Increased Insertion of Glutamate Receptor 2-Lacking α-Amino-3-hydroxy-5-methyl-4-isoxazole Propionic Acid (AMPA) Receptors at Hippocampal Synapses upon Repeated Morphine Administration

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ABSTRACT

Evidence suggests that the long-term adaptations in the hippocampus after repeated drug treatment may parallel its role during memory formation. The neuroplasticity that subserves learning and memory is also believed to underlie addictive processes. We have reported previously that repeated morphine administration alters local distribution of endocytic proteins at hippocampal synapses, which could in turn affect expression of glutamate receptors. Glutamatergic systems, including α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs), are believed to be involved in opiate-induced neuronal and behavioral plasticity, although the mechanisms underlying these effects are only beginning to be understood. The present study further examines the effects of repeated morphine administration on the expression and composition of AMPARs and the functional ramifications. Twelve hours after the last morphine injection, we observed an increased expression of AMPARs lacking glutamate receptor (GluR) 2 in hippocampal synaptic fractions. Immunoblotting studies show that 12 h after morphine treatment, GluR1 subunits are increased at the postsynaptic density (PSD) and at extrasynaptic sites, whereas GluR3 subunits are only increased at the PSD, and they show how this alters receptor subunit composition. In addition, we provide electrophysiological evidence that AMPARs are switched to Ca2+-permeable (GluR2-lacking) at the synapse 12 h after repeated morphine treatment, affecting the magnitude of long-term depression at hippocampal neurons. We propose that morphine-induced changes in glutamatergic synaptic transmission in the hippocampus may play an important role in the neuroadaptations induced by repeated morphine administration.

Addictive processes involve neuronal plasticity (Kauer and Malenka, 2007), thus, repeated exposure to drugs of abuse, such as morphine, produces long-term molecular and neurochemical changes that may underlie drug-induced behavioral responses. Considerable advances have been made toward understanding the anatomical substrates involved in these drug-induced neuroadaptations that may drive addictive behaviors in response to repeated morphine administration (Vanderschuren and Kalivas, 2000). Although it is accepted that the reward pathway (i.e., nucleus accumbens, VTA) is involved, recent reports highlight the role of the hippocampus in the regulation of these neuroadaptations that may underlie opiate-induced behavioral modifications (Shen et al., 2006). The hippocampus is one of the most important subcortical brain regions implicated in memory and learning.

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ABBREVIATIONS: VTA, ventral tegmental area; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; PSD, postsynaptic density; LTD, long-term depression; fEPSP, extracellular field excitatory postsynaptic potential; EPSC, excitatory postsynaptic currents; HFS, high-frequency stimulation; LFS, low-frequency stimulation; D-APV, 2-amino-5-phosphonovalerate; RI, rectification index; Ptx, philanthotoxin; JST, Joro spider toxin; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; PTX, picrotoxin; ANOVA, analysis of variance; IP, immunoprecipitation; FV, fiber volley; PPF, paired-pulse facilitation; CGP55845, 2S-3-[[1S]-1-(3,4-dichlorophenyl)ethyl][amino-2-hydroxypropyl][phenylmethyl]phosphinic acid hydrochloride; QX314, N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium; ACSF, artificial cerebral spinal fluid.
it is also involved in the rewarding effects of abused drugs (Vorel et al., 2001). Long-lasting drug-induced alterations in the hippocampus have the potential to influence the brain reward pathway and are likely to contribute to the intense memories associated with drug use and the propensity for drug relapse (Weiss, 2005). Therefore, the investigation of hippocampal neuroadaptations induced by repeated morphine treatment has great potential to reveal mechanisms contributing to the development of opiate addiction.

We have reported previously that a paradigm of repeated administration with escalating doses of morphine causes a redistribution of endocytic proteins at hippocampal synapses, which in turn may modulate glutamatergic synaptic transmission and plasticity in the hippocampus by altering clathrin-dependent endocytosis of AMPARs (Morón et al., 2007). AMPARs mediate the majority of rapid excitatory neurotransmission in the mammalian central nervous system, and their regulation directly controls synaptic efficacy (Palmer et al., 2004). Subunit composition is critical in determining the function and trafficking properties of resulting channels (Malinow and Malenka, 2002). The majority of AMPARs in hippocampal neurons are GluR2-containing (GluR1/GluR2 and GluR2/GluR3) heteromeric complexes, although there is evidence for GluR1 homomers and GluR2-lacking heteromers (Sprengel, 2006). GluR2-containing AMPARs have low Ca\(^{2+}\) permeability and consequently display linear current-voltage (I-V) relationships, whereas GluR2-lacking AMPARs have high Ca\(^{2+}\) permeability and exhibit inwardly rectifying postsynaptic currents (Cull-Candy et al., 2006). Disruption of AMPAR composition causes critical perturbations in subunit assembly and efficiency in trafficking of the receptor to the membrane (Passafaro et al., 2001). Alterations in these cellular processes have a direct effect on synaptic transmission.

