Chronic Morphine Treatment Alters \(N\)-Methyl-\(D\)-aspartate Receptors in Freshly Isolated Neurons from Nucleus Accumbens

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ABSTRACT

Although there is now evidence of a role for \(N\)-methyl-\(D\)-aspartate (NMDA) receptors in nucleus accumbens (NAcc) neurons in the effects of chronic opiate treatment, the cellular and molecular mechanisms underlying this phenomenon are still unclear. Therefore, we studied the effects of chronic morphine on the pharmacological and biophysical properties of NMDA receptors in freshly isolated medium spiny neurons from NAcc. We found that chronic morphine treatment did not alter the affinity for NMDA receptor agonists such as glutamate, homoquinolinic acid, and NMDA, but decreased the affinity of glycine, the allosteric NMDA receptor coagonist, from 2.24 \(\pm\) 0.15 \(\mu\)M to 5.1 \(\pm\) 1.45 \(\mu\)M. Chronic morphine treatment also altered the affinity of two noncompetitive NMDA receptor antagonists, 7-chloro-kynurenic acid and ifenprodil. However, morphine had no effect on a third antagonist, \(d\)-(−)-2-amino-5-phosphono-pentanoic acid. Single-exponential fits of desensitized NMDA current tails gave tau values ranging from 0.5 to 4 s in neurons from both control and morphine-treated rats. However, a shift to the left of the distribution of tau values after morphine treatment revealed that NMDA current desensitization rate was accelerated in a majority of NAcc neurons. Taken together with our recent molecular studies, our data are consistent with a shift away from NMDA receptor subunit (NR) NR2B and 2C function toward increased NR2A subunit expression or function after chronic morphine, a process that could alter excitability and integrative properties and may represent a neuroadaptation of NAcc medium spiny neurons underlying morphine dependence.

During the past decade the nucleus accumbens, an interface between limbic regions and the extrapyramidal motor system, has emerged as a key structure mediating the acute and chronic behavioral effects of drugs of abuse (Koob et al., 1992). Although behavioral studies conclusively demonstrated a key role for NAcc in opiate self-administration, relatively little is known about the cellular and molecular mechanisms responsible for acute opiate effects, and even more remains to be understood about chronic effects. Recently, NMDA receptors have been recognized as a critical factor underlying tolerance to various drugs of abuse. Trujillo and Akil (1991) and Marek et al. (1991) were the first to report that intraventricular infusion of MK-801 (dizocilpine maleate), a selective noncompetitive NMDA receptor antagonist, could strongly attenuate the morphine withdrawal syndrome, a finding subsequently confirmed by others (Tiseo et al., 1994; Elliott et al., 1995).

Numerous studies established that NMDA receptors, formed by the association of two or more subunits, have multiple binding sites for various agents such as endogenous agonists, competitive antagonists, and Mg\(^{2+}\), Zn\(^{2+}\), or polyamines. To date, five NMDA receptors subunits (NR1, NR2A-D) have been identified. It is believed that, in native NMDA receptors, these subunits are assembled in various combinations to create heteromultimeric receptors. In situ hybridization and immunocytochemical studies established that the NR1 subunit is ubiquitously expressed in the brain (see Mcbain and Mayer, 1994, for review), whereas NR2 subunits show very specific spatial expression. Thus, in mature rats, NR2A and B subunits seem to be preferentially located in the forebrain, whereas NR2C is almost exclusively expressed in cerebellum and NR2D in brainstem and spinal cord.
cord. Several studies (Landwehrmeyer et al., 1995; Standart et al., 1996) reported that the vast majority of NAcc medium spiny neurons express NR2A and NR2B subunits, with a clear predominance for the latter.

