

Subpopulation of Dorsal Horn Neurons Displays Enhanced N-methyl-D-aspartate Receptor Function after Chronic Morphine Exposure

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Background: Morphine tolerance may be attributed to enhancement of glutamatergic neurotransmission, in particular to increased function of the N-methyl-D-aspartate (NMDA) receptor. The cellular mechanisms responsible for these changes remain poorly defined. The authors identified and characterized a specific subpopulation of dorsal horn neurons, displaying NMDA receptor plasticity in response to chronic morphine administration.

Methods: The authors undertook current clamped and voltage clamped recordings of NMDA receptor-mediated responses from cultured rat dorsal horn neurons that were untreated or treated for 7 days with 1 or 100 μM morphine.

Results: Smaller (capacitance ≤ 22 pF), tonic firing neurons showed a significantly enhanced NMDA receptor-mediated peak current after prolonged morphine treatment, whereas larger and phasic firing neurons showed no enhancement. With high-concentration but not low-concentration morphine treatment, Mg^{2+} blockade of NMDA receptors at resting membrane potentials was reduced. Furthermore, the chronic opioid-induced increase in NMDA current was attenuated by pretreatment with either a μ -opioid receptor inhibitor (naloxone) or an NMDA receptor inhibitor (2-amino-5-phosphonovalerate) (low-concentration > high-concentration morphine).

Conclusions: In an electrophysiologically defined subpopulation of dorsal horn neurons, enhanced NMDA receptor function after chronic morphine exposure was shown to be mechanistically dependent on morphine concentration and sensitive to both NMDA and μ -opioid receptor antagonism. Therefore, these changes observed in this population of sensory spinal neurons can be used to study the development and prevention of opioid tolerance described in multiple laboratory and clinical reports.

CHRONIC administration of opioids, in particular morphine, induces tolerance in both humans and animals. Tolerance has been described for all available μ -opioid receptor (MOR) agonists,¹ thereby resulting in problematic long-term therapeutic use of these agents for pain control. Morphine, at clinically relevant concentrations, binds primarily to the μ subtype of G_i/G_o protein-coupled opioid receptors, which regulate adenylyl cyclase,

Ca^{2+} , and K^{+} channels.² The activation of MORs result in reduction of 3'-5'-cyclic adenosine monophosphate, membrane hyperpolarization, and subsequent neuronal depression. Modulation of function in spinal cord neurons by MORs affects the transmission of painful stimuli to the central nervous system. Painful stimuli activate peripheral nociceptors found on dorsal root ganglia cells causing them to release glutamate into synapses with dorsal horn (DH) neurons. This released glutamate activates ionotropic glutamate receptors such as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and N-methyl-D-aspartate (NMDA) receptors in the postsynaptic membrane of the DH neurons, causing membrane depolarization and the firing of action potentials. Glutamatergic neurotransmission is used by sensory neurons to send pain signals through to higher centers in the central nervous system for further processing. MOR modulation of DH neurons depresses this signal transduction causing antinociception and providing pain relief.

Although MOR internalization has been thought to play a role in acute opioid tolerance,³ the exposure of MORs to morphine does not cause internalization and down-regulation of these receptors, as occurs with other more potent and selective μ -opioid agonists.⁴⁻⁶ Prolonged MOR activation results in desensitization of the receptors through G-protein uncoupling and subsequent neuronal excitation. It is the resultant enhancement in neuronal excitation rather than its suppression that is responsible for the paradox underlying opioid or narcotic tolerance. In the same vein, chronic MOR activation-dependent direct increases in glutamatergic neurotransmission can produce neuronal excitation, which may also underlie opioid tolerance. Support for this mechanism has been shown in a multitude of behavioral and *in vitro* studies showing that opioid tolerance can be avoided or rescued by cotreatment with NMDA receptor agonists (reviewed in Trujillo⁷) and that MOR activation can increase protein kinase C (PKC) activity and enhance the function of the NMDA receptor through the reduction of a physiologic Mg^{2+} blockade of its associated channel pore at resting membrane potentials.^{8,9}

The importance of the NMDA receptor in excitatory glutamatergic neurotransmission and in many processes such as pain sensation,¹⁰ learning and memory,¹¹⁻¹³ and development¹⁴ that are subject to plasticity makes this receptor a key player to consider in opioid tolerance. However, the mechanisms responsible for the NMDA receptor plasticity, particularly in response to chronic

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MOR activation, are unclear and understudied. Many recent preclinical⁷ and clinical trials on the prevention of and rescue from opioid tolerance have identified that attenuation of neuronal plasticity occurs by NMDA receptor antagonism. As well, the development of opioid tolerance and dependence and its attenuation by NMDA antagonists has been demonstrated in spinalized animals¹⁵ and after intrathecal or epidural administration.¹⁶ This suggests that cellular mechanisms responsible for opioid tolerance occur in the spinal cord where (1) neural networking is likely more simplified than in the brain and (2) pain processing begins. Therefore, the goal of our research is to use a simplified spinal cord model to investigate the cellular mechanisms responsible for enhanced NMDA receptor activity leading to opioid tolerance.

Using electrophysiologic studies of NMDA receptor-mediated responses from cultured rat DH neurons that were untreated and treated for 7 days with 1 or 100 μM morphine, we determined (1) whether NMDA receptor function in spinal cord DH neurons is modulated by chronic morphine administration; (2) which neurons, classified by size and firing pattern, display this plasticity; (3) whether morphine concentration affects NMDA plasticity; and (4) whether such changes could be attenuated by NMDA antagonism.

Materials and Methods

Experimental protocols were approved by the Animal Care Committee at the Laboratory Animal Services Department of the Hospital for Sick Children (Toronto, Ontario, Canada).

Cell Culture

The primary DH neuron cultures were prepared as previously described.¹⁷ Briefly, fetal Wistar rats (embryonic day 18 or 19) were removed from a time-pregnant female rat (Charles River Laboratories, Wilmington, MA) immediately after cervical dislocation. After decapitation, the fetal spinal cords were then removed by an anterior approach and placed in recently thawed Hank's solution. The meninges were stripped, and each cord was opened by longitudinal incision through the dorsal commissure to the central canal. The opened cord was then pinned onto a Sylgard (Dow Corning Corp., Midland, MI) covered glass dish to allow dissection of the dorsal half of the cord by cuts along the lateral funiculus bilaterally. The dorsal halves of 7–14 spinal cords were pooled and minced and then incubated in Hanks' solution with 0.25% trypsin (Invitrogen Canada Inc., Burlington, Ontario, Canada) at 37°C for 45 min. This tissue was washed in minimum essential medium (Invitrogen Canada Inc.) containing 10% fetal bovine serum (Invitrogen Canada Inc.), 10% horse serum (Invitrogen Canada Inc.),

and 1 U/ml insulin (Novo Nordisk Canada Inc., Mississauga, Ontario, Canada) and then mechanically triturated and plated on collagen in 35-mm Nalgene dishes (Fisher Scientific Co., Ottawa, Ontario, Canada). Approximately one spinal cord dorsal half was plated per dish. After 4 days of incubation, the dishes were treated for 24 h with 5'-fluoro-2-deoxyuridine (13 $\mu\text{g}/\text{ml}$) and uridine (33 $\mu\text{g}/\text{ml}$) (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Beyond 6 days of incubation, the culture media contained minimal essential media with 10% horse serum only. Opioid treatment of the cultures with 1 or 100 μM morphine using a one-time application was started 7 days before recording. Concentrations of morphine were measured by high-performance liquid chromatography and were noted to be stable during the 7 days of incubation. The treated or untreated cultures were used for recording or Western blot at between 14–21 days after plating. This ensured that experiments were undertaken using matured DH neurons with adult complements of NMDA receptors in terms of subunit composition and receptor localization at synapses.¹⁸