In this study we further explore the effects of repeated morphine administration on the expression and subunit composition of AMPARs at hippocampal synapses and how these effects affect AMPAR-mediated synaptic transmission. We use multiple approaches to analyze the changes in AMPARs that were due to morphine treatment, including subcellular fractionation coupled with immunoblotting, and electrophysiology. Biochemical data indicate that there are alterations in both expression and synaptic localization of GluR1 and GluR3 AMPARs subunits 12 h after morphine administration, which consequently leads to changes in AMPAR subunit composition. In addition, we provide electrophysiological evidence that AMPAR subunits are switched to Ca\(^{2+}\) permeable AMPARs (GluR2-lacking) at the synapse resulting in functional changes. These results provide evidence of the synaptic, molecular, and membrane mechanisms of dynamic trafficking of AMPA receptors resulting from repeated morphine administration.

**Materials and Methods**

**Animals and Morphine Treatment.** Experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch, according to the U.S. Department of Agriculture Animal Welfare Act (Public Law 89-544) and the National Institutes of Health’s Guide for the Care and Use of the Laboratory animals (Department of Health, Education, & Welfare publication no. 85-23, revised 1985). Four-week old male wild-type C57BL6 mice (15–20 g) (Harlan, Indianapolis, IN) were maintained on a 12-h light/dark cycle and allowed to acclimate to their environment for 1 week before drug administration. Morphine sulfate (Sigma-Aldrich, St. Louis, MO) was prepared in 0.9% isotonic saline. Mice (n = 6–10 per group) were injected intraperitoneally with saline or morphine; in the latter case, four escalating doses of morphine (5, 8, 10, and 15 mg/kg) every 12 h for 48 h as described previously (Morón et al., 2007) were used. Animals were sacrificed 12 h after the last drug injection. The hippocampi from morphine or saline-treated mice were dissected and stored at −80°C until use.

**Subcellular Fractionation.** Subcellular fractionation was performed as described previously (Morón et al., 2007). In brief, hippocampi (3–5 animals/group) were homogenized in 1.5 ml of 0.32 M sucrose, 0.1 mM CaCl\(_2\) containing protease and phosphatase inhibitors (Sigma-Aldrich). The homogenate was brought to a final concentration of 1.25 M sucrose by adding 2 M sucrose and 0.1 mM CaCl\(_2\). The homogenate was then placed in an ultracentrifuge tube and overlaid with 1 M sucrose and subjected to centrifugation at 100,000g for 3 h at 4°C. The synaptosomal fraction was collected at the 1.25 M/1 M sucrose interface. To obtain synaptic junctions, the synaptosomal fraction was diluted with 20 mM Tris-Cl, pH 6, 0.1 mM CaCl\(_2\), containing 1% Triton X-100 (TX-100), mixed for 20 min at 4°C, and centrifuged at 40,000g for 20 min at 4°C. The pellet containing the isolated synaptic junctions was collected. To separate presynaptic proteins from the PSD, the pellet was resuspended in 20 mM Tris-Cl, pH 8, 1% TX-100, and 0.1 mM CaCl\(_2\). The mixture was again mixed for 20 min at 4°C, and centrifuged at 40,000g for 20 min at 4°C. The insoluble pellet containing the PSD fraction was collected and stored at −80°C until use.

**Immunoblotting.** For immunoblotting, equal amounts of total proteins from fractions obtained from morphine- and saline-treated animals were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by electroblotting. Membranes were then incubated with selective antibodies to GluR1 (1:1000) (C terminus), phosphoGlur1 Ser845 (1:1000), GluR2 (1:1000) (N terminus), or GluR3 (1:500) (N terminus; all from Millipore Bioscience Research Reagents, Temecula, CA). After incubation with appropriate fluorescent secondary antibody (1:10,000–1:20,000) (LI-COR Biosciences, Lincoln, NE), membranes were scanned directly by the Odyssey infrared fluorescent imaging system (LI-COR Biosciences). Blots were reprobed with antibody to tubulin (Sigma-Aldrich) to ensure equal loading and transfer. Band densities were analyzed using Odyssey software. Quantification was performed by measuring the intensity of the band with the protein-specific antibodies and comparing with that of tubulin to control for variation in loading and transfer. These values were normalized to saline control values. Differences between groups were determined by one-way ANOVA followed by Tukey test for multiple comparisons among groups.