Because it was suggested that NMDA receptor subunit expression is very sensitive to drastic changes of brain homeostasis (Follesa and Ticku, 1995, 1996; Riva et al., 1997), in a previous study we explored in a slice preparation the possibility that chronic morphine treatment similarly alters glutamate-mediated synaptic transmission in NAcc by modifying the composition of the NMDA receptor complex. We found that chronic morphine changes the properties of NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) (Martin et al., 1999). We hypothesized from these data that chronic morphine triggered the expression of NR2C and/or NR2D subunits. However, the interpretation of these results was clouded by the difficulty in a slice preparation to ascribe with certainty whether opioid effects were exerted at pre- or postsynaptic sites. Therefore, in the present study, we used freshly isolated neurons to directly examine the chronic effects of morphine on postsynaptic NAcc NMDA receptors. To test the idea that chronic morphine alters NMDA receptor subunit composition, we took advantage of the fact that each NR2 subunit, when coexpressed with NR1, confers distinct pharmacological properties to NMDA receptor-channel complex and acts as a regulatory subunit (see review by Yamakura and Shimoji, 1999). The pharmacological profile of NAcc NMDA receptors, as well as their biophysical properties, suggests an alternative hypothesis to that derived from our slice studies, that chronic morphine increases the expression or influence of NR2A subunits, phenomena that could impair NMDA receptor-mediated synaptic transmission in opiate dependence.

Materials and Methods

Animals, Neuron Preparation, and Experimental Solutions. We used male Sprague-Dawley rats (100–200 g) to prepare NAcc slices and isolated neurons as previously reported (Martin and Siggins, 2002; Martin et al., 2004). The rats were anesthetized with 4% halothane and decapitated, and the brains were rapidly transferred into a cold (4°C) oxygenated, low-calcium HEPES-buffered salt solution: 234 mM sucrose, 2.5 mM KCl, 2 mM NaH2PO4, 11 mM glucose, 4 mM MgSO4, 2 mM CaCl2, 1.5 mM HEPES. We glued a tissue block containing NAcc to a Teflon chuck and cut it transversely (400 μm) by a circulating water bath in the outer chamber of the flask. After enzymatic digestion, we transferred the tissue into a centrifuge tube and rinsed it three to four times with a Na+–isethionate solution. We then filled the tube with 5 ml of Na+–isethionate solution and, after 10 min, triturated the tissue using fire-polished Pasteur pipettes with successively smaller tip diameters. We plated the supernatant onto a 35-mm Petri dish placed on the stage of the inverted microscope. The cells were allowed to attach to the dish for 10 min before replacing the Na+–isethionate solution with normal external solution flowing at a rate of 1.5 ml/min and composed of 142 mM NaCl, 2 mM KCl, 2 mM CaCl2, 23 mM glucose, 15 mM HEPES, 10 mM glycine (pH = 7.35 with NaOH; osmolarity, 300 mosM/l).

Whole-Cell Recordings. We used standard whole-cell recording methods (Hamill et al., 1981). Briefly, we pulled patch electrodes from borosilicate capillary glass (Sutter Instrument Company, Novato, CA) on a Brown-Flaming puller (Sutter Instrument Company) to a final resistance of 1.8 to 2.2 MΩ. We filled the electrodes with a solution that consisted of 120 mM CsF, 10 mM CsCl, 11 mM EGTA, 10 mM HEPES, 0.5 mM CaCl2 (pH = 7.35 with CsOH; osmolarity, 270–275 mosM/l). Although F− has been suggested to alter G-protein-mediated pathways, we felt that such pathways probably would not be involved in our study of NMDA receptor properties (see also the NMDA receptor studies of Kew et al., 1998). The capillaries were first filled through the tip and then backfilled with the internal solution. We recorded in voltage-clamp mode with an Axopatch 1D amplifier and a Digidata 1200 interface from Axon Instruments Inc. (Union City, CA). The recordings were filtered at 5 kHz and digitized at 1 kHz. The series resistance was not compensated. Liquid junction potentials were not corrected but are estimated to be +4 mV.

Superfusion and Drug Application. We applied control and drug-containing solutions by gravity at a rate of 1.5 ml/s, using a rapid three-barrel capillary superfusion device (Warner Instrument, Hamden CT) with the pipette tips placed about 200 μm from the recorded cell. The flow of solutions was controlled by solenoid valves. Each capillary had a tip diameter of 500 μm, and the distance from center to center was 700 μm. The pipette assembly was attached to a motor, allowing fast lateral motions controlled by pClamp 6.0 (Axon Instruments Inc.), the acquisition software. We estimated a drug onset time of 20 ms for the application system by measuring the change in the tip potential of the recording pipette filled with intracellular solution as the perfusion was switched from a normal to a 1:2 dilution of the extracellular recording solution.

