Chronic Morphine Treatment Alters NMDA Receptor-Mediated Synaptic Transmission in the Nucleus Accumbens

Gilles Martin,¹ Serge H. Ahmed,¹ Thomas Blank,² Joachim Spiess,² George F. Koob,¹ and George R. Siggins¹

¹Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037, and ²Department of Molecular Neuroendocrinology, The Max-Planck Institute for Experimental Medicine, Goettingen, Germany

In a study of a possible substrate underlying morphine addiction, we examined NMDA receptor-mediated synaptic transmission of core nucleus accumbens neurons after chronic morphine treatment, using intracellular recording in a slice preparation of rat. We evoked pharmacologically isolated NMDA EPSCs by local stimulation and elicited inward currents by NMDA superfusion. In control slices, Mg^{2+} and phorbol 12,13-diacetate (PDAC), a protein kinase C activator, strongly inhibited and increased, respectively, NMDA EPSC amplitudes. The PDAC effects were likely postsynaptic because PDAC enhanced the currents evoked by superfused NMDA to the same extent that it did the NMDA EPSCs. Chronic morphine treatment significantly decreased NMDA EPSC amplitudes and the sensitivity of NMDA EPSCs to Mg^{2+} and PDAC, as well as the

kinetics of the decay (inactivation rate) of the EPSCs (from 97 ± 2.5 msec in untreated rats to 78.7 ± 1.8 msec in slices from treated rats). One week after withdrawal, the Mg^{2+} and PDAC effects were still significantly less than those in control slices. Interestingly, 1 week of withdrawal led to an increased NMDA EPSC inactivation rate compared with controls. These data demonstrate that chronic morphine treatment significantly alters NMDA receptor-mediated synaptic transmission in the accumbens, and these effects persist 1 week after withdrawal. These long-term effects may represent an important neuroadaptation in opiate dependence.

Key words: NMDA glutamate receptors; electrophysiology; phorbol ester; protein kinase C; chronic morphine treatment; kinetics

The nucleus accumbens (NAcc) is regarded as a pivotal brain structure in drug reinforcement (Koob et al., 1992). It receives massive dopamine input from the ventral tegmental area (VTA) and glutamate input from limbic structures such as the hippocampus, prefrontal cortex, and amygdala (for review, see Zahm and Brog, 1992; Groenewegen et al., 1996; Heimer et al., 1997). Whereas dopamine has long been considered a prime candidate mediating the addictive effects of opioids, other findings support the hypothesis that some opioid effects may be mediated directly by dopamine-independent mechanisms (Koob, 1992). Thus, glutamate-mediated synaptic transmission has emerged recently as another putative opiate substrate. Although non-NMDA (Kest et al., 1997) and metabotropic glutamate receptors (Fundytus et al., 1995, 1997; Fundytus and Coderre, 1997; Martin et al., 1999) have received some attention in opiate dependence and tolerance, the role of NMDA receptors (an ionotropic glutamate receptor subtype) has come under considerable scrutiny. Thus, behavioral studies showed that NMDA receptors may control some aspects of morphine tolerance and dependence (Marek et al., 1991; Trujillo and Akil, 1991, 1994; Trujillo, 1995) as well as sensitization (Jeziorski et al., 1994). Other evidence also suggests that NAcc NMDA receptors may be directly involved in opiate effects. Thus, our laboratory found previously that μ -opioid receptors regulate NAcc NMDA EPSPs both pre- and postsynaptically (Martin et al., 1997a). However, the mechanisms underlying

the possible role of NMDA receptors in opiate-rewarding effects are unknown.

Although still controversial, it is generally believed that NMDA receptors are heteromultimeric channels comprising six subunits (NR1, NR2A–D, and NR3A) identified to date. The NR1 subunit is regarded as the key NMDA receptor subunit (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993; Wang and Thukral, 1996). NR2A–D subunits, when coexpressed with NR1, confer distinct pharmacological properties to the NMDA receptor complex and essentially act as regulatory subunits. For example, recombinant NMDA receptors composed of NR1 and NR2A or NR1 and NR2B are much more sensitive to Mg^{2+} than are those composed of NR1 and NR2C or NR1 and NR2D subunits (Burnashev et al., 1992, 1995; Monyer et al., 1992; Kuner and Schoepfer, 1996). Similarly, the protein kinase C (PKC)-mediated phosphorylation of NMDA receptors (Kutsuwada et al., 1992; Mori et al., 1993), the kinetics of NMDA-evoked currents, and their sensitivity to polyamines (Williams et al., 1994; Gallagher et al., 1996; Takahashi et al., 1996; Flint et al., 1997; Gottmann et al., 1997) all seem to be under the control of NR2A and NR2B subunits.