**Quantitative Coimmunoprecipitation.** Synaptosomal membranes from mice hippocampi were prepared as described previously (Morón et al., 2007) with minor modifications. In brief, hippocampi from individual animals were homogenized using a glass homogenizer in ice-cold 10 mM Tris-HCl, pH 7.4, 5 mM NaF, 1 mM EDTA buffer (TE) containing 350 mM sucrose, and protease and phosphatase inhibitors. The homogenate was then centrifuged at 100,000g for 1 h at 4°C. The supernatant (LP1) containing the PSD fraction was collected. To separate presynaptic proteins from the PSD, the pellet was resuspended in 20 mM Tris-Cl, pH 8, 1% TX-100, and 0.1 mM CaCl\(_2\). The mixture was again mixed for 20 min at 4°C, and centrifuged at 40,000g for 20 min at 4°C. The insoluble pellet containing the PSD fraction was collected and stored at −80°C until use.

**Immunoprecipitation.** Synaptosomal membranes from mice hippocampi were prepared as described previously (Morón et al., 2007) with minor modifications. In brief, hippocampi from individual animals were homogenized using a glass homogenizer in ice-cold 10 mM Tris-HCl, pH 7.4, 5 mM NaF, 1 mM EDTA buffer (TE) containing 320 mM sucrose, and protease and phosphatase inhibitors. The homogenate was then centrifuged at 100,000g for 1 h at 4°C. The insoluble pellet containing the PSD fraction was collected and stored at −80°C until use.
pelleted by centrifugation at 1000g for 30 s and washed three times with 0.1% TBS/Triton X-100. Synaptosomal membranes (100 μl) were then added to the beads and incubated overnight with gentle rocking at 4°C. The samples were then centrifuged at 1000g for 30 s to pellet the agarose-bound antibody. We obtained two fractions, the bound (pellet) and the unbound (supernatant). The unbound fraction was then subjected to a second round of immunoprecipitation. After the final round of immunoprecipitation, the unbound sample was quantified using the BCA assay kit from Pierce. Equal amounts of unbound proteins (12 μg) were run on 7.5% gels, transferred to nitrocellulose membranes and immunoblotted as described above. The percentage of total AMPA receptor remaining in the unbound fraction was calculated based on the standard curve (i.e., 14 μg (100%), 7 μg (50%), and 0.7 μg (5%)) created from control IgG immunoprecipitated sample.

Electrophysiology. Mice were sacrificed 12 h after receiving their last injection of saline or morphine as described above. Hippocampal slices were prepared by following standard procedures. In brief, brains were removed rapidly and sliced to 400 μm thickness in an ice-cold cutting solution containing 240 mM sucrose, 2.5 mM KCl, 1.25 mM NaH2PO4, 25 mM NaHCO3, 0.5 mM CaCl2, and 7 mM MgCl2, and bubbled continuously with 95% O2/5% CO2. After incubation for at least 30 min at room temperature, the slices were transferred and submerged in the recording chamber. Both incubating and recording chambers contained artificial cerebrospinal fluid (ACSF): 119 mM NaCl, 3.0 mM KCl, 1.2 mM NaH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 25 mM NaHCO3, and 11.5 mM glucose. The temperature of the recording chamber was kept at 32 ± 1°C. Synaptic responses were evoked through a concentric bipolar electrode placed in the Schaffer collateral fiber bundles. Extracellular field excitatory postsynaptic potential (fEPSP) measurements were recorded using a glass Ag/AgCl electrode filled with ACSF in the presence of 100 μM picrotoxin (PTX), 2 μM (S)-3-[(1S)-(3,4-dichlorophenyl)ethyl] amine-2-hydroxypropyll[phenylmethyl]phosphinic hydrochloride (CGP55845), and 50 μM D-APV. Stimulation parameters consisted of a square-wave current pulse of 150-μs duration. Stimulus-response curves were performed at the beginning of each experiment, and pulses at an intensity eliciting 50% of a maximum slope were used. In the paired-pulse paradigm, the first conditioning response (fEPSP1) and the second, or test response (fEPSP2), were elicited at intervals of 30 to 140 ms. Peak amplitude of the paired fEPSP responses was measured. The NMDAR-mediated EPSC was obtained by digital subtraction of the AMPAR EPSC from the dual EPSC. AMPA-evoked whole-cell currents were recorded by bath application of S-AMPA in the presence of 1 μM tetrodotoxin.

Data Analysis. Results are expressed as the mean ± S.E.M. Statistical comparisons were made using one-way ANOVA with Tukey’s test for multiple comparisons, two-way ANOVA, or unpaired t test where appropriate. Significance was set at p < 0.05. Statistical analyses of data were generated by using Prism software (ver. 5.0; GraphPad Software Inc., San Diego, CA).