Electrophysiologic Recording

Immediately before electrophysiologic recording, the control and 100 μM morphine-treated cultured DH neurons were washed with three near-complete exchanges of extracellular solution containing 140 mM NaCl, 1.3 mM CaCl_2 , 5.4 mM KCl, 25 mM HEPES, 33 mM glucose, 0.003 mM glycine, and 0.0003 mM tetrodotoxin, buffered to a pH of 7.4 with NaOH and adjusted to an osmolality of 320–325 mOsm. This ensured the removal of morphine in the treated dishes. High-performance liquid chromatographic measurements of morphine concentration showed that $76 \pm 6\%$ of the morphine was removed after only one partial (two-thirds volume) replacement of the extracellular solution.

All electrophysiologic recordings were made at room temperature (20°–22°C). Patch electrodes were pulled from thin-walled borosilicate glass (1.5-mm OD; World Precision Instruments, Sarasota, FL) using a two-stage vertical puller (Narashige PP-830; Tokyo, Japan) with a series resistance of 3–8 M Ω . Initial voltage clamped whole cell recordings of 1,000 μM NMDA-evoked current were made using cesium fluoride (CsF)-containing electrodes which consisted of 140 mM CsF, 35 mM CsOH, 10 mM HEPES, 2 mM MgCl_2 , 11 mM EGTA, 2 mM tetraethylammonium chloride, 1 mM CaCl_2 , and 4 mM magnesium adenosine 5'-triphosphate, buffered to pH 7.4 with cesium hydroxide and adjusted to 290–310 mOsm. Electrodes containing CsF used in these experiments further eliminated MOR G protein-coupled activity and confounding morphine effects. Later experiments required a KCl-based electrode (see Firing Pattern Determinations below) with which the maximal current evoked by 1,000 μM NMDA was of lower amplitude than with the CsF-containing electrode, as predicted. However, the

differences between control and treated neurons remained.

Whole cell potentials and currents were recorded using the Multiclamp 700A amplifier (Axon Instruments Inc., Union City, CA), and data were filtered (2 kHz), digitized using the Digidata 1322A (Axon Instruments Inc.), and acquired on-line at a sampling frequency of 10 kHz using the pCLAMP8 program (Axon Instruments Inc.).

Firing Pattern Determinations. The cultured DH neurons were initially patched in voltage clamp at a holding potential of -60 mV. After the neuron was determined to be stable by a small and unchanging leak current (between 0 and -100 pA), the head stage was changed to a current clamped configuration. Firing pattern determinations were undertaken using current clamped whole cell recordings of the control and morphine-treated DH neurons. Tetrodotoxin and glycine were eliminated from the extracellular solution during current clamped recordings. The electrodes were filled with intracellular solution consisting of 140 mM KCl, 10 mM HEPES, 2 mM MgCl_2 , 10 mM EGTA, and 4 mM magnesium adenosine 5'-triphosphate. This solution was buffered to a pH of 7.4 using KOH, and the osmolality was adjusted to 290–300 mOsm. The resting membrane potential was recorded, and firing patterns were determined in response to hyperpolarizing and depolarizing current injections (50-pA steps from -350 to $+200$ pA) in 500-ms intervals. Further hyperpolarizing protocols with current injections in 200-pA steps from $-1,000$ to $+200$ pA and depolarizing protocols with injections in 100-pA steps from 0 to $+1,000$ pA were run on some neurons to ensure that higher thresholds of activity were not missed. Only neurons that maintained a small leak current and looked healthy under light microscopy were further studied under voltage clamp.

Neuronal Size Determinations. Small to medium neurons in the dorsal root ganglia have been shown to vary in their expression of MORs.¹⁹ Therefore, to determine whether DH neuronal size correlates with NMDA receptor plasticity after chronic morphine exposure, we measured NMDA current amplitude in control and treated cells that had varying membrane capacitances.

As a measure of neuronal size, membrane capacitance of the DH neuron (Cap_m) under study was estimated from the area under the capacitance transient $[(\text{AUC})I_{\text{ct}}]$ evoked by a hyperpolarizing 10-mV step and was used as an approximation of neuronal size.

$$\text{Cap}_m = (\text{AUC})I_{\text{ct}} \times \Delta V_m$$

The frequency distribution of the membrane capacitance measurements from 127 consecutively studied control and treated DH neurons was then plotted and fit visually and by the least sums of squares of the variance. It was determined that this frequency distribution was best fit by three distinct gaussian distributions (fig. 1) as

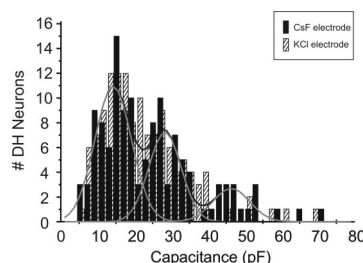


Fig. 1. Frequency distribution of dorsal horn (DH) neuron capacitances measured in pooled control and morphine-treated neurons. Neurons recorded using a cesium fluoride (CsF)-containing electrode (filled bars) or a KCl-containing electrode (striped bars) had a similar capacitance distribution that was best fit with three gaussian curves with intersects at approximately 22.0 and 38.0 pF.

determined by chi-square fitting (least chi-square = 3.55 compared with 2-gaussian chi-square = 4.82 and 1-gaussian chi-square = 5.00) using the Levenberg-Marquardt algorithm (Origin 5.0; OriginLab, Northampton, MA). The points of intersection of these three curves defined the ranges of capacitances by which the DH neurons were arbitrarily grouped according to size and further analyzed. Recordings were made using both CsF- and KCl-based electrodes and were compared with respect to size groupings (fig. 1).