Interestingly, several findings have suggested that NMDA receptor subunit expression is very sensitive to antipsychotic drugs, as well as to drugs of abuse like alcohol (Follesa and Ticku, 1996; Snell et al., 1996; Chen et al., 1997; Kalluri et al., 1998). Recently, Fitzgerald et al. (1996) reported that chronic morphine treatment increased the levels of NR1 subunits in the VTA. The involvement of discrete NMDA receptor subunits in opiate dependence is supported by a report that antisense oligonucleotides directed against the NR1 subunit attenuated morphine withdrawal signs (Zhu and Ho, 1998). Therefore, in the present study, we tested the hypothesis that chronic morphine treatment alters NMDA

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Correspondence should be addressed to Dr. George Robert Siggins, Department of Neuropharmacology, CVN-12 The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

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receptor properties, using intracellular voltage-clamp recording in NAcc slices and pharmacological and kinetic assays designed to help discriminate between different compositions of heteromeric NMDA receptor subunits.

MATERIALS AND METHODS

Animals and slice preparation. We used male Sprague Dawley rats (100–170 gm) to prepare NAcc slices from fresh brain tissue, as described previously (Martin et al., 1997b, 1999). The brains were rapidly removed and transferred to cold (4°C), oxygenated artificial CSF (ACSF) of the following composition (in mM): NaCl, 130; KCl, 3.5; NaH₂PO₄, 1.25; MgSO₄·7H₂O, 1.5; CaCl₂, 2; NaHCO₃, 24; and glucose, 10. We glued a tissue block containing NAcc to a Teflon chuck and cut it transversely with a vibroslice cutter (Campden Instruments) and immediately incubated the slices (400 μ m thick) in the recording chamber. During initial incubation in an “interface” configuration, the tops of the slices were exposed to a mixture of O₂ (95%) and CO₂ (5%). After 30 min, we completely submerged and superfused the slices with warm (34°C), carbogenated ACSF, at a rate of 3–4 ml/min. Slices from morphine-treated rats were maintained in 1 μ M morphine throughout experimentation.

Recording. We pulled sharp glass microelectrodes from borosilicate capillary glass (1.2 mm outer diameter and 0.8 mm inner diameter) on a Brown-Flaming puller (Sutter Instruments) and filled them with 3 M KCl. Tip resistances were 60–100 M Ω . We used an Axoclamp 2B amplifier (Axon Instruments) to record neurons in discontinuous single-electrode voltage-clamp mode. Throughout all experiments we continuously monitored electrode settling time and capacitance neutralization on a separate oscilloscope. Current and voltage levels were monitored and stored on polygraph paper, digitized by a Digidata interface (Axon Instruments), and acquired to a 486 personal computer using Clampex 6.0 software (Axon Instruments). The digitized records then were analyzed with Clampfit and Axograph software (Axon Instruments). We recorded neurons within the core NAcc just ventrally to the anterior commissure. For most of the cells we constructed three-step current–voltage curves in voltage-clamp mode with the first voltage step –20 mV negative to the holding potential (–80 mV), with an increment of +20 mV for the two subsequent steps.

Synaptic stimulation. We studied the NMDA component of EPSCs in voltage-clamp mode, using an *I–V* protocol (400 msec step duration) to measure EPSC amplitudes evoked at different membrane potentials. The NMDA EPSCs were elicited by local (“focal” or “proximal”) stimulation (see below) triggered 100 msec after the onset of, and superimposed on, the voltage pulse. We averaged two traces for each voltage-step size with superimposed NMDA EPSCs. To minimize the influence of stimulation artifacts on the NMDA current, we injected a 2 msec duration pulse into the amplifier-blanking circuitry 1 msec before the stimulation.

We evoked synaptic components through a tungsten bipolar stimulating electrode with a tip separation of 1 mm, placed in the NAcc within 1 mm of the recording electrode. Stimulation parameters (7–14 V; 50 msec pulse duration; 0.1 Hz) were chosen to generate sizable (near half-maximal) and reproducible NMDA EPSCs without spiking, and the stimulus intensity then was maintained constant throughout the recording period. The analysis of the NMDA EPSC amplitudes involved setting one cursor 1 msec before the stimulation artifact and the other at the peak of the response. We measured the inactivation rate via Clampfit software (Axon Instruments), by setting the first cursor at the peak amplitude and the second one 260 msec later. A single exponential (Chebyshev method) was used to fit the response and obtain the time to decay.

Drug administration. To isolate the NMDA EPSC component pharmacologically, we superfused the slices with antagonists specific for non-NMDA (kainate and AMPA) glutamate receptors (10 μ M CNQX) and GABA_A receptors (15 μ M bicuculline), for at least one-half hour before study. The identity of the isolated component was determined by superfusing the NMDA receptor antagonist D-APV (60 μ M) at the end of some experiments (see Martin et al., 1997a, 1999). In other studies, to test for postsynaptic phorbol 12,13-diacetate (PDAC) effects, we superfused NMDA in the presence of 1 μ M TTX to minimize presynaptic effects (in addition to CNQX and bicuculline). We purchased CNQX and PDAC from Research Biochemicals (Natick, MA) and D-APV and NMDA from Sigma (St. Louis, MO).