Our standard drug testing procedure was as follows. After recording a stable current, NAcc medium spiny neurons were exposed for 5 s to increasing concentrations of agonists (NMDA, glutamate, homoquinolinic acid) in the presence of a saturating (100 μM) concentration of glycine. To avoid current inactivation, drugs were applied every 30 s. Antagonists (7-chloro-kynurenic acid and ifenprodil) were coapplied with saturating concentrations of NMDA (200 μM) and glycine (100 μM). We purchased glycine, glutamate, NMDA, homoquinolinic acid, 7-chloro-kynurenic acid, and ifenprodil from Sigma-Aldrich (St Louis, MO).

We fitted dose-response curves with a Hill equation as follows: \( I = I_{\text{max}}/[1 + (EC_{50}/C)^n] \), where \( I \), \( I_{\text{max}} \), \( C \), \( EC_{50} \), and \( n \) are agonist-elicited current, maximal agonist-activated current, agonist concentration, the concentration for 50% of the response, and the Hill coefficient, respectively. We measured NMDA current desensitization on NMDA currents evoked by coapplication (5 s) of 200 μM NMDA and 100 μM glycine at −60-mV holding potential. We fitted the current decay using a single exponential (Chebyshev method, pClamp 6.0), and we used the tau value to construct a histogram with a 0.5-s bin width. All results are expressed as mean ± S.E.M.

Statistics. We expressed all averaged values as mean ± S.E.M. We tested for statistically significant differences between placebo and morphine-treated groups using a t test analysis. We assessed changes in NMDA current desensitization with the nonparametric Kolmogorov-Smirnov test. We considered \( p < 0.05 \) statistically significant.
Chronic Morphine Treatment. As described by Gold et al. (1994), rats were made dependent by subcutaneous implantation of morphine pellets (75 mg of 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 testing was performed 4 to 6 days after pellet implantation.

Results

Because NMDA currents have been shown to undergo a strong run-down in some experimental conditions (Rosenmund and Westbrook, 1993), we first determined whether NMDA currents in the NAcc neurons could undergo similar alterations. We recorded currents evoked by coapplication of 200 μM NMDA and 100 μM glycine every 20 s for 10 min at a membrane potential of −60 mV (Fig. 1A). We found that NMDA currents were remarkably stable over this period of time and that no run-down (i.e., a decrease of NMDA current amplitude over time) occurred (Fig. 1B).

Chronic Morphine Does Not Alter the Affinity of Agonists on NAcc NMDA Receptors. In a previous study we found that chronic morphine treatment decreased the current amplitude of postsynaptic NMDA receptor-mediated events in a slice preparation (Martin et al., 1999), suggesting that morphine depressed glutamate synaptic transmission in NAcc either by decreasing the amount of glutamate released

or by altering postsynaptic NMDA receptor properties. In the present study, we tested the latter possibility by studying properties of postsynaptic NMDA receptors from freshly isolated medium spiny neurons. We first asked whether the depressant effects of morphine on NMDA receptor-mediated synaptic transmission was due to a decrease of glutamate affinity, a property shown to be controlled by NR2 subunits. Thus, the glutamate EC_{50} for NR2C is lower than that for NR2A or NR2B (Wafford et al., 1993; Laurie and Seeberg, 1994), making this agonist suitable for identification of a possible alteration of NMDA receptor subunit composition after chronic treatment. In NAcc neurons of placebo rats, we evoked NMDA currents with glutamate (0.01 μM to 1 mM) in the presence of 15 μM CNQX to block non-NMDA glutamate receptors. Current amplitudes were maximal with 0.5 mM glutamate and rapidly declined with lower concentrations (Fig. 2A). In NAcc neurons from morphine-treated rats, NMDA response amplitudes evoked with a similar range of glutamate concentrations were identical to those recorded in placebo neurons (Fig. 2A). In dose-response curves constructed by averaging glutamate-evoked NMDA response peak amplitudes from seven and eight NAcc medium spiny cells from placebo and morphine-treated rats, respectively, the glutamate EC_{50} for placebo rats (4.7 ± 0.7 μM) was equivalent to that from morphine-treated rats (5.6 ± 0.5 μM) and was not statistically different.