NMDA Current Recordings. The cultured DH neurons were held in voltage clamp at a membrane potential of -60 mV. The DH neuron under study was perfused continuously with extracellular solution containing glycine and tetrodotoxin delivered *via* a three-barrel capillary tube system with each barrel attached to a 7-ml reservoir, height-adjusted to deliver perfusion fluid at a rate of 1 ml/min. A rapid exchange of solutions (approximately 30 ms) between barrels by lateral movement of the capillary tube system was achieved using the SF-77BLT Perfusion Fast-Step (Warner Instruments Corp., Hamden, CT) and allowed exposure of the neuron to different concentrations of NMDA, morphine, or both.

Initial voltage clamped whole cell recordings were made during a 1-s application of $1,000 \mu\text{M}$ NMDA. Peak NMDA current amplitude was then correlated to neuronal size and firing pattern for both control and morphine-treated DH neurons.

Following studies using DH neurons selected for smaller size and tonic firing capabilities were undertaken to examine 100 and $1,000 \mu\text{M}$ NMDA-evoked current in control and morphine-treated neurons.

To determine whether there were any changes in the function of the NMDA receptors after chronic morphine exposure, we examined the concentration-response and current-voltage relations of these receptors. NMDA at different concentrations (3, 10, 30, 100, 300, and $1,000 \mu\text{M}$) was applied using the fast perfusion system described. A measurable current was evoked by $3 \mu\text{M}$ NMDA, but an open channel block of the NMDA receptor occurred at concentrations above $1,000 \mu\text{M}$. There-

fore, 1,000 μM NMDA was used as the saturating concentration for concentration-response relations and maximal NMDA-evoked current comparisons between control and morphine-treated DH neurons. As well, NMDA-evoked currents desensitized to a steady state amplitude; therefore, both the maximal and steady state current amplitudes were measured using pCLAMP (Axon Instruments Inc.), and the data were plotted using GraphPad Prism (Graph Pad, San Diego, CA). The concentration of NMDA that produced 50% of the maximal and steady state responses ($\text{EC}_{50\text{max}}$ and $\text{EC}_{50\text{ss}}$) and the respective Hill coefficient (n_{Hmax} and n_{Hss}) were determined according to the equations

$$I = I_{\text{max}} \times \frac{1}{1 \oplus \frac{\text{EC}_{50\text{max}}}{[\text{ligand}]^{n_{\text{Hmax}}}}}$$

and

$$I = I_{\text{ss}} \times \frac{1}{1 \oplus \frac{\text{EC}_{50\text{ss}}}{[\text{ligand}]^{n_{\text{Hss}}}}},$$

where I_{max} is the maximal response and I_{ss} is the steady state response observed at a saturating concentration of the agonist.

To assess the physiologic Mg^{2+} blockade of NMDA receptors, NMDA (100 μM) current-voltage relations in the control and morphine-treated DH neurons were examined at holding potentials between -120 and 80 mV (in 20-mV increments) in extracellular solution with 0 and 30 μM MgCl_2 .

MOR-1 Western Blots

Cultured DH neurons were grown, as described above (Cell Culture section), in 100-mm dishes (6 or 7 spinal cord dorsal halves per dish) and incubated for 14 days without or with 1 μM morphine, 100 μM morphine, or 1 μM [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin acetate (DAMGO) added for the last 7 days before the Western blots were performed. The DH neurons were gently washed off the dishes after incubating for 5–6 min at 37°C with 0.01% trypsin-EDTA in phosphate-buffered saline solution. The tissue was transferred to a glass tube with lysis buffer consisting of 50 mM Tris buffer, 150 mM NaCl, and 5 mM EDTA at a pH 7.4 and mechanically homogenized with a fitted plastic pestle (VWR International, Mississauga, Ontario, Canada) attached to a Cafamo Stirrer Type RZR2000 (Warton, Ontario, Canada). Cellular fractionation²⁰ of the tissue into membrane and cytosolic fractions was performed by differential centrifugation of the tissue homogenate in lysis buffer. Each fraction was then resuspended in the same lysis buffer with 0.5% Nonidet P-40, 1% Triton-X, 0.5 mM phenylmethylsulfonyl fluoride, and 5 $\mu\text{g}/\text{ml}$ each of aprotinin, pepstatin A, and leupeptin (Sigma-Aldrich Canada Ltd.)

added. After a 1-h incubation period at 4°C, 0.5% sodium deoxycholate was added, and each fraction was shaken for 10 min at the same temperature. For Western blots, 7.5 μg protein was loaded for each lane, subjected to a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membrane for probing with 1 $\mu\text{g}/\text{ml}$ anti-MOR-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Oxidized luminol was used for detection, and the nitrocellulose was exposed to x-ray film, which was scanned using Kodak Image Station 2000R (Eastman Kodak Co., New Haven, CT). Using Kodak 1D Image Analysis Software (Eastman Kodak Co.), band intensities were quantified and compared.

Statistical Analyses

All results were reported as mean \pm SEM. The mean NMDA peak amplitudes measured in control and morphine-treated neurons were compared using the two-tailed Student *t* test with significance reported at $P < 0.05$. The EC_{50} and Hill coefficients for NMDA concentration-response relations in both groups of neurons were similarly compared using the Student *t* test. In the control and morphine-treated neurons that were grouped by size, comparisons between the groups of same-sized neurons were made using a one-way analysis of variance. Data sets for which tests of normality failed were analyzed with corresponding nonparametric tests.

Results

NMDA Plasticity in Chronic Morphine-treated DH Neurons

Many recent studies indicate that the DH of the spinal cord consists of a heterogeneous population of neurons with differing expression of μ -opioid and other receptors as well as membrane activity.^{19,21–25} Therefore, we investigated NMDA receptor responses in control and chronic morphine-treated DH neurons that were characterized by size, morphology, and action potential firing pattern.

Enhanced NMDA Responses in Small-capacitance Chronic Morphine-treated DH Neurons. *N*-methyl-D-aspartate receptor-mediated increases in intracellular Ca^{2+} during chronic morphine administration, which results in “darkened neurons,”²⁶ were not observed in our preparation. The cultured DH neurons treated with morphine were indistinguishable by light microscopy from controls. Patch clamped neurons, including those in the high-morphine group, did not show an increased leak current or premature demise even with repeated exposure to NMDA. All neurons studied showed NMDA-evoked responses.