Induction of morphine dependence and withdrawal. Rats were made dependent by subcutaneous implantation of morphine pellets (75 mg of

Figure 1. Chronic morphine treatment reduces the amplitude of NMDA EPSCs. Input–output curves. Mean NMDA EPSC amplitudes in NAcc neurons from untreated ($n = 12$) and morphine-treated ($n = 10$) rats over an equivalent range of stimulus intensities are shown. Note that the reduction of the amplitude is relatively homogenous throughout the range of stimulus intensities, although only the effects at the two lowest intensities (8 and 10 V) were significant (** $p < 0.001$; * $p < 0.05$).

base) provided by the National Institute on Drug Abuse (Bethesda, MD). We implanted control rats with placebo pellets. Two pellets (either morphine or placebo; wrapped in nylon) were implanted in each rat under light halothane anesthesia (halothane–oxygen mixture; 1–1.5% halothane). All electrophysiological testings were limited to 4–6 d after pellet implantation. To study the persistence of the effects of withdrawal, we removed morphine and placebo pellets 1 week before the withdrawal experiments, under light halothane anesthesia.

Statistics. We expressed all group values as mean $\pm$ SEM. We determined statistical significance between control, drug, and washout conditions within each group of cells using one-factor ANOVA for repeated measures, with a *post hoc* analysis by Newman–Keuls or Fisher PLSD comparison tests. Analysis of the statistical differences, expressed as percent of control within and between groups of cells from untreated, morphine-treated, and sham-operated rats, was done by one-way ANOVA between subjects. We considered p values of <0.05 statistically significant.

RESULTS

Chronic morphine treatment reduces synaptic input–output relationships

We tested the hypothesis that morphine treatment could change NMDA receptor-mediated synaptic transmission first by measuring the amplitude of NMDA EPSCs evoked by equivalent stimulus intensities before and after chronic treatment. The amplitudes of NAcc NMDA EPSCs from untreated rats increased with increasing stimulus intensities (Fig. 1). Intensities of >18–20 V triggered spikes (data not shown in the graph). After morphine treatment, the amplitudes of the synaptic currents, within the same stimulus range, were smaller than those in slices of untreated rats. Statistical analysis showed that the difference was significant at the lowest intensities, that is, at 8 V [$F_{(1,25)} = 79.46$; $p < 0.001$] and 10 V [$F_{(1,24)} = 4.26$; $p < 0.05$]. These data suggest that morphine treatment alters NMDA receptor-mediated synaptic transmission either by decreasing the responsiveness of NMDA receptors for glutamate or by decreasing glutamate release. Although the magnitude of this inhibition seems smaller in chronically treated rats than that for acute μ agonists (Martin et al., 1997a), it is difficult in our experimental conditions to determine whether this difference can be accounted for by alteration of the pre- or postsynaptic effects we observed in naive animals. We are now addressing this issue by assessing NMDA responsivity in

Figure 2. Time course of the 5 μ M PDAC-evoked enhancement of NMDA EPSCs. *A*, Current traces recorded at -63 mV (left) and -101 mV (right) holding potentials from an NAcc neuron before (Control) and after PDAC superfusion (10, 15, 30, and 40 min). At both potentials, PDAC enhanced the NMDA EPSC amplitudes with a maximum effect at 10 min. *B*, Mean ($n = 7$) effect of PDAC on NMDA EPSC amplitudes measured at -100 and -60 mV as a function of time. For NMDA EPSCs measured at -60 mV, the peak effect is reached 10 min after PDAC superfusion. See text for $**p$ values. In this and subsequent figures, downward-pointing arrows in the current traces indicate the time of local stimulation to evoke EPSCs.

a freshly isolated NAcc cell preparation that will allow the examination of changes of NMDA receptor affinity.

Chronic morphine decreases PDAC enhancement of NMDA EPSCs

As noted in the introductory remarks, the NMDA receptor sensitivity to PKC activators is determined by NR2A and/or NR2B subunits. Therefore, we tested the effects of the PKC activator PDAC (5 μ M) on NMDA EPSC amplitudes in NAcc slices from untreated rats, evoked at approximately -60 and -100 mV before and after PDAC superfusion (applied for 5 min). The enhancement of NMDA EPSCs by PDAC peaked 10 min after the onset of the superfusion (Fig. 2*A*). Thirty minutes later, NMDA EPSC amplitudes slowly returned to control values. Although the reason for this decay is not completely understood, some evidence suggests that it is caused by the action of phosphatases that counteract the PKC effect (Lieberman and Mody, 1994; McBain and Mayer, 1994; Wang et al., 1994; Blank et al., 1997). We observed a similar pattern of action for responses recorded at the more hyperpolarized potential (Fig. 2*B*), even though the overall potentiation was smaller (Fig. 2*A*, right). Averaged over seven NAcc cells from untreated rats, 5 μ M PDAC significantly increased NMDA EPSC amplitudes by $157 \pm 21\%$ of control 10 min after superfusion when measured at approximately -60 mV and by $109 \pm 13\%$ at approximately -100 mV (Fig. 2*B*). Statistical analysis showed that NMDA EPSC amplitudes evoked at -60 mV and measured 5, 10, and 15 min after