We then examined the affinity of NAcc NMDA receptors for homoquinolinic acid (HQ) and NMDA, two agonists reported to discriminate among NMDA receptor subunits. Thus, the affinity of recombinant NR1/NR2C or NR2D receptors for NMDA was higher than that of NR1/NR2A or NR2B (Prestley et al., 1995; Buller and Monaghan, 1997). By contrast, the EC_{50} for HQ is higher for NR2A and B than for NR2C. Effects of both HQ and NMDA were tested in the presence of 100 μM glycine. In NAcc neurons from placebo rats, HQ evoked threshold currents at around 1 μM and maximum transient current amplitudes at concentrations between 0.5 and 1 mM (Fig. 3A), with an EC_{50} of 32 ± 4 μM (Fig. 3B). Chronic morphine treatment failed to significantly (p < 0.1) alter the HQ EC_{50} (38 ± 3 μM). However, as is apparent in Fig. 3B, the desensitization rate is faster after chronic morphine treatment, a phenomenon clearly attributable only to HQ because no such effect was observed for the other compounds tested. We also examined the effects of chronic morphine on NMDA-mediated currents. As for glutamate and HQ, the largest current amplitudes were evoked with 1 mM NMDA. Chronic morphine had no effect on NMDA currents, since the NMDA EC_{50} was 37 ± 3 and 38 ± 4 μM in neurons from placebo and morphine-treated rats, respectively (Fig. 3C).

Chronic Morphine Treatment Alters Glycine Affinity for NAcc NMDA Receptors. Glycine, the allosteric NMDA receptor coagonist, has been shown to have a differential affinity for the various NMDA receptor subunits. Thus, the NR1/NR2A affinity for glycine is lower than that of NR2B or NR2C (Kutsuwada et al., 1992; Wafford et al., 1993; Laurie and Seeberg, 1994; Buller and Monaghan, 1997). Therefore, we evoked glycine responses at −60 mV in the presence of 200 μM NMDA and increasing concentrations of the glycine (0.03–100 μM). Under these conditions, glycine EC_{50} was shifted to the right from 2.24 ± 0.15 μM in placebo rats to 5.1 ± 1.45 μM after chronic morphine treatment (Fig. 4). NMDA current amplitudes were statistically different at al-

Fig. 1. NMDA current amplitudes of NAcc medium spiny neurons evoked over a long period of time are stable. NMDA currents were evoked every 20 s for 10 min at −60 mV by long (5-s) applications of 200 μM NMDA and 100 μM glycine. A, typical NMDA currents every 100 s for about 7 min. Baseline currents preceding the current onset are aligned for clarity. The dashed line is set at the peak amplitude of the current recorded at time 0 (t0) for comparison to subsequent NMDA currents. B, mean peak currents averaged from six neurons.
most all concentrations ($p < 0.05$ at 1 $\mu$M and $p < 0.01$ at 3, 10, and 30 $\mu$M glycine).