Pooled data from voltage clamped recordings of randomly selected treated or untreated cultured DH neurons revealed no significant difference between the con-

Table 1. NMDA-Evoked NMDA Receptor-mediated Current Amplitude

	I_{\max} , pA, 1 mM NMDA	Cap, pF	I_{\max}/Cap , pA/pF	I_{ss} , pA	I_{ss}/Cap , pA/pF	I_{ss}/I_{\max}
Control (n = 70)	1,418 ± 109	22 ± 1	74 ± 6	765 ± 80	37 ± 4	0.49 ± 0.02
100 μM morphine treated (n = 57)	1,873* ± 146	27† ± 2	83 ± 8	1,007 ± 97	41 ± 4	0.49 ± 0.02
P value	0.01*	0.04†	0.40	0.05	0.47	0.84

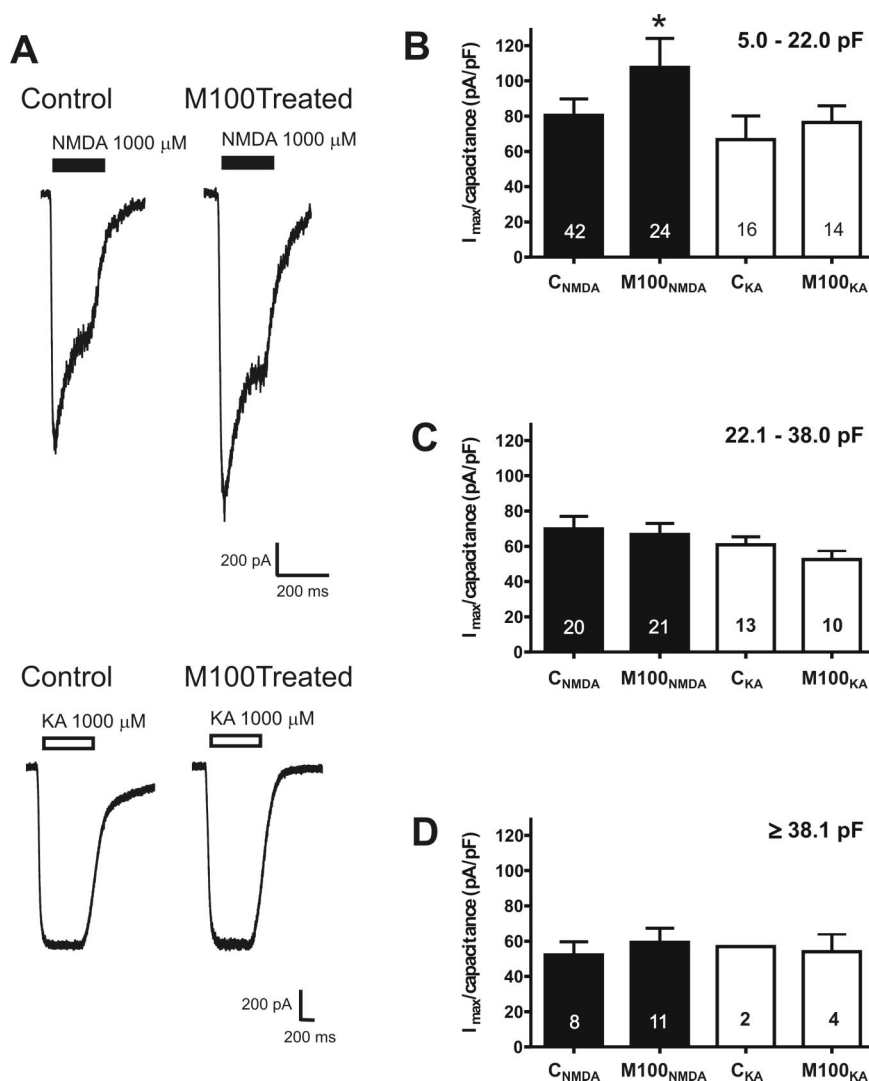
N-methyl-D-aspartate (NMDA)-evoked NMDA receptor-mediated current measurements in 70 control dorsal horn neurons and 57 dorsal horn neurons treated with 100 μM morphine. Average maximal current (I_{\max}), neuronal capacitance (Cap), maximal current normalized to capacitance (I_{\max}/Cap), steady state current (I_{ss}), steady state current normalized to capacitance (I_{ss}/Cap), and steady state current normalized to maximal current (I_{ss}/I_{\max}) are tabulated for control and morphine-treated neurons. (Student *t* test, *† $P < 0.05$.)

control and morphine-treated NMDA-evoked peak current amplitude when normalized to capacitance (table 1). However, after the DH neurons were grouped by size, the difference in NMDA-evoked current amplitude was evident in 100 μM morphine-treated neurons compared with control in a subset of smaller DH neurons with membrane capacitance measures between 5 and 22 pF (1,388 ± 928, n = 24, *vs.* 983 ± 527, n = 42; $P = 0.02$; figs. 2A and B). This difference in the NMDA-induced peak amplitude did not exist in larger neurons with membrane capacitance measures of 22.1–38 pF ($P =$

0.92 and > 38 pF; $P = 0.34$; figs. 2C and D). In the neurons with smaller capacitances, there were no differences in the ratio of steady state to peak NMDA current between control and morphine-treated neurons ($P = 0.46$), suggesting that desensitizing responses of the NMDA receptor are unchanged after morphine treatment.

Classification of DH Neurons Based on Firing Pattern. Previous studies have shown no correlation between morphology and μ -opioid responses,²⁷ but only tonic firing neurons in the DH were shown to hyperpolarize in response to DAMGO, a specific MOR agonist.²¹

Fig. 2. N-methyl-D-aspartate (NMDA), 1,000 μM -evoked maximal current amplitudes in control (C_{NMDA}) and 100 μM morphine-treated ($M100_{\text{NMDA}}$) dorsal horn neurons grouped according to neuronal size. (A) Representative traces of 1,000 μM NMDA and 1,000 μM kainate (KA)-evoked current recorded from small (low-capacitance) dorsal horn neurons. (B) NMDA, 1,000 μM -evoked maximal current amplitudes normalized to capacitance ($I_{\max}/\text{capacitance}$) in smaller neurons with capacitance of 5.0–22.0 pF. (C) Medium neurons with capacitance of 22.1–38.0 pF. (D) Large neurons with capacitance of 38.1 pF or greater. In control experiments, 1,000 μM kainate-evoked AMPA receptor mediated responses were also recorded and did not show any differences in $I_{\max}/\text{capacitance}$ between various sized dorsal horn neurons that were untreated (C_{KA}) and morphine treated ($M100_{\text{KA}}$). Numbers within the bars indicate the number of dorsal horn neurons from which the data were averaged. (Student *t* test, * $P < 0.05$).