Figure 3. Comparison of the effects of PDAC on NMDA EPSCs in NAcc neurons from untreated, morphine-treated, and sham-operated rats. *A*, Representative traces of NMDA EPSCs from three different neurons (all held at -60 mV) from the three treatment groups, before (Control) and 10 and 40 min after PDAC superfusion. In the neurons from untreated and sham-operated rats, 5 μ M PDAC increased the amplitudes of NMDA EPSCs by 150% after 10 min. This effect disappeared 30 min later. After chronic morphine treatment, the PDAC-mediated enhancement is markedly reduced to only 66% of control levels. *B*, Mean PDAC effects on NMDA EPSC amplitudes measured at -100 and -60 mV, 5 and 10 min after PDAC superfusion. In NAcc neurons from untreated rats, the PDAC-induced potentiation is voltage-dependent; the NMDA EPSC is almost doubled at -60 mV versus that at -100 mV. After chronic morphine treatment, the potentiation is significantly reduced [$F_{(2,18)} = 5.62$; $p < 0.0014$] for the -60 mV condition only (i.e., in a voltage-dependent manner), 10 min after PDAC superfusion. The statistics were equivalent for sham versus untreated rats. $*p < 0.05$; $**p < 0.01$.

PDAC superfusion were significantly higher than that in control conditions ($p < 0.0042$, 0.0002 , and 0.0004 , respectively).

We also compared representative NMDA EPSCs recorded at -60 mV in three different cells from untreated, morphine-treated, and sham-operated rats before and 10 and 40 min after superfusion of PDAC (Fig. 3*A*). NMDA EPSC amplitudes were strongly enhanced in untreated and sham-operated rats 10 min after PDAC superfusion, but this effect was less pronounced in neurons from a morphine-treated rat (Fig. 3*A*). On average, PDAC boosted the mean NMDA EPSC amplitudes at both holding potentials (-100 and -60 mV) and time points (5 and 10 min) in slices from untreated and morphine-treated rats (Fig. 3*B*). However, this effect was markedly reduced after morphine treatment; at approximately -60 mV, PDAC-elicited augmentation of NMDA EPSC amplitudes dropped to 34 ± 7 and $74 \pm 18\%$ of control levels in neurons from morphine-treated rats, 5 and 10 min after PDAC superfusion, respectively. The PDAC effect, at -60 mV and 5 min after superfusion, was significantly different between untreated and morphine-treated rats ($p < 0.007$) and between treated and sham rats ($p < 0.001$). However, there was no significant difference between the untreated and sham groups. A similar pattern was observed 10 min after PDAC superfusion.

Because the effect of phorbol ester could be caused by PDAC acting presynaptically (increasing glutamate release) as well as

Figure 4. Studies on the site and specificity of PDAC actions. *A*, Inward currents elicited by rapid superfusion of NMDA (initiated at the onset of records; shown by solid horizontal bar) onto NAcc slices from untreated rats in the presence of 15 μ M bicuculline, 10 μ M CNQX, and 1 μ M TTX to block presynaptic release and GABA and non-NMDA receptors. Superimposed polygraph recordings of membrane currents in the same cell held at -68 mV before and 5 min after superfusing PDAC are shown. Downward deflections are current responses to brief voltage commands used to monitor membrane conductance. PDAC more than doubled the amplitude of the NMDA current. *B*, Traces of NMDA EPSCs evoked at -62 mV from the same cell before (Control), 10 min after sphingosine (Sphing), and 10 min after sphingosine and PDAC together. Note that sphingosine by itself weakly increased NMDA EPSC amplitude but also strongly attenuated PDAC-mediated enhancement of this current, compared with PDAC without sphingosine (compare with Fig. 2).

postsynaptically, we further examined the locus of its interaction in control slices by rapidly superfusing 60 μ M NMDA in the presence of 1 μ M TTX to block glutamate release from terminals. Membrane potential was held at approximately -65 mV to reduce the voltage-dependent NMDA receptor blockade by Mg^{2+} . PDAC, 5 min after the beginning of superfusion, enhanced the NMDA-elicited inward currents (Fig. 4*A*). When averaged over three cells, PDAC increased the mean NMDA current by $90 \pm 13\%$. The extent of this PDAC effect is almost equivalent to that for NMDA EPSCs evoked by local stimulation (see Fig. 2*B*). We also assessed the selectivity of the PDAC effect with sphingosine, a specific PKC inhibitor. Sphingosine (20 μ M) alone slightly increased NMDA EPSC amplitudes (Fig. 4*B*). When superfused together with sphingosine, 5 μ M PDAC enhanced NMDA EPSCs by only $10 \pm 13\%$, an effect that was not significantly different from that of sphingosine alone. Although we cannot completely rule out some presynaptic action, these data suggest that PDAC enhances NMDA EPSC amplitudes mostly via a postsynaptic mechanism.