**Chronic Morphine Treatment Alters the Affinity of Antagonists of NAcc NMDA Receptors.** We assessed the inhibition of NMDA currents by ifenprodil, a polyamine site antagonist, and 7-CI-kyn on currents evoked by NMDA (200 $\mu$M) with glycine (100 $\mu$M). Although ifenprodil was reported to block NMDA currents evoked by recombinant NMDA receptors expressing NR1 and NR2A, B, or C, the affinity of NR1/NR2B recombinant receptors for this antagonist is much stronger than that for NR1/NR2A and NR2C (Williams, 1993; Avenet et al., 1997). Therefore, any decrease of ifenprodil affinity could be interpreted as an increase of NR2A or NR2C expression. We evoked NMDA currents at −60 mV with saturating concentrations of NMDA (200 $\mu$M) and glycine (100 $\mu$M) in the presence of increasing concentrations of ifenprodil (0.01 $\mu$M to 1 mM). Ifenprodil began to block NMDA currents at low concentrations (1 $\mu$M) in both placebo and morphine-treated rats (Fig. 5A). Interestingly, ifenprodil inhibition of NMDA currents was strongly attenuated after chronic morphine treatment for concentrations of 10 to 300 $\mu$M (Fig. 5, A: gray traces, and B). Although there was no detectable difference at the highest concentrations tested (0.5 and 1 mM), the difference between the two groups at 1, 10, 30, 100, and 250 $\mu$M was statistically significant (Fig. 5, A and B). In some neurons of morphine-treated rats, NMDA currents recorded in the presence of effective concentrations of ifenprodil (10–500 $\mu$M) showed a slower desensitization than in neurons of placebo rats (Fig. 5A), a phenomenon not observed in other experimental conditions (compare with Fig. 2A). In placebo rats, ifenprodil blocked NMDA currents with an IC$_{50}$ of 32 ± 2.2 $\mu$M. After chronic morphine treatment, the ifenprodil IC$_{50}$ tripled to 102 ± 2.4 $\mu$M (Fig. 5B).

Avenet et al. (1997) and Priestley et al. (1995) reported that the ability of 7-CI-kyn (which blocks the glycine-binding site) to block NMDA currents also depended upon the type of NR2 subunits coexpressed with NR1: the affinity of NR1/NR2A recombinant NMDA receptors for 7-CI-kyn was higher than that of NR1/NR2B and NR1/NR2C. Therefore, we again evoked NMDA currents by local application of saturating concentrations of NMDA and glycine (see above) in the presence of increasing concentrations of 7-CI-kyn (0.01 $\mu$M to 1 mM). In neurons of both placebo and morphine-treated rats, the lowest effective concentration of 7-CI-kyn in blocking NMDA currents was 1 $\mu$M, with an almost total inhibition reached at 1 mM 7-CI-kyn. Chronic morphine treatment enhanced the 7-CI-kyn block of NMDA currents at concentrations of 1 $\mu$M and higher (Fig. 5, C: gray traces, and D): the 7-CI-kyn IC$_{50}$ was 27 ± 5.8 $\mu$M in neurons of placebo rats and 9.8 ± 1.58 $\mu$M after chronic morphine treatment (Fig. 5B). The difference between the placebo and morphine groups was statistically significant at all the concentrations of 7-CI-kyn but the lowest (Fig. 5D).

We also tested the effects of chronic morphine on the action of d-AP5, a selective competitive NMDA receptor antagonist. Although there is little difference in d-AP5 affinity for NR1/NR2A and NR1/NR2B NMDA recombinant receptors, its affinity for NR2C and NR2D is substantially lower (Priestley et al., 1995; Buller and Monaghan, 1997). In the present study, we found that in NAcc neurons of placebo rats, the d-AP5 IC$_{50}$ was 4.45 ± 1.85 $\mu$M. Chronic morphine treatment did not clearly alter d-AP5 affinity for NAcc medium spiny neurons (6.45 ± 2.83 $\mu$M; Fig. 5D, inset).

We also examined the effects of chronic morphine on the Mg$^{2+}$-mediated block of NMDA receptors, because we had previously shown that chronic morphine decreased this block in a slice preparation. To directly address the postsynaptic locus of action of this effect, we again tested the effects of magnesium in placebo and morphine-treated rats. We found that the Mg$^{2+}$-IC$_{50}$ was only slightly higher (154 ± 10.8 $\mu$M) after chronic treatment compared with placebo rats (147 ± 9.26 $\mu$M).

**Chronic Morphine Alters NMDA Receptor Desensitization.** Among the different biophysical properties of NMDA receptors, deactivation and desensitization have been extensively studied in neurons and expression systems. Thus, it was reported that both inactivation and desensitization are controlled by NR2 subunits with a faster decay of the current when NR2A is coexpressed with the NR1 subunit. Because we found in a previous study (Martin et al., 1999) that chronic morphine treatment accelerated NAcc NMDA current deactivation, we extended this study by examining the effects of morphine on NMDA current desensitization. Of