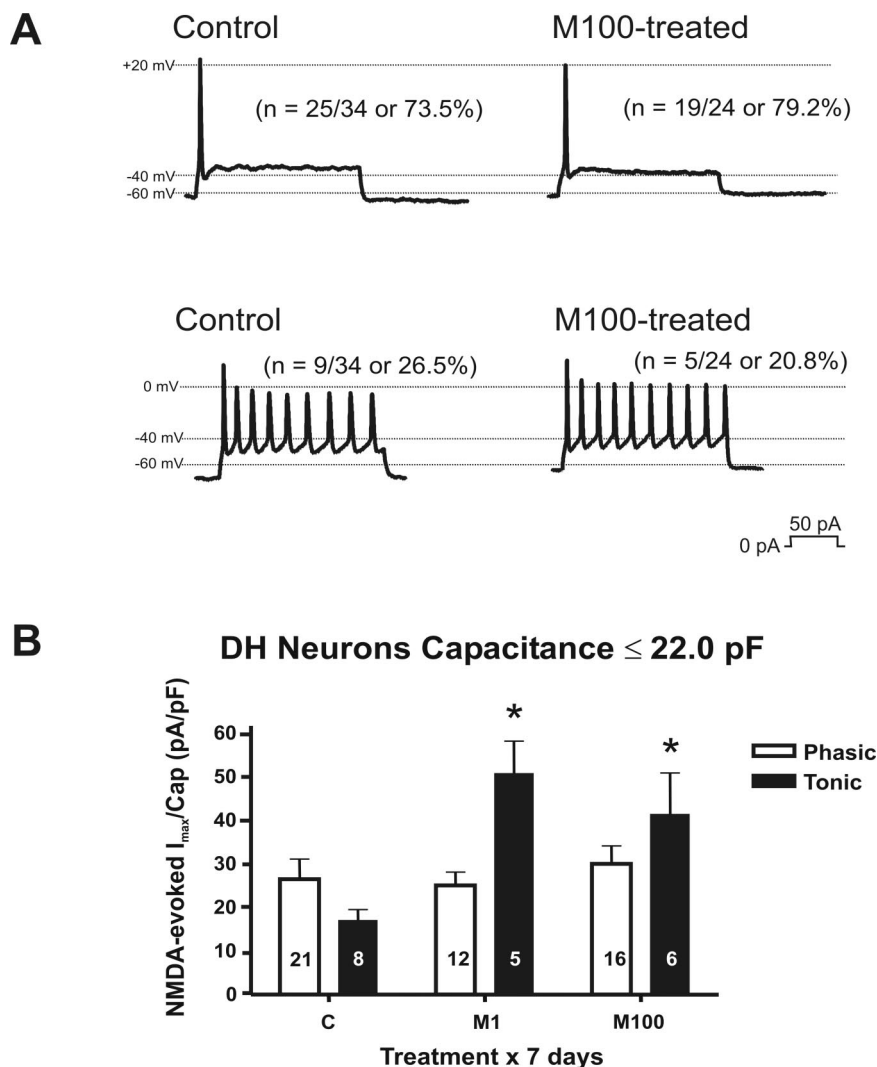


Fig. 3. (A) Representative traces from phasic (top) and tonic (bottom) firing dorsal horn (DH) neurons during a 50-pA current injection. Most of the neurons were phasic firing, with no difference in prevalence between control and morphine (M100) treated. (B) *N*-methyl-D-aspartate (NMDA)-evoked peak current normalized to capacitance (I_{\max}/Cap) in small DH neurons with cell capacitance of 22.0 pF or less. Tonic firing (black bars) DH neurons showed an increase in NMDA-evoked I_{\max}/Cap after 1 μ M (M1) and 100 μ M (M100) morphine treatment for 7 days, whereas phasic firing (white bars) cells did not. Numbers within the bars indicate the number of DH neurons from which the data were averaged. (One-way analysis of variance with Bonferroni multiple comparisons posttest, * $P < 0.05$).

In an initial study, current injection of a random sample of neurons from the cultured rat DH showed that 25 of 34 control DH neurons (73.5%) were phasic firing and 9 (26.5%) fired tonically (fig. 3A). Similarly, 19 of 24 neurons (79.2%) chronically treated with 100 μ M morphine were phasic firing, whereas the remainder (20.8%) were tonic firing (Fisher exact test, $P = 0.76$; fig. 3A).

A previous study of lamina I neurons suggested a correlation between firing pattern and morphology of the neurons.²² Therefore, we compared the DH neurons, control and treated, identified by phasic or tonic firing patterns with their morphology determined under light microscopy (chi-square test, $P = 0.14$). Pyramidal neurons accounted for 35% of phasic and 60% of tonic firing cells, bipolar neurons accounted for 44% of phasic and 10% of tonic firing cells, and multipolar neurons accounted for 21% of phasic and 30% of tonic firing cells. *Post hoc* analysis of all neurons in the study (including those chosen for size and firing pattern; see next section) showed that pyramidal neurons accounted for 62 of 140 phasic (44%) and 33 of 68 tonic firing cells (49%), bipolar neurons accounted for 54 of 140 phasic (39%) and 22 of 68

tonic firing cells (32%), and multipolar neurons accounted for 24 of 140 phasic (17%) and 13 of 68 tonic firing cells (19%) ($P = 0.98$). In this preparation, there was no correlation between DH neuron morphology and firing pattern in either control or morphine-treated conditions.

Of the tonic firing neurons, all showed a reduction to phasic or nonfiring patterns after an acute exposure to 100 μ M morphine in both control and chronic morphine-treated groups. Only a very small fraction ($< 5\%$) of phasic firing neurons responded with a change in firing pattern to acute morphine exposure.

Enhanced NMDA Responses in Small, Tonic Firing DH Neurons after Chronic Morphine Exposure.

Of the smaller neurons (between 5 and 22 pF) treated with morphine, it was determined through consecutive current and voltage clamped recordings that tonic firing morphine treated neurons had a higher NMDA-induced peak current amplitude than tonic firing control neurons (-672 ± 388 pA, $n = 11$, vs. -293 ± 126 pA, $n = 8$; $P = 0.01$), whereas neurons that were phasic firing were no different in their NMDA current than controls (-388 ± 203 pA, $n = 28$, vs. -365 ± 277 pA, $n = 22$;