Chronic morphine decreases Mg^{2+} inhibition of NMDA EPSCs

As with the facilitation of NMDA receptors by PKC, the inhibition by Mg^{2+} is known to be under the control of NR2 subunits (Monyer et al., 1992, 1994; Ishii et al., 1993; Kawajiri and Dingle, 1993). To determine the effect of Mg^{2+} , we evoked NMDA EPSCs at holding potentials of approximately -60 mV, first in the presence of a low Mg^{2+} concentration (0.3 mM) for 10 min and then 10 min after switching to ACSF containing 1.5 mM

Figure 5. Chronic morphine treatment reduces the Mg^{2+} sensitivity of NMDA EPSCs in NAcc neurons. *A*, Representative traces of NMDA EPSCs grouped by equivalent amplitudes and similar holding potentials (-59 and -62 mV), with superfusion of ACSF containing either low (0.3 mM) or high (1.5 mM) Mg^{2+} concentrations. *B*, Mean data from four to five NAcc neurons for each condition, showing significantly reduced Mg^{2+} -mediated inhibition of EPSCs from morphine-treated rats [$F_{(2,13)} = 9.31$; $p < 0.004$]. There was no significant difference between neurons of untreated and sham rats. $**p < 0.01$.

Mg^{2+} . As before, we adjusted the stimulus intensity to evoke control NMDA EPSCs with similar amplitudes across different slices (Fig. 5*A*). Ten minutes after superfusing 1.5 mM Mg^{2+} , the mean amplitude of NMDA EPSCs of untreated and sham-operated rats decreased by 68 ± 5 and $60 \pm 5\%$, respectively, of those in 0.3 mM Mg^{2+} (Fig. 5*B*). After morphine treatment, the inhibition by 1.5 mM Mg^{2+} was only $41 \pm 6\%$. The differences between untreated and morphine-treated rats [$F_{(1,7)} = 15.02$; $p < 0.006$] and between sham-operated and morphine-treated rats [$F_{(1,8)} = 11.22$; $p < 0.01$] were both significant, whereas the difference between untreated and sham-operated rats was not ($p = 0.5$).

Because slices from morphine-treated rats were constantly exposed to 1 μ M morphine in the bath to prevent withdrawal, we tested the possibility that the difference in Mg^{2+} effects between untreated and morphine-treated rats was caused by acute exposure (2–3 hr) of slices to morphine and not by chronic treatment. When the same neuron from an untreated rat (Fig. 6*A*) was superfused for 2–3 hr with 1 μ M morphine in the presence of low (0.3 mM) and high (1.5 mM) Mg^{2+} concentrations at -59 mV, Mg^{2+} (1.5 mM) reduced the amplitude of the NMDA EPSCs by 48% of control (0.3 mM). When averaged over four cells (Fig. 6*B*), the mean high Mg^{2+} -mediated inhibition was $55 \pm 5\%$ of that in 0.3 mM Mg^{2+} , an effect intermediate between that in untreated (68%) and morphine-treated (41%) rats. Although the difference between untreated and acute morphine conditions was not statistically significant ($p = 0.06$), that between acute morphine- and chronic morphine-treated rats was significant ($p = 0.03$). These data suggest that acute exposure to morphine tends to alter

Figure 6. Effects of Mg^{2+} on NMDA EPSCs from slices of untreated rats after acute exposure (2–3 hr) to morphine ($1 \mu M$). *A*, Individual superimposed traces of NMDA EPSCs recorded in the presence of morphine at -59 mV during 0.3 and 1.5 mM Mg^{2+} superfusion. *B*, Mean effects of Mg^{2+} on NMDA EPSC amplitudes, comparing acute morphine effects with that of untreated and chronic morphine-treated conditions.

NMDA receptor properties but not to the same significant extent that chronic morphine treatment does.

Prolonged morphine withdrawal on Mg^{2+} and PDAc effects

We examined the persistence of the effects of chronic morphine by testing for the effects of the high Mg^{2+} concentration and 5 μM PDAc on NMDA EPSCs 1 week after withdrawal from chronic morphine treatment. The inhibition elicited by Mg^{2+} was still reduced 1 week after withdrawal compared with that in sham rats (Fig. 7*A*). When averaged over nine and eight cells, Mg^{2+} decreased NMDA EPSC amplitudes from placebo rats by $44 \pm 5\%$ and in NAcc slices from rats withdrawn from morphine for 1 week by $27 \pm 6\%$, respectively (Fig. 7*B*). The difference between these values was significant [$F_{(1,18)} = 5.14$; $p = 0.038$]. Similarly, the enhancement of NMDA EPSC amplitudes by PDAc was still attenuated 1 week after withdrawal compared with that in placebo rats (Fig. 7*C*). Thus, PDAc increased the mean NAcc NMDA EPSC amplitudes of placebo rats by $136 \pm 17\%$ of control versus only $89 \pm 10\%$ in morphine-withdrawn rats. Again, although the difference of the PDAc-elicited enhancement of mean NMDA EPSC amplitudes between the placebo and withdrawn rats is smaller than that between untreated and chronic morphine-treated rats, nevertheless this difference is statistically significant [$F_{(1,15)} = 4.91$; $p = 0.043$; Fig. 7*D*].