Results

Morphine Administration Results in Increased Levels and Phosphorylation of GluR2-Lacking Receptors in Synaptic Fractions. Exposure to drugs of abuse, such as morphine, has been shown to alter the levels and composition of AMPARs at the synapse (Zhang et al., 2008). Regulation of the number of postsynaptic AMPARs at a given synapse is recognized as an integral feature of rapid long-term changes in synaptic strength (Kauer and Malenka, 2007) that may be responsible for the long-term behavioral modifications elicited by drug administration. Using the morphine administration paradigm described above, we specifically examined the effects of morphine on the expression and phosphorylation of AMPAR subunits in synaptic fractions 12 h after morphine treatment to elucidate neuroadaptations in response to morphine. By coupling subcellular fractionation with immunoblotting, we were able to assess changes in both expression and location of the receptors within the synapse, with focus on the PSD (Morón et al., 2007; Billa et al., 2009).

Hippocampi were dissected, and subcellular fractionation was performed as reported previously (Morón et al., 2007; Billa et al., 2009). Quantification of phosphoGluR1, GluR1, GluR2, and GluR3 subunits was performed by Western blot analysis in synaptic fractions from saline- and morphine-treated animals, and results obtained are shown in Fig. 1. Data show that morphine treatment produced a robust increase in the total levels of GluR1 in the hippocampal homogenate and synaptosomes (*, p < 0.05; **, p < 0.01; one-way ANOVA with Tukey’s test). In addition, morphine treatment also increased GluR1 levels at the PSD, albeit to a lesser extent (*, p < 0.05; one-way ANOVA with Tukey’s test) (see Fig. 1A). Because expression of GluR1 at the cell surface is regulated by phosphorylation (Ehlers, 2000), we next examined the effect of morphine treatment on the phosphorylation of GluR1 at serine 845, a site known to be phosphorylated by protein kinase A (Colledge et al., 2000). Repeated morphine treatment led to a significant increase in GluR1 phosphorylation in the total homogenate, synaptosomes, and synaptic junctions (*, p < 0.05; **, p < 0.01; one-way ANOVA with Tukey’s test) (Fig. 1B). Although we also observed an increase in GluR1 phosphorylation at the PSD after morphine treatment, this was not significant. To determine whether the increase in pGluR1 was due to an elevation in total GluR1 levels, we analyzed whether the ratio of pGluR1/total GluR1 was altered. This ratio was calculated using the normalized band intensities for pGluR1 and GluR1, respectively, for each fraction. A significant increase in the ratio of pGluR1/total GluR1 (180 ± 15%) was found at the synaptic junctions (p < 0.01; one-way ANOVA with Tukey’s test).
Morphine administration resulted in an increase in GluR1 and GluR3 expression without affecting levels of the GluR2 subunit, we wanted to directly examine whether morphine administration had an effect on AMPAR subunit composition. To this end, we performed quantitative coimmunoprecipitation studies in hippocampal synaptosomal membranes from morphine- and saline-treated mice; results are shown in Fig. 2. Two independent experiments showed that repeated morphine administration decreased both the association between GluR1 and GluR2 and the expression of GluR2 at hippocampal synapses (Fig. 1C), showed that repeated morphine administration did not alter relative to saline-treated animals. In addition, these data indicate an increase in GluR2-lacking receptor levels at 12 h upon repeated morphine exposure.

**Morphine Administration Alters AMPAR Subunit Composition.** Given the observation that repeated morphine administration decreased both the association between GluR1 and GluR2 and the expression of GluR2 at hippocampal synapses (Fig. 1C), we wanted to directly examine whether morphine administration had an effect on AMPAR subunit composition. To this end, we performed quantitative coimmunoprecipitation studies in hippocampal synaptosomal membranes from morphine- and saline-treated mice; results are shown in Fig. 2. Two independent experiments showed that repeated morphine administration decreased both the association between GluR1 and GluR2 and the expression of GluR2 at hippocampal synapses (Fig. 1C), showed that repeated morphine administration did not alter relative to saline-treated animals. In addition, these data indicate an increase in GluR2-lacking receptor levels at 12 h upon repeated morphine exposure.
between GluR1 and GluR3; in contrast, morphine increased the association between GluR2 and GluR3: 1) after GluR1 immunoprecipitation (IP), morphine-treated mice showed an increase in unbound GluR2 (24% in saline-versus 42% in morphine-treated mice) and GluR3 (56% in saline-versus 75% in morphine-treated mice), indicating an increase in GluR2 or GluR3 not associated with GluR1; and 2) after GluR2 IP, morphine-treated mice showed a decrease in GluR3 remaining in the unbound fraction (82% in saline-versus 72% in morphine-treated mice) and an increase in unbound GluR1 (26% in saline-versus 43% in morphine-treated mice), indicating an increase in GluR3 associated with GluR2 and a decrease in GluR1 associated with GluR2, respectively. In addition, the increase in the absolute amount of GluR1 in both the synaptosomal fraction and the PSD shown above (see Fig. 1) that does not seem to be associated with GluR2 or GluR3 may indicate an increase in homomeric GluR1.