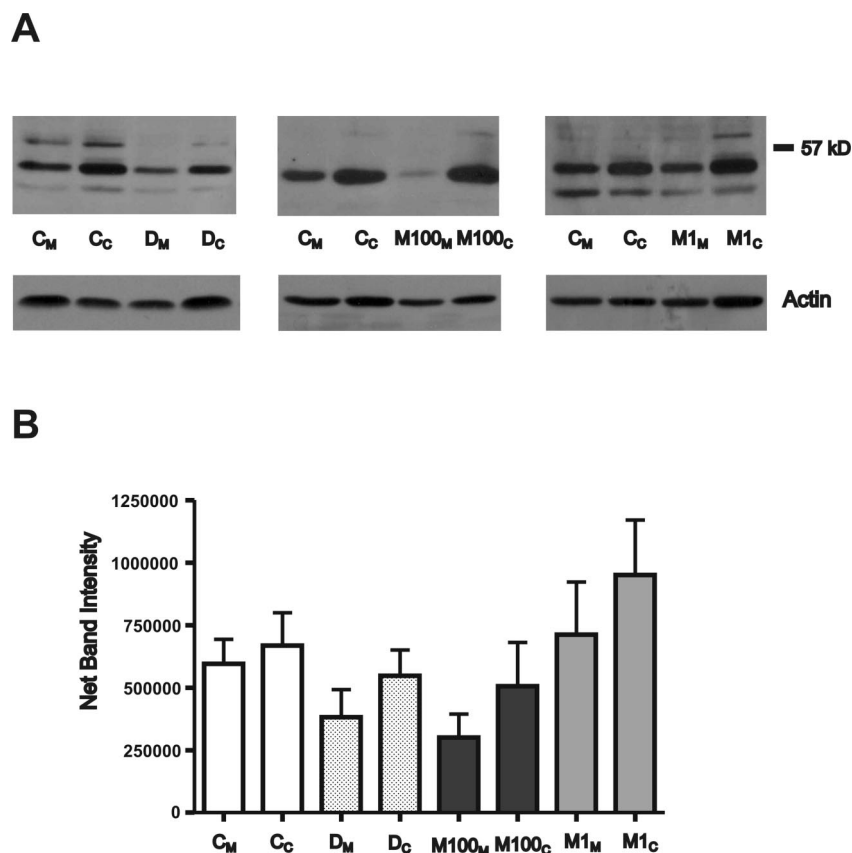


Fig. 4. (A) Western blots labeled with anti-MOR1 of membrane (subscript M) and cytosolic (subscript C) fractions of dorsal horn neuron cultures untreated (C) and treated with 1 μ M morphine (M1), 100 μ M morphine (M100), or 1 μ M DAMGO (D) for 7 days. The detected MOR1 protein was approximately 50 kd in size. The 57-kd marker is shown. (B) Surface expressions of MOR1 in dorsal horn neurons treated for 7 days with 1 μ M DAMGO, 100 μ M morphine, and 1 μ M morphine were not significantly reduced. (One-way analysis of variance).

$P = 0.75$). The increase in NMDA current was not demonstrated in the larger DH neurons (≥ 22.1 pF). Peak current normalized to capacitance recorded from low-concentration morphine (1 μ M)-treated neurons (38.4 ± 20.4 pA/pF, $n = 8$) and high-concentration morphine (100 μ M)-treated neurons (37.3 ± 18.3 pA/pF, $n = 5$) were not significantly different from in controls (30.1 ± 13.6 pA/pF, $n = 16$) ($P = 0.32$ and $P = 0.24$, respectively).

There was a more prominent effect after chronic exposure to low-concentration morphine (I_{\max} /capacitance: 50.8 ± 16.4 pA/pF, $n = 5$, vs. 17.1 ± 6.7 pA/pF, $n = 8$ controls; $P = 0.01$) than after higher-concentration morphine exposure (41.4 ± 23.0 pA/pF, $n = 6$, vs. 17.1 ± 6.7 pA/pF, $n = 8$ controls; $P = 0.05$; fig. 3B). This may be due to the trend to a lesser reduction in surface expression of MOR1 receptors after a 7-day exposure to 1 μ M than 100 μ M morphine as shown by Western blot, although MOR1 levels after chronic morphine treatments were not significantly different from controls (fig. 4). The MOR splice variant MOR1 is highly expressed in the DH cultures and is still expressed even after chronic treatment with 1 μ M DAMGO, 1 μ M morphine, and 100 μ M morphine.

NMDA Receptor Affinity Unchanged after Chronic Morphine Treatment

Subunit composition of NMDA receptors determines activation and desensitization kinetics of these receptors (reviewed in Cull-Candy *et al.*²⁸). Therefore, to investi-

gate whether expressed NMDA receptors have been altered with chronic morphine treatment, we measured receptor affinity and cooperativity for NMDA agonist using concentration-response recordings and subsequent calculations of effective concentration for activation at 50% maximal current (EC_{50}) and the Hill coefficient (n_H) of the peak and steady state NMDA-induced current. These recordings were obtained in selected small, tonic firing DH neurons. There were no significant differences in EC_{50} or n_H calculated for peak or steady state NMDA receptor responses in low- and high-concentration morphine-treated neurons compared with control neurons (figs. 5A and B). These values were no different from randomly selected DH neurons that were untreated (I_{\max} : $EC_{50} = 39.99 \pm 1.08$ μ M, $n_H = 1.13 \pm 0.08$; steady state: $EC_{50} = 16.64 \pm 1.09$ μ M, $n_H = 2.06 \pm 0.28$; $n = 11$) and treated with 100 μ M morphine (I_{\max} : $EC_{50} = 43.71 \pm 1.04$ μ M, $n_H = 1.08 \pm 0.04$; steady state: $EC_{50} = 16.91 \pm 1.08$ μ M, $n_H = 1.80 \pm 0.22$; $n = 9$). Therefore, these results suggest a uniformity of expressed NMDA receptors that does not affect agonist affinity for activation and in the desensitized state of these receptors even after morphine treatment.

Reduced Physiologic Mg^{2+} Blockade of NMDA Receptors after a High Concentration but Not a Low Concentration of Chronic Morphine Treatment
DAMGO, 1 μ M, has been shown to induce a PKC-dependent reduction in the physiologic Mg^{2+} blockade