Effect of chronic morphine treatment and withdrawal on the NMDA EPSC inactivation rate

The kinetics of the NMDA EPSC inactivation also has been shown to be controlled by NR2 subunits (Takahashi et al., 1996; Flint et al., 1997; Gottmann et al., 1997). Therefore, we estimated the kinetics by fitting the decay of the NMDA synaptic responses

Figure 7. Effect of 1 week withdrawal from morphine on Mg^{2+} and PDAc effects on accumbens NMDA EPSCs. *A*, *B*, Chronic withdrawal from morphine treatment reduces the Mg^{2+} sensitivity of NMDA EPSCs in NAcc neurons. *A*, Representative superimposed traces of NMDA EPSCs at equivalent holding potentials (-60 mV) after superfusion of ACSF containing either low (0.3 mM) or high (1.5 mM) Mg^{2+} concentrations. Mg^{2+} -induced inhibition is still attenuated 1 week after withdrawal compared with that in placebo rats. *B*, Mean Mg^{2+} -mediated inhibition of NMDA EPSC amplitudes averaged from eight to nine NAcc neurons for each condition. Compared with that in placebo rats, this reduced Mg^{2+} effect was significant 1 week after withdrawal [$F_{(1,16)} = 5.1$; $*p = 0.038$]. *C*, Individual traces of superimposed NMDA EPSCs at equivalent holding potentials (-60 mV) with superfusion of 5 μM PDAc. PDAc-evoked enhancement was attenuated 1 week after withdrawal compared with that in placebo rats. *D*, Mean PDAc-mediated facilitation of NMDA EPSC amplitudes from seven to nine NAcc neurons for each condition (legend same as in *B*). Compared with that in placebo rats, this effect is still significant 1 week after withdrawal [$F_{(1,14)} = 4.91$; $*p = 0.043$]. *Morph*, Morphine.

between the peak and 260 msec after the stimulation (see Materials and Methods). To draw a meaningful comparison between experimental conditions, we divided the neuronal sample into four groups (Fig. 8*A*), according to their mean NMDA EPSC amplitudes (100, 200, 300, and 500 pA). The inactivation time constant (τ) of NMDA EPSCs of neurons from untreated rats was very similar across the four groups, with τ values ranging between 80 and 100 msec (Fig. 8*B*); the mean τ was 97 ± 2.5 msec. In the chronically treated group, τ ranged from 68 to 84 msec with a mean of 78.7 ± 1.8 msec. This decrease by morphine treatment was significant for mean NMDA EPSC amplitudes at approximately 200, 300, and 500 pA ($p = 0.004$, 0.0001, and 0.031, respectively), but the difference was not significant ($p = 0.0504$) for the smallest group (100 pA). The representative traces of normalized NMDA EPSCs in Figure 8, *C* and *D*, exemplify the faster inactivation in neurons from morphine-treated rats.

We also examined the persistence of the effects of chronic morphine treatment on the inactivation rate of NMDA EPSCs 1

Figure 8. Chronic morphine treatment decreases the NMDA EPSC inactivation rate (τ). *A*, Mean NAcc NMDA EPSC amplitudes from untreated and morphine-treated rats grouped over four different EPSC amplitudes (100, 200, 300, and 500 pA). *B*, Mean NMDA EPSC inactivation rates measured from the same cells shown in *A*. Note that the τ inactivation rates are reduced by ~15–20% after chronic morphine treatment compared with that of controls, regardless of initial EPSC sizes. *C*, *D*, Representative traces of NMDA EPSCs from two different neurons, recorded at approximately -60 mV using equivalent stimuli and holding potentials and grouped by EPSC size (scaled or normalized EPSCs on the right). Morphine ($1 \mu\text{M}$) was superfused to prevent withdrawal. * $p < 0.05$; ** $p < 0.01$.

week after morphine withdrawal, measured from neurons grouped according to their mean NMDA EPSC amplitude (100 and 200 pA; Fig. 9*A*). Surprisingly, the mean inactivation τ values of neurons from withdrawn rats (80–100 msec) were bigger than those of placebo rats (68–72 msec) for both groups of EPSC sizes (Fig. 9*B*), but only the increase in the first group (100 pA) was significant ($p < 0.034$).

