Published in final edited form as:

J Neurophysiol. 2007 February ; 97(2): 1485–1494.

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

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Abstract

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, 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. (2) MOR-GFP is clustered in dendritic spines in most hippocampal neurons but is concentrated in axon-like processes in striatal and corticostriatal non-spiny neurons. It suggests that MORs might mediate pre- or post-synaptic effects depending upon cell types. (3) Neurons were cultured from MOR knock-out mice and were exogenously transfected with GFP-tagged MORs (MOR-GFP). Chronic treatment with morphine suppressed mEPSCs only in neurons that contained postsynaptic MOR-GFP, indicating that opioids can modulate excitatory synaptic transmission postsynaptically. (4) Morphine acutely decreased mEPSC amplitude in neurons expressing exogenous MOR-GFP, but had no effect on neurons expressing GFP. It indicates that the low level of endogenous MORs could only allow slow opioid-induced plasticity of excitatory synapses under normal conditions. (5) A theoretical model suggests that morphine might affect the function of spines by decreasing the electrotonic distance from synaptic inputs to the soma.

INTRODUCTION

Many previous studies show that opioids can increase the excitability of neuronal circuits by inhibiting the release of pre-synaptic vesicles in GABAergic neurons (Wimpey and Chavkin, 1991; Madison and Nicoll, 1988; Jin and Chavkin 1999; Williams et al., 2001). AMPA receptors, particularly GluR1 subunits, are reported to be important for morphine tolerance and dependence (Vekovischeva, et al., 2001; Stephens and Mead, 2003), 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; Kennedy 1997, 2000; Hering and Sheng, 2001; Nimchinsky et al., 2002). A recent study showed that selfadministration 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 weeks 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 (von Zastrow et al., 2003; Dang and Williams, 2004 and 2005). 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; Minnis et al., 2003; Sternini et al., 1996; von Zastrow, 2001; Whistler and von Zastrow, 1998; Yu et al., 1997; Koo et al., 1998). 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 “non-internalizing” 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 (Kelley 2004; Nestler 2002; Biala et al., 2005; Vorel et al., 2001; Fan et al., 1999). 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.

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 and striatum at postnatal day 1-2 were prepared as previously described (Liao et al., 2001 and 1999; Brustovetsky et al., 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 1×10^6 per dish (each type contained 0.5×10^6). 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 1×10^6 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 (Lin et al., 2004).

Electrophysiology:

Miniature excitatory postsynaptic currents (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 2 neurons were attempted from the same culture dish. Miniature EPSCs were recorded at holding potentials of $-55 \sim -60$ mV and filtered at 1 KHz. Input and series resistances were checked before and after the recording of mEPSCs, which lasted about 10-30 minutes. There were no significant difference in the series resistances and input resistance among various groups of experiments. One recording sweep lasting 200 milliseconds was sampled for every 1 sec. Miniature EPSCs were recorded in cultured dissociated neurons in standard Earle’s Balanced Salts Solution (EBSS) at room temperature with 200 μ M APV (NMDA receptor blocker; D, L-form, the concentration is equivalent to active form at 100 μ M), 1 μ M TTX (sodium current blocker) and 100 μ M picrotoxin (GABA receptor antagonist), gassed with 95% air and 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 100 mM cesium gluconate, 0.2 mM EGTA, 0.5 mM MgCl₂, 2 mM ATP, 0.3 mM GTP and 40 mM HEPES (pH 7.2 with CsOH).

All mEPSCs were analyzed with the Mini!Analysis 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 timecourse 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 time and decay time 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 (Figures 1 and 5) or MOR-GFP and DsRed (Figure 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 in order to compare the distribution of MORs between these two types of cultures (Figure 2). For a further comparison, the distribution of MORs in cortico-striatal neurons was also examined (Figure 2). All digital images were analyzed with the MetaMorph Imaging System (Universal Imaging Co.). 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 non-spine 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. In order 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 Mean $\pm$ Standard Error. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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 (Cox, 2005; Celver et al., 2004). 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. Miniature EPSCs were recorded in 3-week-old GFP-labeled neurons with clearly visible dendritic spines (Figure 1A). Chronic treatment with morphine (10 μM) caused mEPSCs to become smaller and faster than untreated control (Figure 1B) and significantly decreased the amplitude, frequency, rise time and decay time of mEPSCs (Figure 1C-F, $n = 10$ in each group; black bars and black circles). In contrast, DAMGO (1 μM) and etorphine (1 μM) had no significant effect on the amplitude and time-kinetics of mEPSCs (Figure 1C-D and F) but significantly increased their frequency (Figure 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.