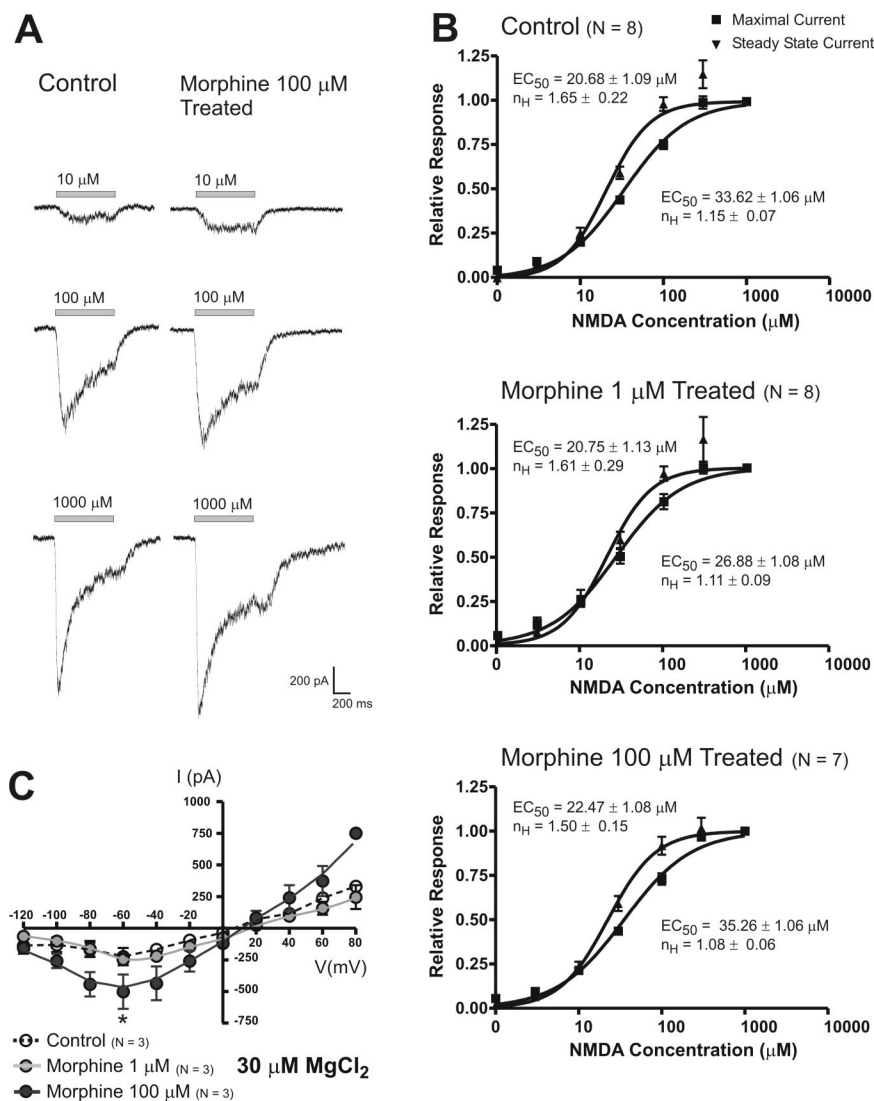


Fig. 5. *N*-methyl-D-aspartate (NMDA)-evoked NMDA receptor responses in control and 1 or 100 μM morphine-treated small-capacitance, tonic firing dorsal horn neurons. **(A)** Representative current traces from a control neuron and morphine-treated neuron in response to 10, 100, and 1,000 μM concentrations of NMDA. **(B)** Concentration-response relation normalized to maximal peak (squares) and steady state (triangles) currents evoked by 1,000 μM NMDA in control and morphine-treated neurons. The calculated EC₅₀ and Hill coefficient (η_{H}) for NMDA-evoked peak responses were similar in control and morphine-treated neurons. There was also no difference in EC₅₀ and η_{H} of the NMDA-evoked steady state responses between control and treated neurons. **(C)** Current-voltage relation of currents evoked by 1,000 μM NMDA recorded in extracellular solution containing 30 μM MgCl₂ in control (open circles), 1 μM morphine-treated (gray circles), and 100 μM morphine-treated (black circles) neurons. These show a greater amplitude response in 100 μM morphine-treated neurons at membrane holding potentials less than 0 mV, suggesting a reduction in Mg²⁺ blockade. (One-way analysis of variance with Bonferroni multiple comparisons posttest for amplitudes of NMDA current at holding potentials less than 0 mV, * P < 0.05; no significant differences for amplitudes of NMDA current at holding potentials of 0 mV or greater).

of NMDA receptors in spinal trigeminal neurons.^{8,9} With 30 μM extracellular MgCl₂, the NMDA current-voltage relation shows a reduced inward rectification at resting membrane potentials after high-concentration but not low-concentration morphine exposure compared with control (fig. 5C). This suggests that a reduction in the Mg²⁺ inhibition of NMDA receptors may be a mechanism by which high-dose morphine administration may further enhance NMDA responses. Because of the lack of extracellular Mg²⁺ in recordings of NMDA current previously described (table 1 and figs. 2 and 3), the chronic opioid-induced NMDA receptor up-regulation may be due to other mechanisms yet undetermined.

NMDA Responses Enhanced by Chronic Morphine Exposure Are Attenuated by NMDA and Opioid Receptor Antagonists

To determine whether this up-regulated NMDA response in the small, tonic firing DH neurons is attenu-

ated by NMDA and opioid receptor antagonism, we added 10 μM 2-amino-5-phosphonovalerate (APV) or 1 μM naloxone in the control and morphine-treated cultures for 7 days. Naloxone is an opioid antagonist that, applied at 1 μM , is more selective for the μ subtype of opioid receptor.¹ The APV or naloxone and morphine were completely washed out before current and NMDA-evoked responses were recorded.

With APV, there was a greater reduction in NMDA-evoked responses in the neurons treated with a low concentration (1 μM) of morphine (42.1 ± 13.4 pA/pF, $n = 6$, vs. 38.5 ± 8.7 pA/pF, $n = 7$) than in those treated with a high concentration (100 μM) of morphine (76.8 ± 44.8 pA/pF, $n = 6$, vs. 38.5 ± 8.7 pA/pF, $n = 7$) when compared with control neurons treated with the NMDA antagonist (fig. 6). However, naloxone attenuated the increase in NMDA-evoked responses at both lower and higher morphine concentrations (48.6 ± 28.0 pA/pF, $n = 6$, and 63.2 ± 17.8 pA/pF, $n = 5$, vs. 58.2 ± 35.0 pA/pF, $n = 7$) (fig. 6).

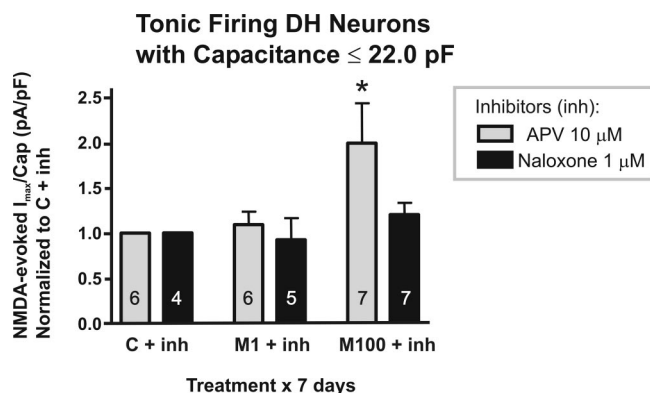


Fig. 6. *N*-methyl-D-aspartate (NMDA), 1,000 μM -evoked current amplitude in 1 μM morphine (M1) and 100 μM morphine (M100)-treated small, tonic-firing dorsal horn (DH) neurons after cotreatment with 10 μM 2-amino-5-phosphonovaleate (APV; gray bars) or 1 μM naloxone (black bars) for 7 days. Current amplitudes have been normalized to NMDA current recorded from control (C) neurons similarly exposed to the inhibitors (inh) APV or naloxone, respectively. Numbers within the bars indicate the number of DH neurons from which the data were averaged. (One-way analysis of variance with Bonferroni multiple comparisons posttest, * $P < 0.05$). $I_{\text{max}}/\text{Cap}$ = maximal current normalized to capacitance.

Discussion

We found that small, tonic firing DH neurons show an up-regulation of NMDA receptor responses after chronic morphine exposure, which is not seen in larger, phasic firing neurons. This enhancement of NMDA receptors was due to a reduction in the physiologic Mg^{2+} blockade of these receptors only after high-concentration morphine exposure and was inhibited by pretreatment with the opioid receptor antagonist naloxone and the NMDA antagonist APV.