**Repeated Morphine Increases Basal Synaptic Transmission.** The changes in AMPAR expression and subunit composition shown above have direct consequences on synaptic function. To investigate the functional impact on glutamatergic synaptic transmission within the hippocampus, we used a series of electrophysiological approaches. First, we examined the effects of morphine treatment on glutamatergic transmission at the Schaeffer collateral to CA1 synapse. We performed these experiments in the presence of D-APV (50 μM) to block NMDA receptors. Changes in the slope of the input-output relationship provide a measure of efficacy of information transfer and synaptic gain. Input-output relationships were measured by varying stimulus input and measuring fEPSP slope as a function of the presynaptic fiber volley (FV) amplitude in slices from mice sacrificed 12 h after receiving their last injection of saline or morphine as described above. fEPSP slope magnitude significantly increased with increasing stimulus intensity and was greater in the morphine-treated population suggesting an increase in synaptic transmission ($F_{1,126}^* = 9.25; *, p < 0.05$, two-way ANOVA) (Fig. 3B). Furthermore, the relationship between FV amplitude and stimulus intensity was similar in the two groups, suggesting that presynaptic mechanisms may not be involved in morphine’s effect (Fig. 3C). Finally, the relationship of FV versus fEPSP showed that morphine treatment significantly increased fEPSP magnitude at similar presynaptic FV amplitudes (Fig. 3D) (linear regression comparison of the fit of two curves, $F_{3,294}^* = 31.8, p < 0.0001$). The slope of the input-output curve in neurons from morphine-treated animals ($6.18 \pm 0.28$ ms$^{-1}, n = 14$) was significantly greater than that in neurons from saline-treated animals ($4.39 \pm 0.25$ ms$^{-1}, n = 10, p < 0.001$). These data suggest an increase in synaptic efficacy in hippocampal CA1 neurons from morphine-treated animals. In addition, we examined PPF, which is a sensitive measure of changes in the probability of neurotransmitter release. Figure 3E shows that PPF was unchanged after morphine administration, indicating that morphine-induced increase in syn-

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**Fig. 3.** Morphine increases fEPSP but not fiber volley magnitude. A, traces of FVs and fEPSPs were recorded 12 h after discontinuation of treatment in hippocampal slices from saline- and morphine-treated animals. B, fEPSP magnitude increased as a function of stimulus intensity and was greater in morphine-treated animals ($F_{1,126}^* = 9.25, *, p < 0.05$, two-way ANOVA). C, relationship between FV amplitude and stimulus intensity did not differ between saline- and morphine-treated animals. D, input (FV)-output (fEPSP) shows that morphine treatment increased fEPSP magnitude at similar presynaptic fiber volley amplitudes (linear regression comparison of the fit of two curves, $F_{3,294}^* = 31.8, p < 0.0001$). E, PPF does not differ between saline- and morphine-treated animals.
aptic gain within the hippocampal circuitry involves postsynaptic events.

Our biochemical data show that morphine administration, besides increasing synaptic GluR1 and GluR3 levels, also produces a significant increase in the expression of extrasynaptic GluR1 (Fig. 1). To examine whether some of this extrasynaptic GluR1 pool could be responsible for the observed effects on synaptic transmission, we recorded whole-cell responses to bath application of AMPA, which activates AMPARs through the cell, including somatic and dendritic extrasynaptic AMPARs as well as synaptic AMPARs. We found that bath application of AMPA did not affect the size of currents in morphine-treated animals compared with those of saline-treated animals (Fig. 4, A and B). These results suggest that although we observe an increase in extrasynaptic receptors in our biochemical studies, this pool of receptors is not involved in the synaptic responses obtained with our electrophysiological approaches.

The relative contribution of AMPAR and NMDAR to excitatory synaptic currents is believed to play an important role in synaptic plasticity (Malinow and Malenka, 2002). We next measured the ratio of AMPAR- to NMDAR-mediated EPSCs in both morphine- and saline-treated animals. Figure 4D shows that the AMPAR/NMDAR ratio increased significantly 12 h after morphine administration (saline, 0.95 ± 0.04, n = 9; morphine, 1.59 ± 0.07, n = 9; p < 0.0001), indicating that the enhancement in synaptic transmission induced by morphine treatment is specific to the AMPAR component.