The 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 post-synaptic 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 (Figure 2A) or the striatum of rats (Figure 2B-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 (Figure 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 (Figure 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 non-spiny neurons in primary striatal cultures after 21 DIV (only 24% of MOR-GFP-expressing neurons were spiny neurons; n=10 dishes; Figure 2B-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 non-spiny neurons in cultured hippocampal neurons. Interestingly, MORs were exceptionally abundant in axon-like processes in these non-spiny 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 post-synaptic changes in specific types of neurons. The diverse MOR distribution in various types of neurons might provide reconciliation for many previously reported pre- or post-synaptic 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 pre-synaptic inputs (Segal et al., 2003). It is possible that the strong expression of MOR-GFP in axon-like processes in non-spiny neurons (Figure 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 non-spiny neurons (Figure 2F, n=14 dishes). Consistent with results in Figure 2A-D, MOR-GFP molecules were clustered in dendritic spines in spiny neurons (see the arrows in Figure 2E, right) and were strongly expressed in axon-like processes in non-spiny neurons (see the triangle in Figure 2E, left). These results indicate that the differential distribution of MOR-GFP in spiny and non-spiny 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 non-spiny 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 (Figure 2). The relative homogeneity of these hippocampal neurons gave us a useful tool to test postsynaptic effects of opioids. To address the issue of post-synaptic MOR activities, two plasmids encoding GFP-tagged μ -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; Figure 3). Miniature EPSCs were recorded to examine the function of dendritic spines (see the recording pipette in Figure 3A, left; mEPSC traces in Figure 4), DsRed was used to label dendritic morphology (Figure 3A, middle), and MOR-GFP was used to rescue the MOR deficit in cultured neurons (Figure 3A, right). Similar to cultured rat neurons (Figure 2), MOR-GFP molecules also clustered in dendritic spines in mouse cultures (Figure 3B 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, Figure 3C and F). In contrast, DAMGO, another MOR agonist, significantly increased the density of dendritic protrusions and spines after 3-6 days of treatment (Figure 3D 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 Figure 3A, left for configuration; Figure 4A for sample traces). The transfected neurons (left 3 averaged traces in Figure 4B) contain exogenous MORs whereas the untransfected neurons contain neither endogenous nor exogenous MORs (right trace in Figure 4B). Morphine decreased the frequency, amplitude, rise time and decay time in neurons that expressed exogenously introduced MORs (Figure 4A-F; $n=10$ in each group) but did not suppress excitatory synaptic transmission in neurons that did not express MORs (untransfected neurons; Figure 4B-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 (Figure 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 (Figure 5A-B; $n=10$ in each group). Morphine significantly decreased the amplitude, frequency, rise time and decay time of mEPSCs (Figure 5C-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 (Figure 5C-F). Data in Figures 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 Figure 6A). In the control group, no morphine was applied to the GFP-labeled neurons during the recording of mEPSCs (Figure 6A, left). In the other two groups, mEPSCs were recorded in neurons expressing GFP or MOR-GFP before and after treatment with 10 μ M morphine (Figure 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 minutes of treatment (Figure 6B-D and 6F). Morphine had no significant effect on mEPSC frequency, rise time and decay time in neurons over-expressing MOR-GFP (Figure 6E-G), indicating that morphine might regulate these parameters through either a different mechanism or the same mechanism but in different temporal phase. Interestingly, morphine had no effect on neurons expressing GFP alone (Figure 6), indicating that the endogenous level of MORs is relatively low under normal conditions, which normally allow only slow opioid-induced plasticity of dendritic spines.

DISCUSSION

Differential Effects of “Internalizing” and “Non-Internalizing” 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 (Robinson et al., 2002; Robinson and Kolb, 2004; Liao et al., 2005). In a proposed “RAVE” (receptor activity versus 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; Minnis et al., 2003; Sternini et al., 1996; von Zastrow, 2001; Whistler and von Zastrow, 1998; Yu et al., 1997; Kovoov et al., 1998). In contrast, DAMGO and etorphine cause robust internalization and desensitization of opioid receptors (Kieffer and Evans, 2002; von Zastrow et al., 2003; Dang and Williams, 2004). 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 "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

The 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 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. $m = n \cdot p$, 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 central nervous system (CNS), $F_m = n \cdot p'$, 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 pre-synaptic termini, 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).