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**Fig. 2.** Chronic morphine does not alter NMDA receptor-mediated currents evoked by glutamate. A, representative NMDA currents from NAcc neurons from a placebo rat (black traces) and a morphine-treated rat (gray lines) evoked by long (5–s) applications of increasing concentrations of glutamate (1, 10, 100, and 500 $\mu$M) at 30-s intervals. NMDA currents were evoked in the presence of 15 $\mu$M CNQX. Intervening baseline traces have been omitted for clarity. NMDA current traces from the morphine-treated rat were normalized to NMDA currents evoked by 500 $\mu$M glutamate in NAcc neurons from a placebo rat for comparison. B, dose-response plot of peak glutamate currents averaged over seven and eight NAcc cells from placebo and morphine-treated rats, respectively. The fit of the dose-response curves was monophasic using a two-binding site model and yields an EC$_{50}$ of 4.7 ± 0.7 $\mu$M and 5.6 ± 0.5 $\mu$M in NAcc neurons of placebo and morphine-treated rats, respectively.
the three different types of desensitization that have been identified to date (glycine-dependent, $\text{Ca}^{2+}$-dependent, and $\text{Ca}^{2+}$-independent), we studied the effects of morphine on the latter type, because this was an appropriate test for differentiating NR2A from NR2B subunits (Yamakura and Shimoji, 1999).

We evoked NMDA currents around a holding potential by coapplication of 200 $\mu$M NMDA with saturating concentrations of glycine (100 $\mu$M). Current decay was best fit by a single exponential (see Fig. 6A, solid lines). We constructed the histogram of Fig. 6A by distributing the tau values across 0.5-s bin widths. We found that in neurons from both placebo and morphine-treated rats, tau values were unevenly distributed (Fig. 6B). However, taus of NMDA currents from placebo rats were predominantly (18 of 32 neurons) distributed between 1.5 and 2.5 s with a peak at 2 s (Fig. 6B). In contrast, in rats chronically treated with morphine, taus were distributed between 0.5 and 2 s in a majority of neurons (27 of 32 neurons), with a peak at 1.5 s (Fig. 6B). The fit of tau value distributions with a Gaussian function gave mean values of $1.99 \pm 0.08$ s and $1.38 \pm 0.03$ s for placebo and morphine-treated rats, respectively. The nonparametric Kolmogorov-Smirnov test showed that the difference between the two groups was statistically significant ($p < 0.05$).

**Discussion**

Taken together with previous data, our present results suggest that chronic morphine treatment modifies the properties of NAcc NMDA receptors by altering the expression, heterogeneity, or functionality of NR2 subunits (see Table 1).

**NAcc NMDA Receptors in Naive Rats.** Early reports suggested that NMDA receptor heterogeneity originated from the fact that several different subunits (NR1, NR2A-D) are necessary to form functional NMDA receptors. Specific antibodies raised against these subunits and in situ hybridization techniques revealed that each subunit followed a
unique temporal and spatial pattern of expression. NR1, the functional core NMDA receptor subunit, is ubiquitously expressed in the brain, whereas NR2A-D expression is highly variable, with a predominance of NR2A and B in frontal brain structures, NR2C mostly in cerebellum, and NR2D in lower brain regions. In the ventral and dorsal striatum, NR2C is scarce (Standaert et al., 1999) and appears only in interneurons (Standaert et al., 1999). Similarly, although NR2D subunits have been detected in striatum, they are expressed at very low levels, and again, exclusively, in interneurons (Landwehrmeyer et al., 1995; Dunah et al., 1996; Standaert et al., 1996), and are absent in medium spiny projection neurons (Landwehrmeyer et al., 1995; Küppenbender et al., 2000), the focus of our study.

By contrast, medium spiny projection neurons display high levels of mRNA coding for NR2A and NR2B subunits in NAcc and dorsal striatum (Landwehrmeyer et al., 1995; Küppenbender et al., 2000). Our preliminary single-cell reverse transcription-polymerase chain reaction data (Siggins et al., 2003) support these findings, because in placebo rats, NR2A and/or NR2B mRNAs appeared in almost all the NAcc medium spiny neurons tested. Interestingly, we also found that fewer control medium spiny neurons expressed NR2A subunit mRNA than that of NR2B, in line with the recent findings by Landwehrmeyer et al. (1995) and Küppenbender et al. (2000).