Repeated Morphine Treatment In Vivo Induces Inwardly Rectifying AMPAR EPSCs at Hippocampal Synapses. Next, we studied whether the observed increase in synaptic gain after repeated morphine administration was mediated through an alteration in RI [i.e., switching from linear Ca2+-impermeable (GluR2-containing) receptors to inwardly rectifying Ca2+-permeable (GluR2-lacking) receptors]. To this end, 12 h after the last injection, hippocampal neurons from saline- and morphine-treated animals were recorded with patch electrodes. AMPAR EPSCs were isolated by superfusing PTX (100 μM), CGP55845 (2 μM), and D-APV (50 μM) in ACSF; in addition, spermine (100 μM) and QX314 (5 mM) were included in the electrode solution. In CA1 neurons from saline-treated animals, the AMPAR EPSCs displayed an approximately linear I-V relationship with inward and outward currents of equivalent magnitude (Fig. 5A). In contrast, neurons from morphine-treated animals showed inward rectification (Fig. 5B). This finding suggests that morphine treatment induces an increase in the number of inwardly rectifying AMPARs at hippocampal synapses.
treated animals exhibited only limited ability to conduct outward AMPAR EPSCs and demonstrated inward rectification, a marker for the presence of GluR2-lacking receptors (Fig. 5B). Furthermore, the RI [calculated as EPSC (−60+/−40 mV)] was significantly increased in morphine-treated animals (2.62 ± 0.16) compared with saline-treated animals (1.44 ± 0.15) (**, p < 0.01, unpaired t test; Fig. 5C). Because the RI is a measure of Ca2+-permeable AMPARs, these data suggested that, although homomers may affect both basal synaptic transmission and plasticity, repeated morphine treatment has no impact on hippocampal LTP but reduces LTD at CA1 synapses.

The above data suggest increased insertion of GluR2-lacking receptors (Ca2+-permeable) at hippocampal synapses upon repeated administration of morphine. To further confirm this effect, we analyzed fEPSP in the presence of a polyamine toxin that specifically blocks Ca2+-permeable receptors (Tóth and McBain, 1998). Recording of fEPSP in the presence of 10 μM Phtx in slices from saline- and morphine-treated animals during basal synaptic transmission was performed as described above. Figure 6 shows that 12 h after discontinuation of morphine treatment, fEPSPs in hippocampal neurons showed a significant increase in the inhibitory effect of Phtx 15 min after infusion of the toxin, whereas fEPSP recorded in slices from saline-treated animals did not exhibit appreciable sensitivity to Phtx (***, p < 0.01, unpaired t test). These data provided further evidence of a greater insertion of Ca2+-permeable AMPA (GluR2-lacking) receptors after morphine treatment.

Repeated Morphine Treatment Has No Impact on Hippocampal LTP but Reduces LTD at CA1 Synapses. Activity-dependent insertion of AMPA-type glutamate receptors is believed to underlie the mechanisms of LTP (Plant et al., 2006; Derkach et al., 2007). A transient increase in postsynaptic GluR2-lacking AMPA receptor ion channels is required for the stabilization of LTP (Plant et al., 2006). Because morphine-induced dynamic trafficking of AMPARs may affect both basal synaptic transmission and plasticity, we next examined the effect of repeated morphine administration on LTP at Schaffer collateral fiber synapses in CA1 region. As shown in Fig. 7A, HFS produced a robust induction of LTP in slices from both saline- and morphine-treated rats. Comparing two groups at 60 min after HFS, the slopes of fEPSP increased by 168.5 ± 8.9% of baseline in the saline group and 159.0 ± 13.9% in the morphine group, respectively (p > 0.05, unpaired t test). These results indicate that repeated morphine treatment has a significant affect on the induction and maintenance phases of HFS-induced LTP in hippocampal synapses.

The data above indicated an increase in the expression and synaptic insertion of GluR2-lacking AMPA receptors (Figs. 1–3, 5, and 6), which have been suggested to be important for synaptic transmission (Chung et al., 2003). However, it was found that LTP was not altered after morphine treatment (Fig. 7A). We further tested whether LTD, another form of synaptic plasticity, had been altered. To this end, we measured the magnitude of LTD induced by LFS (1 Hz, 15 min) of the Schaffer collateral to CA1 synapses in hippocampal slices from saline- and morphine-treated mice. Figure 7B shows that LFS delivery produced a significant depression of fEPSP lasting at least 50 min in both saline- and morphine-treated animals. It is interesting that the magnitude of LTD was significantly decreased in morphine-treated mice compared with saline-treated mice (fEPSP slopes 50 min after LFS: saline, 80.5 ± 3.4% of baseline; morphine, 89.4 ± 3.8% of baseline; p < 0.05, unpaired t test). These data suggest that repeated morphine administration decreases the magnitude of LTD in the CA1 region. Finally, we further tested whether this effect was due to an increased insertion of GluR2-lacking (Ca2+-permeable) AMPA receptors. To this end, we analyzed morphine-induced alteration of LTD in the presence of 10 μM Phtx or 500 nM JST, polyamine toxins that specifically block Ca2+-permeable receptors, in slices from saline- and morphine-treated animals. Figure 7B shows that infusion of Phtx or JST completely reversed the morphine-induced decrease in LTD magnitude (fEPSP slopes 50 min after LFS: morphine, 89.4 ± 3.7% of baseline; Phtx-morphine, 78.8 ± 1.5% of baseline; JST-morphine, 80.1 ± 3.0% of baseline).