DISCUSSION

We have studied the properties of NMDA receptor-mediated synaptic transmission in NAcc neurons before and after chronic morphine treatment to uncover possible mechanisms underlying the involvement of this glutamate receptor subtype in some aspects of morphine addiction (Marek et al., 1991; Trujillo and Akil, 1991, 1994; Elliott et al., 1994; Tiseo et al., 1994). The

Figure 9. Reversal of chronic morphine effects on EPSC inactivation 1 week after withdrawal. *A*, Mean NMDA EPSC amplitudes from placebo and withdrawn rats grouped over two different EPSC amplitudes (100 and 200 pA). *B*, Mean NMDA EPSC inactivation rates (τ values) measured from the same cells shown in *A*. Note that the τ values for the morphine-withdrawn EPSCs are now reversed to be larger than that of the sham controls. $n = 4$ –5 for each group; * $p < 0.05$.

pharmacological and kinetic data presented here suggest that chronic morphine treatment decreases: (1) NMDA receptor-mediated synaptic transmission, (2) the enhancement of NMDA EPSC amplitudes elicited by a PKC activator, (3) Mg^{2+} -elicited inhibition of NMDA EPSCs, and (4) NMDA EPSC inactivation τ values.

Comparison of native and recombinant NMDA EPSCs

Early anatomical studies reported that the NR1 subunit is present throughout the brain, whereas NR2A and NR2B subunits are expressed differentially in forebrain structures with almost no expression in other brain regions. Although NR2A expression has been observed in the NAcc, some data suggest that projection neurons of this brain region, as well as those from dorsal striatum, preferentially express NR2B subunits (Buller et al., 1994; Landwehrmeyer et al., 1995). It also is now well established that NR2C and NR2D, expressed mostly in the cerebellum and pons, respectively, are not expressed in NAcc under normal conditions. These anatomical observations suggest that native NAcc NMDA receptors are composed of NR1 and NR2B and maybe to a lesser extent of NR1 and NR2A subunits, although a combination of these three subunits (Chazot and Stephenson, 1997; Luo et al., 1997) is also possible.

The expression of NR2A and NR2B subunits is supported by our electrophysiological data showing that NAcc NMDA EPSCs of naïve rats are markedly enhanced by PDAC, a PKC activator, and strongly inhibited by Mg^{2+} . The vast bulk of our current knowledge on electrophysiological properties of NMDA receptors derives from studies on various expression systems such as

oocytes and human embryonic kidney cells that provide the advantage of dissecting receptor properties via construction of various combinations of the NMDA receptor subunits (NR1 and NR2A–D) expressed in brain. Thus, several groups have shown that binary NR1/NR2A and NR2B receptors expressed in oocytes display a high sensitivity to PKC activators (Kutsuwada et al., 1992; Meguro et al., 1992). Similarly, Mg^{2+} inhibition of NMDA currents (Nowak et al., 1984) is almost exclusively displayed by receptors containing NR1/NR2A and NR2B subunits, with no influence of NR1/NR2C and NR2D (Kutsuwada et al., 1992; Monyer et al., 1992).

NAcc NMDA EPSC decays measured in naïve rats also support the absence of NR2C or NR2D subunits in naïve NAcc as indicated by all anatomical studies (see above). Thus, the average decay time (97 ± 2.5 msec) of NAcc NMDA EPSCs was closer to that of recombinant NR1/NR2A NMDA receptors (83 msec) expressed in human embryonic kidney cells than to that of NR1/NR2C receptors (319 msec) (Monyer et al., 1992). However, estimation of native receptor subunit composition from decay values alone requires caution, because they vary for the same heteromeric recombinant NMDA receptors from study to study (Monyer et al., 1992; Flint et al., 1997; Gottmann et al., 1997).

Chronic morphine treatment on NMDA receptor-mediated synaptic transmission

We found that chronic morphine treatment significantly decreased NMDA EPSC amplitudes, Mg^{2+} -elicited inhibition, and PDAC-elicited enhancement of NMDA EPSCs. Previous studies have established that weak Mg^{2+} block and reduced PKC-mediated enhancement of NMDA currents correlated with the presence of NR2C and NR2D subunits (Mori et al., 1993; Grant et al., 1998). As noted above, in normal rat brain neither NR2C nor NR2D is expressed in naïve adult NAcc neurons. Only one study reported that NAcc neurons (somatostatinergic and cholinergic NAcc interneurons, but not projecting GABA neurons) express measurable levels of NR2D (Standaert et al., 1996). One explanation for our result is that chronic morphine treatment could trigger the expression of “dormant” NR2C and/or NR2D subunits that are not expressed in the NAcc of naïve rats. However, our finding of decreased NMDA EPSC inactivation times during chronic treatment apparently contradicts this assumption; as discussed above, reduction of inactivation times is thought to be associated with expression of NR2A rather than NR2C and NR2D. Another explanation is that chronic morphine treatment has multiple effects, inducing the expression of NR2C or NR2D subunits but also increasing the expression of preexisting NR2A subunits. However, Zhu et al. (1999) found no effect of intracerebroventricular chronic morphine treatment on NR2A mRNA expression. Several groups found in NAcc as well as in dorsal striatum that NR2A expression is much less abundant than that for NR2B (Buller et al., 1994; Landwehrmeyer et al., 1995; Wang et al., 1995) or is absent as in human NAcc (Rigby et al., 1996). This predominance of NR2B in naïve animals and the postulated shift toward NR2A could account for the increase of the NMDA EPSC inactivation times after chronic morphine treatment. However, the possible switch to these subunits (NR2A, NR2C, and NR2D) might lead to opposite effects. To address such questions, we are now examining the expression of mRNAs coding for NR2A–D subunits of NAcc medium spiny neurons using single-cell reverse transcription-PCR in an acutely isolated cell preparation.