**Does Chronic Morphine Treatment Alter NMDA Receptor Subunit Composition in NAcc?** In a previous report on NAcc medium spiny neurons in a slice preparation, we proposed that chronic morphine caused the switch to NR2C or D subunits in postsynaptic NMDA receptors. However, the present findings do not support our initial hypothesis. Indeed, the expression of NR2C or D subunits after chronic morphine should have reduced the glutamate and

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**Fig. 5.** Chronic morphine alters the affinity of ifenprodil and 7-Cl-kyn in NAcc neurons. A, NMDA currents in representative medium spiny neurons from a placebo rat (black traces) and a morphine-treated rat (gray lines) evoked by long (5-s) coapplications of NMDA (200 μM) and increasing concentrations of ifenprodil (0.01–1000 μM). NMDA currents were evoked at 30-s intervals. Intervening baseline traces have been omitted for clarity. B, plot of ifenprodil-mediated inhibition of NMDA currents averaged over 8 and 10 NAcc cells from placebo and morphine-treated rats, respectively. C, representative NMDA currents evoked by coapplication of NMDA (200 μM) and 7-Cl-kyn (0.01–1000 μM). Note the stronger inhibition of NMDA currents by 7-Cl-kyn after chronic morphine treatment. D, mean inhibition of NMDA current peak amplitudes as a function of 7-Cl-kyn concentration. Concentration-response curves of NMDA peak currents averaged over nine NAcc neurons each from placebo and morphine-treated rats. E, the mean NMDA current amplitudes in neurons from naive and morphine-treated rats in the presence of increasing concentrations of d-AP5. Note the lack of effect of chronic morphine on the block by this antagonist of NMDA currents. *, p < 0.05, and **, p < 0.01.
NMDA EC\textsubscript{50} values, because the affinity of NR1/NR2C or NR2D recombinant NMDA receptors for these two agonists is higher than those of NR1/NR2A or NR2B (Wafford et al., 1993; Laurie and Seeburg, 1994; Priestley et al., 1995) (see Table 1). Similarly, the presence of NR2C or D subunits should have increased NAcc NMDA receptor affinity for its coagonist glycine (Kutsuwada et al., 1992; Wafford et al., 1993; Laurie and Seeburg, 1994). However, we found that NAcc NMDA receptor affinity for glycine actually decreased.

Our results with NMDA receptor antagonists also argue against our original hypothesis. Again, had chronic morphine led to the formation of postsynaptic NMDA receptors containing NR2C or NR2D subunits, their affinity for 7-Cl-kyn should not have been affected since recombinant NR1/NR2B (the predominant subunits in medium spiny neurons) and NR1/NR2C have a similar affinity for this antagonist (Priestley et al., 1995; Avenet et al., 1997). By contrast, we found a clear decrease of the IC\textsubscript{50} for 7-Cl-kyn. A possible explanation is that chronic morphine augments the expression or function of the NR2A subunit, which increases the affinity of NMDA receptors for 7-Cl-kyn (Priestley et al., 1995; Avenet et al., 1997). Increased NR2A expression could also account for the decrease of NAcc NMDA receptor IC\textsubscript{50} for ifenprodil (which has a higher affinity for NR2B) that we observed, although substitution of NR2C subunits could lead to a similar conclusion (Williams, 1993; Avenet et al., 1997). However, this latter possibility is weakened by our data on decay rate of NMDA currents; chronic morphine shifted the distribution of NMDA current-desensitizing rates toward faster values, a phenomenon commonly associated with increased expression of the NR2A subunit (see Table 1; Kohr et al., 1994; Monyer et al., 1994). Although the difference in ifenprodil IC\textsubscript{50} values between naive and morphine-treated groups is small (3-fold, but still significant) compared with that between NR2A- and NR2B-containing NMDA receptors in heterologous systems (about 500-fold), this apparent discrepancy may derive from the fact that native NAcc NMDA receptors are unlikely to be purely composed of NR2A or NR2B, as suggested by our single-cell PCR findings (see Siggins et al., 2003). In fact, in control neurons, the NR2 components of these receptors are likely to be di- or trimeric. In addition, the stoichiometry may also fluctuate from cell to cell, dampening the difference observed with NMDA receptors containing only one NR2 subunit. Furthermore, although subunit combinations of native NMDA receptors have
been found to show the same general properties as those in recombinant expression systems (Flint et al., 1997; Gottmann et al., 1997; Kew et al., 1998), pharmacological sensitivities of native receptors in the normal milieu of the neuronal membrane may be quantitatively different from those in expression systems. The proposed up-regulation of NR2A subunit expression or function by chronic morphine would also explain the faster deactivation rate of NMDA-EPSCs of NAcc medium spiny neurons after morphine treatment (Martin et al., 1999), since deactivation is regulated in a similar way by NR2 subunits (Flint et al., 1997; Gottmann et al., 1997).