**Fig. 5.** Morphine treatment results in the insertion of Ca2+-permeable AMPA receptors. A, left, traces of EPSCs; right, linear current-voltage relationship obtained by plotting EPSC amplitude as a function of holding potential indicates presence of GluR2-containing AMPARs. B, Same as in A but recorded in hippocampal neurons from morphine-treated animals. Note that current-voltage relationship deviates from linearity, a marker of Ca2+ passing AMPARs not containing the GluR2 subunit. C, RI calculated as EPSC (−60+/−40 mV) was increased by morphine indicating the removal of GluR2 (***, p < 0.01, unpaired t test). The patch electrode contained 100 μM spermine to saturate polyamine binding sites.
baseline; \( p < 0.05 \), unpaired \( t \) test). These data confirm that the alteration in LTD observed after morphine administration is associated with an increase in the insertion of GluR2-lacking receptors.

**Discussion**

Behavioral evidence demonstrates that drugs of abuse produce changes in brain function that can be persistent (Kauer and Malenka, 2007). In this study, our goal was to examine these neuroadaptations in response to repeated morphine administration. In the present study, our biochemical and electrophysiological data indicate that morphine administration elicits changes in glutamate receptors and synaptic neurotransmission 12 h after discontinuation of the treatment.

It is known that GluR2-containing receptors are prevalent in the hippocampus during basal conditions (Sprengel, 2006). Here, we report an increase in the expression of the GluR1 subunit of AMPARs 12 h after repeated morphine treatment both at extrasynaptic and synaptic hippocampal sites. In addition, we found that morphine treatment also increased phosphorylation of GluR1 subunit at serine 845, the protein kinase A site (Lee et al., 2000), at synaptic fractions, which is in agreement with the effects observed with total GluR1. Up-regulation in the phosphorylation state of the GluR1 subunit in response to drug treatment has been reported previously. For example, an increase in the phosphorylation state of GluR1 at serine 845 in the dorsal hippocampus has been observed during cocaine-conditioned place preference behavior (Tropea et al., 2008). This effect has also been observed during withdrawal from heroin self-administration in the amygdala and the VTA (Edwards et al., 2009). Furthermore, previous studies obtained from our laboratory have shown that the extinction of morphine-conditioned place preference behavior is associated with an increase in the phosphorylation of GluR1 at hippocampal synapses (Billa et al., 2009).

In addition, we find that repeated morphine administration led to an increase in the expression of the GluR3 subunit at the PSD. In contrast, no changes in GluR2 levels were observed in any synaptic fraction upon morphine administration. GluR2 levels are not altered by morphine treatment because insertion of this subunit does not depend on synaptic activity (Shi et al., 2001). In addition, morphine-induced increase in GluR3 receptors may compete with the recycling of GluR2-containing receptors to the cell membrane (Shi et al., 2001). Overall, these data suggest that morphine administration may lead to changes in both the expression and location of AMPARs.

Subunit composition of AMPARs is also critical in determining the functional properties of resulting channels (Malinow and Malenka, 2002). Our data show that morphine administration could replace GluR1/GluR2-containing receptors for GluR2/GluR3-containing receptors, without affecting the total levels of GluR2. These results are similar to those reported by Conrad et al. (2008) during withdrawal from cocaine self-administration. In addition, these data are in agreement with the subcellular fractionation data that show a dramatic increase in GluR1 levels with no changes in GluR2 levels. Furthermore, the coimmunoprecipitation data indicate that this increased GluR1 does not seem to be associated with any other subunit, and therefore, this could re
fect an increase in homomeric GluR1, which would lead to an overall increase in GluR2-lacking receptors.