The change of NMDA receptor-mediated synaptic transmis-

sion could occur either pre- or postsynaptically. However, the fact that the PDAC-elicited enhancement of NMDA EPSCs was nearly identical to that of currents evoked by NMDA superfusion in the presence of TTX argues in favor of a postsynaptic locus, at least for the PDAC effect. Nonetheless, we cannot rule out the possibility that decreased glutamate release accounts for some portion of the chronic morphine effects on NMDA EPSC amplitudes and kinetics.

Persistence of chronic morphine effects

The effects of chronic morphine persisted for 1 week after withdrawal, suggesting that they cannot be explained by the presence of morphine in the bath during the experiments. These long-lasting alterations of NMDA receptor properties could contribute to the neuroadaptations known to persist long after the end of opiate intake. It is also interesting that the change in NMDA EPSC kinetics is reversed after withdrawal, whereas inhibitions of Mg^{2+} -evoked inhibition and PDAC-evoked facilitation are still present, although smaller. This divergence of persisting actions may support the hypothesis that Mg^{2+} - and PDAC-mediated effects reflect a true modification of postsynaptic NMDA receptor subunit composition, whereas the biphasic alteration of EPSC kinetics may derive from summated pre- and postsynaptic effects: with early chronic morphine, presynaptic effects may predominate, whereas long withdrawal leads to a loss of presynaptic effects to reveal an underlying long-term alteration of postsynaptic NMDA receptor subunit composition.

Physiological significance of alterations in NMDA receptor properties

Chronic morphine treatment has long been known to alter cellular homeostasis. Thus, it downregulates the expression of μ opiate receptors, stimulates immediate early gene (IEG) expression, and increases cAMP levels. This latter phenomenon may represent a crucial aspect of the effects of chronic morphine, because cAMP is at the crossroads of numerous biochemical processes that control cell homeostasis. There is evidence that increases of cAMP levels may be under the control of an IEG such as that for cAMP response-binding protein (CREB) (Lane-Ladd et al., 1997). Interestingly, the expression of IEGs like c-jun, jun-B, and CREB may be controlled in part by NMDA receptor activation (Cole et al., 1989; Szekely et al., 1990; Morgan and Linnoila, 1991). Thus, it is tempting to speculate on the control that NMDA receptors could exert on the cAMP upregulation after morphine. In that perspective, the inhibition of NMDA receptor-mediated transmission suggested by the present findings might be regarded as a compensatory mechanism that ultimately diminishes NMDA receptor stimulation of CREB synthesis and therefore the increase of cAMP levels induced by chronic morphine treatment. These effects may occur in concert with the postulated recomposition of NAcc NMDA receptors.

Regardless of the mechanisms involved, the overall effect of chronic morphine should be decreased glutamatergic transmission, exerted both presynaptically (Martin et al., 1999) and now postsynaptically as well. The hypothesis of a depression of the glutamatergic synaptic transmission in NAcc is further substantiated by the recent data of Rogers et al. (1999) showing that the deficit of decision making by human opiate abusers can be correlated with damage of the orbitofrontal subregion of the prefrontal cortex that contributes most of the NAcc glutamate afferents. This reduced glutamatergic transmission, along with the increased release of GABA reported for accumbens neurons

(Chieng and Williams, 1998), would depress neuronal excitability. These concerted effects of chronic morphine on glutamate- and GABA-mediated synaptic transmission could underlie some of the changes that occur during the transition to drug addiction (Koob et al., 1998) as well as the rewarding properties of opioids.

In view of the role of NAcc in drug reward, it is tempting to suggest that these long-lasting changes in excitability of NAcc neurons may contribute to various phenomena such as increased opiate self-administration observed recently in dependent rats (Carrera et al., 1999), as well as other forms of enhanced behavioral responsiveness to opiates (Shippenberg et al., 1996). Thus, rats may work to inhibit their accumbens neurons, and chronic administration of opioids may facilitate such inhibition. Because the enhanced reward associated with opiate dependence could derive from both sensitization and tolerance, further studies are necessary to determine whether the effects observed in this study account for either phenomena.

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