The Kinetics of mEPSC: 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 by morphine-induced shortening of dendritic spines (see the theoretical model in Figure 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 when the electrotonic distance increases as described by the equation: $V(X)/V_o = \cosh(L -$

X)/cosh L (continuous cable model; Rall and Segev, 1985; Carnevale and Johnston, 1982). 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 (Tsay and Yuste, 2004; Segev and Rall, 1988; Johnston and Wu, 1995). 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 (Figure 7A). When the length of the spine stem is decreased by morphine (Figure 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 ~20% and a decrease in R_i' (resistivity of the spine neck) might account for another ~10% reduction (Figure 7D). The most critical assumption in our theoretical model is that the resistivity of spine neck (R_i') is much higher than that in the dendrite (R_i). 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 (Figure 7; due to decreases in R_i' and R_i), although other factors such as spine size and glutamate uptake may also play minor roles.

Compared with previous studies (Thiagarajan et al., 2005; Stellwagen 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 week-old neurons is much slower than that from 2 week-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 Figure 7, the kinetics of mEPSCs from a dendritic spine should be slower than that from a non-spiny 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 (Figure 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 upon 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 (Luo, 2000; Lippman and Dunaevsky, 2005). 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 Post-Synaptic 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 pre-synaptic release of GABA (the “disinhibition” theory; Wimpey and Chavkin, 1991; Madison and Nicoll, 1988; Williams et al., 2001). As shown in Figure 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 post-synaptic effects if different cell types were examined. However, we believe that MOR-mediated effects on hippocampal pyramidal neurons are mainly post-synaptic 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-administrated 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 (Figure 2). (5) In neurons cultured from MOR knock-out mice, chronic treatment with morphine only suppressed mEPSCs in neurons expressing exogenous MOR-GFP (Figures 3-4).

Conclusions:

This study has contributed two key pieces of knowledge explaining normal opioid neurophysiology and pathology. Firstly, 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 over-activated. The normal level of endogenous MORs allows only slow opioid-induced plasticity of dendritic spines. Secondly, this study demonstrates a correlation between receptor internalization and opioid-induced plasticity of excitatory synaptic transmission by showing that non-internalizing 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.

Acknowledgements

Acknowledgements: We thank Drs. Janet Dubinsky, George Wilcox, David Redish, Robert Miller for their helpful comments, and Mr. Eric Nordstrom and Mr. Paul Haggins for their technical support. This study is supported by a grant from NIDA (R01DA020582), a grant from the Whitehall Foundation, a grant from McManus Trust for Drug

Abuse and a grant from the Minnesota Medical Foundation to DL. This study is also supported by NIDA grants DA01806, DA007339, DA000564, DA016674, K05-DA70554 and K05-DA000513 to HHL and PYL respectively.

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Figure 1

Figure 1.

The effects of DAMGO and etorphine on mEPSCs are distinctly different from morphine.

A. Miniature EPSCs were recorded in neurons cultured from the hippocampus of rats and transfected with GFP alone. *Upper*, 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 (open), morphine-treated (black), DAMGO-treated (gray) and etorphine-treated neurons (slashed), plotted as histograms. **D.** Cumulative frequency curves of mEPSC amplitudes from untreated (gray circle), morphine-treated (black circle), DAMGO-treated (black triangle) and etorphine-treated neurons (gray triangle). Bin size = 0.5 pA. **E** and **F.** The frequency and time-kinetics of mEPSCs in the above 4 groups of neurons are plotted as histograms.

Figure 2

Figure 2.

