inputs (Segal et al. 2003). It is possible that the strong expression of MOR-GFP in axon-like processes in nonspiny neurons (Fig. 2C) might result from the lack of glutamatergic inputs. Therefore we further examined the distribution of MOR-GFP in neurons of cortico-striatal cultures (50% neurons from the frontal and parietal cortex; 50% from the striatum; see Experimental procedures), which should contain abundant glutamatergic inputs (Segal et al. 2003). In cortico-striatal cultures, 45% of MOR-GFP-expressing neurons were spiny neurons and 55% were nonspiny neurons (Fig. 2F, \( n = 14 \) dishes). Consistent with results in Fig. 2, A–D, MOR-GFP molecules were clustered in dendritic spines in spiny neurons (see the arrows in Fig. 2E, right) and were strongly expressed in axon-like processes in nonspiny neurons (see \( \Delta \) in Fig. 2E, left). These results indicate that the differential distribution of MOR-GFP in spiny and nonspiny neurons is unlikely to result from the lack of glutamatergic inputs. Nevertheless, further in vivo experiments are needed to test whether endogenous MORs are indeed differentially distributed in spiny and nonspiny neurons in intact animals.

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...
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, nearly 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 (bin size = 0.5 pA). E and F, frequency, rise time and decay time of mEPSCs in the same four groups of neurons: untreated control (○), morphine-treated (□), DAMGO treated (△) and untransfected (■).

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

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.
MOR-GFP but had no effect on GFP-labeled cultured rat hippocampal neurons expressing neuron with no morphine application; neurons before and after morphine application. cation (gray trace). were continuously recorded for another 25 min after morphine applica-
recorded in GFP-expressing neurons for 5 min before morphine application (black trace) and were continuously recorded for another 25 min after morphine applica-
A: 3 groups of experiments were performed to examine the acute effect of morphine: Left: as control, mEPSCs of GFP-expressing neurons were continuously recorded for 30 min and no morphine was applied during the recording. The early 5 min were used as baseline (black trace, average of mEPSCs from a sample neuron) and the later 25 min were used for comparison (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 were 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 < 0.01) 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 in all experimental groups. Open bars, GFP-expressing neurons with no morphine application; black bars, morphine-treated GFP-expressing neurons; gray bars, morphine-treated MOR-GFP-expressing neurons.

**RESULTS**

**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 addictive 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 cause different changes in excitatory synaptic transmission, which might account for various addictive liabilities among opiates.

**Morphological changes and mEPSCs**

**AMPLITUDE AND FREQUENCY OF mEPSCS.** 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
The attenuation at “d” should be similar to “c.”

FIG. 7. A theoretical model showing that morphine can significantly decrease the electrotonic distance from the soma to the head of spines. A: dendrite is represented by an equivalent cable of finite length with sealed ends and a dendritic spine is located at point “b” in the middle of the cable (Rall and Segev 1985) (also see Kinetics of mEPSCs). Points “a” and “c” are the beginning and the end of the cable, respectively. The electronic circuit from the soma (point a) to the head of spine (point c) is another cable with an electrotonic length $L' = (abA' + bcA')$. $\lambda$ is the space constant of the dendrite and $A'$ is the space constant of the spine stem. Because the effect of increasing cable length from the soma to the head of spine requires the same, the EPSC kinetics should be mainly determined by the electrotonic distance from the soma to the spine neck (Spruston et al. 1993). Therefore the attenuation of EPSC kinetics at point c should be similar to point d. A and C: assuming morphine decreases the length of the spine stem from 2 to 0.5 $\mu$m, the electrotonic distance from the spine head to the dendrite shaft should be decreased by $1.5A'$. b: assumed shortening in the spine stem leads to a decrease in electrotonic distance by 11% (see the calculation in the left). Based on previous simulation data (see Fig. 6 in the paper by Spruston et al. 1993), this decrease in electrotonic distance would decrease the rise time by 20%. In addition, morphine might also decrease $R_\text{ia}$ because the obstruction of free ion movement might decrease during the collapse of spines. This decrease in $R_\text{ia}$ might lead to an extra 10% decrease in the rise time of mEPSCs.

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 $R_\text{ia}$), 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 nonsynaptic spine. 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 collapse 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.

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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