However, an increase of NR2A subunit expression cannot account for two other findings in the NAcc slice preparation: chronic morphine-attenuated Mg\(^{2+}\) blockade and phorbol ester-mediated enhancement of NMDA-EPSC amplitudes (Martin et al., 1999, see Table 1). We initially hypothesized that NR2C or NR2D expression accounted for these effects, but this is now countered by our pharmacological and molecular (Siggins et al., 2003) data from isolated neurons. These effects, believed to be postsynaptic, may in fact reflect, at least partially, a presynaptic locus of action. Although some phorbol effects appeared to be postsynaptic (Martin et al., 1999), we cannot rule out the possibility that a major target for phorbol esters may take place on the presynaptic side (Oleskevich and Walsmey, 2000). Similarly, because Mg\(^{2+}\) is well known to alter neurotransmitter release, it is conceivable that the decrease of Mg\(^{2+}\)-mediated block by chronic morphine may reflect a presynaptic site of action. Finally, NMDA-EPSCs that originate mostly from the dendritic arborization in a slice preparation may be different from the NMDA currents evoked predominantly from somata in the present study. In support of this idea, we recently reported that dendrites and somata of NAcc medium spiny neurons express calcium-activated potassium (BK) channels that have disparate subunit compositions (Martin et al., 2004). Although the importance of dendrites, which represent 90% of neuronal membrane surface, may not be as critical for mRNA as it is for proteins, it is worth noting that mRNAs have also been found in neurites (see Job and Eberwine, 2001).

A recent study by Zhu et al. (1999) found that neither NR2A nor NR2B mRNA levels were affected after chronic morphine, suggesting that the hypothesized up-regulation of NR2A subunits may take place at the post-transcriptional or post-translational (e.g., subunit phosphorylation) level. It also seems possible that the change from heteromultimeric to homomeric NR2 expression seen in the isolated NAcc neurons with chronic morphine (Siggins et al., 2003) could account for the physiological changes. Interestingly, a recent study on transgenic mice by Inoue et al. (2003) further supports the role of NR2A subunits in chronic morphine effects by showing that NR2A knockout mice showed a significant loss of tolerance to and dependence on morphine analgesic properties by morphine. NAcc neurons are generally silent in an in vitro preparation, but they display a bi-stable membrane potential in vivo that varies between $-80$ and $-60$ mV and that presumably is controlled by convergent electrical activity coming from limbic structures such as hippocampus, amygdala, and prefrontal cortex (O’Donnell et al., 1999). This convergence should enable a blend of various types of information (affective, mnemonic, and cognitive). Although the mechanisms of integration of this information by medium spiny neurons is still unknown, it is likely that any persistent alteration of the physiology of receptors and channels will impact NAcc electrical activity and, ultimately, its integrative properties. A faster desensitization and inactivation brought about by NR2A subunits may impair spatial and temporal integrative properties of NAcc medium spiny neurons and therefore may disrupt the way signals from limbic structures are processed. Similarly, the decrease of NMDA receptor affinity for glycine, which could partially account for the reduction of NMDA-EPSC amplitudes after chronic morphine treatment seen in the NAcc slice, suggests that the ability of NMDA receptors to reach spike threshold and to bring the membrane potential to an “up state” would be hampered, further impacting the integrative properties of NAcc projection neurons.

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**NMDA Receptors in Accumbens and Chronic Morphine**