The distribution of 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 (arrows). **B-C.** MOR-GFP is highly concentrated in axon-like processes in non-spiny striatal neurons. *Arrows:* The axon in C is continued from B at this location. **D.** An enlarged image from the same neuron as in B and C shows that this is a non-spiny neuron. **E.** Cortico-striatal cultures contain both spiny (left) and non-spiny neurons (right). The arrows denote that MOR-GFP is clustered and concentrated in dendritic spines (left). The triangle denotes a strong expression of MOR-GFP in axon-like processes in non-spiny neurons (right). **F.** The proportion of "spiny" neurons versus total MOR-GFP-expressing neurons in hippocampal (H), striatal (S) and cortico-striatal (CS) cultures. The visual field was moved to approximately the center of a culture dish and randomly moved to search for MOR-GFP-expressing neurons. The first encountered 8 neurons were photographed and a neuron that contains > 5 spine-like protrusions per 100 μ m dendrite is defined as a "spiny"

neuron. The number of “spiny” neurons is divided by 8 to estimate the percentage of “spiny” neurons in each dish (the Y-axis in F) and each experimental group contains 10-14 dishes (see the Results). Both striatal and cortico-striatal cultures contain significantly fewer “spiny” MOR-GFP-expressing neurons than hippocampal cultures.

Figure 3

Figure 3.

Opioids can directly act on postsynaptic neurons, causing morphological plasticity of dendritic spines in cultured hippocampal neurons. **A.** Miniature EPSCs 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. Arrows show the location of the patch electrode. **B.** Zoom-in images from **A** (denoted by the triangle in **A**) including DsRed (left), MOR-GFP (middle) and overlay (right). *Arrows* denote the clustering of MORs in dendritic spines. **C-D.** Similar neurons had been treated with morphine and DAMGO for >3 days. **E.** The ratio of fluorescence intensity in the spines versus adjacent dendrites was estimated in untreated neurons. *Open bar*, a ratio calculated from images taken in red fluorescence channel. *Solid bar*, 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%. **F.** The density of

dendritic protrusions and spines in untreated, morphine-treated and DAMGO-treated neurons in DsRed images.

Figure 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.** The amplitudes of mEPSCs from the above 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-F.** Frequency, rise time and decay time of mEPSCs in the same four groups of neurons: untreated control (open), morphine-treated (black), DAMGO treated (gray) and untransfected (slashed).

Figure 5

Figure 5.

The 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 in G) or DAMGO (right in G) for > 3 days. Untreated neurons were used as control (left in G). **B.** The density of dendritic spines in the three groups of neurons plotted as histograms (open, untreated; black, morphine-treated; gray, DAMGO-treated). **C.** Amplitudes of mEPSCs plotted as histograms. **D.** The cumulative frequency curves of mEPSC amplitudes plotted. Bin size = 0.5 pA. **E-F.** The frequency, rise time and decay time of mEPSCs plotted as histograms.

Figure 6

Figure 6.

Morphine acutely decreased mEPSC amplitude in neurons expressing MOR-GFP, but had no effect on GFP-labeled cultured rat hippocampal neurons. **A.** Three 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 minutes and no morphine was applied during the recording. The early 5 minutes were used as baseline (black trace, average of mEPSCs from a sample neuron) and the later 25 minutes were used for comparison (gray trace). *Middle*, mEPSCs were recorded in GFP-expressing neurons for 5 minutes before morphine application (black trace) and were continuously recorded for another 25 minutes after morphine application (gray trace). *Right*, mEPSCs were recorded in MOR-GFP-expressing neurons before and after morphine application. **B.** The upper example traces were from a GFP-expressing neuron with no morphine application and the lower example 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-tests show that the mean mEPSC amplitudes are not significantly changed during the period of recording (see D). The gray curves were slight 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-E.** The 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.

Figure 7

Figure 7.

A theoretical model showing that morphine can significantly decrease the electrotonic distance from the soma to the head of spines. **A.** 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 *The Kinetics of mEPSCs* in the Discussion). Points "a" and "e" 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 $L' = (ab/\lambda + bc/\lambda')$. λ is the space constant of the dendrite and λ' is the space constant of the spine stem. Because the effect of increasing cable length on the kinetics of synaptic current is small if the position of the synapse remains the same, the EPSC kinetics should be mainly determined by the electrotonic distance from the synapse to the soma (Spruston et al., 1993). Therefore, the attenuation of EPSC kinetics at point "c" should be similar to point "d". **B-C.** Assuming morphine decreases the length of the spine stem from 2 μm to 0.5 μm , the electrotonic distance from the spine head to the dendritic shaft should be decreased by $1.5/\lambda'$. **D.** The

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 Figure 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_i' since the obstruction of free ion movement might decrease during the collapse of spines. This decrease in R_i' might lead to an extra 10% decrease in the rise time of mEPSCs.