Therefore, we hypothesized that morphine exposure may increase the insertion of GluR2-lacking AMPARs at hippocampal synapses. Studies characterizing the electrophysiological mechanisms contributing to AMPAR subunit composition at glutamatergic synapses during basal synaptic transmission after repeated morphine administration were performed. We found that morphine treatment increased the fEPSP magnitude without altering fiber volley magnitude, which would be expected with a postsynaptic increase in synaptic transmission. This was further confirmed with our assay of PPF that showed no effect on presynaptic release after morphine treatment. This would be in agreement with our hypothesis that morphine-induced changes in glutamatergic transmission are related to changes at the postsynaptic terminal. Next, we examined whether changes in the gain of synaptic transmission after repeated morphine administration were mediated through an alteration in rectification index switching from linear Ca²⁺-impermeable receptors (GluR2-containing) to inwardly rectifying, GluR2-lacking, Ca²⁺-permeable AMPA receptors. We found that repeated morphine administration significantly increased the rectification index, a measure of GluR2-containing receptors (Washburn et al., 1997), reflecting an increased rectification in the current-voltage relationship and indicating an altered AMPAR composition at the synapse. This effect has also been observed in the VTA after a single injection of cocaine (Bellone and Lüscher, 2006). In addition, we also examined fEPSP sensitivity to Phtx as a measure of insertion of Ca²⁺-permeable AMPA receptors under basal conditions. The data obtained indicate that repeated morphine administration led to an increased sensitivity of basal fEPSPs to Phtx, which further validates the idea that morphine treatment promotes the insertion of GluR2-lacking receptors during basal synaptic transmission by altering the dynamics of AMPAR trafficking at the synapse.

Our biochemical data showed that in addition to increased synaptic levels of GluR1, morphine administration induced a dramatic increase in extrasynaptic GluR1. However, data with whole-cell recordings and bath application of AMPA, which activates both synaptic and extrasynaptic receptors, did not show an increase in AMPA responses after morphine administration. These data do not agree with the observed increase in extrasynaptic GluR1 obtained in our biochemical studies. One possible explanation is that these increased GluR1 receptors at extrasynaptic sites are not functionally available, particularly because morphine may also affect the rate of receptor endocytosis at the synapse (Morón et al., 2007).

At first sight, one would expect that an increase in the AMPA/NMDA ratios would be associated with an increase in LTP; however, our data show that morphine administration does not affect LTP. In addition, this lack of effect on LTP does not seem to agree with the biochemical results or with the electrophysiological recordings performed at baseline. One possible explanation is that if a synapse is already potentiated, the synapse may not show further potentiation, whereas the observed increase in GluR1 or GluR3 levels after morphine administration would be reflected as an increase in AMPA/NMDA ratio during baseline recordings. On the other hand, we should consider the existing controversy regarding the involvement of GluR2-lacking receptors on expression of hippocampal LTP. In this regard, some reports show that blockers of GluR2-lacking receptors prevent LTP in hippocampal slices (Plant et al., 2006; Lu et al., 2007), although this effect seems to be age-dependent. In contrast, another report shows that insertion and activation of GluR2-lacking receptors are not involved in LTP at excitatory synapses in the hippocampal CA1 region (Gray et al., 2007). AMPAR modulation of LTP after cocaine exposure has also been studied in other brain areas such as the VTA (Argilli et al., 2008) with different results to those obtained in our study. Again, it is quite likely that AMPAR systems may respond specifically to a particular drug of abuse and administration paradigm and that this response may be different depending on the brain structure studied.

On the other hand, the present study shows that repeated morphine administration decreases the magnitude of LTD and that this effect is related to an increased insertion of GluR2-lacking (Ca²⁺-permeable) receptors. More specifically, this effect on LTD is quite likely to be associated with the observed increase in the phosphorylation state of the GluR1 subunit at Ser845 after morphine administration. In this regard, it has been reported that dephosphorylation of GluR1 at Ser845 is an expression mechanism of LTD (Lee et al., 2000). By increasing phosphorylation of GluR1 at Ser845, morphine could alter LTD and thus affect its magnitude. Likewise, it is known that increased phosphorylation at this site is associated with stabilization of Ca²⁺-permeable (GluR2-lacking) receptors (He et al., 2009). Another possible explanation is that LTD depends on clathrin-dependent endocytosis (Wang, 2008). We have previously reported that the paradigm of morphine administration used in the present study may affect endocytosis of the GluR1 subunit (Morón et al., 2007). Therefore, morphine could also affect LTD by impairing clathrin-dependent endocytosis of GluR1-containing AMPARs.

Overall, data presented in this study provides electrophysiological and biochemical evidence that AMPAR subunits are switched to Ca²⁺-permeable AMPARs 12 h after repeated morphine treatment. This suggests that repeated use of opiates may lead to maladaptive changes in hippocampal synaptic transmission and plasticity, such as formation of GluR2-lacking receptors. Modulation of specific AMPAR subtypes may provide a viable therapeutic intervention in the development of opiate addiction.

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References