In the spinal cord, MORs are found in abundance in superficial layers of the DH, substantia gelatinosa (lamina II),¹⁹ which receive input from nociceptive C fibers projecting from the dorsal root ganglia. MORs are located predominantly at somatodendritic sites in these DH neurons and likely function postsynaptically. Recently, two separate groups^{21,27,29} reported that only a subset of neurons in the substantia gelatinosa of rat spinal cord slice showed a hyperpolarizing current response to the selective μ -opioid agonist DAMGO. Santos *et al.*²¹ demonstrated that the DAMGO-induced hyperpolarization only occurred in tonic firing neurons and not in neurons showing other firing patterns. These opioid responsive neurons are most likely excitatory interneurons.¹⁹ Consistent with these previous reports, we showed that in DH neuronal cultures, only small tonic firing neurons, presumably excitatory interneurons, showed an enhancement of NMDA receptor current after prolonged morphine exposure. Santos *et al.*²¹ also showed that tonic firing was sensitive to inhibition through membrane hyperpolarization by opioid exposure, whereas other firing patterns were maintained. Similarly, in our DH neuronal cultures, tonic firing neu-

rons were more susceptible to inhibition of action potentials by acute exposure (or reexposure) to 100 μM morphine. Firing pattern-associated NMDA receptor plasticity and hyperpolarization secondary to MOR activation in our experiments suggest the preservation of DH neuron receptor and ion channel expression and function in culture. Therefore, this preparation may be useful for further study of the mechanisms involved in opioid tolerance development.

Although neuronal size has not been associated with the expression of MOR and NMDA receptors, we report that DH neurons with capacitances of 22.1 pF or greater did not show enhanced NMDA current despite demonstrating tonic firing patterns. Our preliminary results (table 1) suggested that the morphine-treated neurons had larger capacitance measurements on average; however, subsequent comparisons through construction of the capacitance histograms for control and treated neurons did not reveal distribution differences contrary to the three identified gaussian curves used to identify our neurons according to size. Recently, morphine administration has been shown to cause the retraction and reduction of dendritic spines and branches in cortical, hippocampal, and nucleus accumbens neurons.^{30,31} However, no observed changes in soma size were noted under light microscopy in our preparation. The retraction of preexisting dendritic spines were shown to reduce synaptic AMPA receptor-mediated responses,³⁰ however, NMDA receptor responses were not studied.

Mu-opioid receptors and NMDA receptors have been shown to colocalize in individual neurons in many areas of the central nervous system.³²⁻³⁴ Therefore, the output of neurons that express both MOR and NMDA receptors depend on dual modulation by these receptors. Their proximity may also contribute to interactions between these receptors through intracellular second messengers that may important in the development of opioid tolerance. Current opinion (reviewed in Mao and Mayer²⁶) is that MOR activation initiates several intracellular cascades that result in PKC γ -subtype translocation. PKC activation is mediated also by an increase in intracellular Ca^{2+} as occurs with NMDA receptor activation.³⁵ The enhancement NMDA receptor function by PKC involves the reduction of the physiologic Mg^{2+} block of its associated channel pore^{8,9} possibly through the activation of PKC-upregulated nonreceptor tyrosine kinases such as the proto-oncogene Src and cell adhesion kinase β .³⁶ This was noted to occur in small, tonic firing DH neurons exposed to a high concentration of morphine, but not to a low concentration of morphine despite showing similarly up-regulated NMDA receptor activity. Therefore, another mechanism must exist for the enhanced NMDA receptor function after low concentration opioid exposure that has yet to be identified and does not involve the PKC-dependent reduction in the NMDA physiologic Mg^{2+} block.

Reciprocally, NMDA receptor activation has been shown to attenuate μ -, κ -, and δ -opioid receptor function by facilitating Ca^{2+} entry and PKC phosphorylation of the $\text{G}_{i\alpha 2}$ protein, resulting in opioid receptor-G protein uncoupling.³⁷ Uncoupling of the MOR-G protein, rather than receptor internalization, has been implicated as the mechanism for tolerance to morphine.³⁸ Possible inhibition of NMDA receptors, thereby reducing MOR-G protein uncoupling, may enhance morphine analgesia and prevent the development of tolerance. Therefore, the interactions between MOR and NMDA receptors are complex, and activation of the NMDA receptor complex is required for the development of plasticity under the cellular conditions maintained by chronic MOR activation.

Multiple animal studies have shown opioid tolerance and dependence to be dependent on NMDA receptor activity and can be attenuated by NMDA antagonists of all classes,⁷ including competitive (D-AP5,³⁹ LY274614^{40,41}), noncompetitive (d-methadone,⁴² MK-801,⁴³ ketamine, dextromethorphan, phencyclidine⁴⁴), glycine site antagonist (ACEA-1328^{45,46}), channel pore inhibitor (magnesium⁴⁷), and NR1 subunit antisense.⁴⁸ These studies show that the concurrent administration of NMDA antagonist is required for the attenuation of opioid tolerance.

Consistent with these data, our experimental model did show a failure of chronic morphine treatment to enhance NMDA-evoked current in small, tonic firing DH neurons treated for the duration of exposure with APV. However, this was only observed in the low-morphine (clinically relevant concentrations) and not in the high-morphine-treated neurons, suggesting a mechanistic difference in the NMDA receptor up-regulation depending on the concentration of the opioid. Interestingly, the baseline NMDA maximal current measured after APV treatment in control neurons was greater than in neurons not exposed to APV. Although APV applied for 2 days has not been noted to change NMDA-mediated excitatory postsynaptic current area after washout, there are increases in these responses after 6-cyano-7-nitroquinoline-2,3-dione (AMPA/kainate inhibitor) and tetrodotoxin (Na^+ channel inhibitor). These data suggest an activity dependence of NMDA response scaling.⁴⁹ With longer APV application, it is possible that NMDA responses may be further increased with withdrawal of the receptor inhibitor. We also recorded higher baseline NMDA current with application of naloxone alone, which may be a result of a different, as yet undefined, mechanism. Intrathecally administered naloxone alone can result in hyperalgesia in animals.^{50,51} Despite chronic cellular changes with prolonged administrations of APV and naloxone, which are mechanistically not well understood but have been previously observed, we still demonstrated an absence of NMDA-evoked response enhancement by inhibition of the NMDA receptor or MOR, respectively. As well, APV 10 μM was not able to inhibit the enhanced NMDA responses recorded after high mor-

phine application, suggesting that the mechanisms responsible for these changes remain intact despite the higher baseline measures.

Therefore, this study has identified, through their electrophysiologic properties, a subgroup of DH neurons that show NMDA receptor plasticity after prolonged morphine treatment. Consistent with animal studies, this plasticity is attenuated by pretreatment with NMDA receptor antagonist. Importantly, this is a functional cellular model of chronic opioid exposure-related changes that can be studied over time, developmental stages, and with the interaction of other drugs. Therefore, our *in vitro* model may be used in future studies to further elucidate the mechanisms responsible for possible changes underlying the development of opioid tolerance.

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