Enhancement of Spinal N-Methyl-D-aspartate Receptor Function by Remifentanil Action at δ-Opioid Receptors as a Mechanism for Acute Opioid-induced Hyperalgesia or Tolerance

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Background: Intraoperative remifentanil infusions have been associated with postoperative opioid-induced hyperalgesia and tolerance. Using a previously identified subpopulation of spinal neurons that displays an augmentation in N-methyl-D-aspartate (NMDA) receptor current after chronic morphine, investigations were undertaken to determine whether remifentanil induces acute increases in NMDA responses that are concentration dependent and receptor subtype dependent.

Methods: Electrophysiologic recordings of NMDA current were made from cultured rat dorsal horn neurons treated with remifentanil at various concentrations for 60 min. Selective μ- or δ-opioid receptor inhibitors and agonists were used to determine the site of action of remifentanil.

Results: Remifentanil at 4, 6, and 8 nM, but not higher or lower concentrations, caused significant mean increases in NMDA peak current amplitude of 37.30% (P < 0.001), 30.19% (P < 0.001), and 23.52% (P = 0.025), respectively, over control conditions. This occurred by 36 min of remifentanil perfusion and persisted throughout its washout. Inhibition by 100 nM naloxone or 1 nM naltrindole attenuated the remifentanil-induced NMDA response increase. Selective δ-opioid agonists [D-Pen²,D-Pen⁵]enkephalin and deltorphin II displayed a similar bell-shaped concentration–response relation for the enhancement of NMDA responses, and 10 nM deltorphin II occluded the effects of 4 nM remifentanil on NMDA responses.

Conclusions: Clinically relevant concentrations of remifentanil induce rapid, persistent increases in NMDA responses that mirror the development of remifentanil-induced hyperalgesia and tolerance. NMDA enhancement by remifentanil is dependent on the activation of both μ- and δ-opioid receptors and is inducible solely by δ-opioid receptor activation. Therefore, selective δ-opioid inhibition may attenuate acute paradoxical increases in pain and tolerance to opioids.

OPIOID administration has been associated with the development of paradoxical, pathologic pain that presents as opioid-induced hyperalgesia (OIH) or tolerance. Although the prevalence of these states among patients treated with opioids remains unknown, hyperalgesia and tolerance seem to occur more frequently and predictably with the administration of the potent, short-acting opioid remifentanil.1–7 Remifentanil is primarily a μ-opioid receptor agonist that causes antinociception or analgesia by activating receptor-associated G_{i/o} proteins.8,9 But similar to other opioids, remifentanil may also have activity at other subtypes of opioid receptors.10 Perioperative trials have reported the existence of remifentanil-induced tolerance as measured by an increase in postoperative morphine requirements for adequate analgesia.2–4 As well, studies in human volunteers1,5–7 and surgical patients4 that used standardized response measures demonstrate the presence of hyperalgesia or allodynia and tolerance after a 60- to 90-min remifentanil intravenous infusion at rates between 0.05–0.3 μg · kg⁻¹ · min⁻¹. Acute OIH associated with remifentanil occurs rapidly and, consequently, offers a unique opportunity for a real-time study of the cellular changes underlying its development.

A pharmacologic study of remifentanil distribution in dogs during intravenous administration has revealed a high penetration of the drug into cerebrospinal fluid, equal to 74% of venous levels.11 Therefore, pharmacodynamic effects of remifentanil in the spinal cord are relevant for the study of mechanisms underlying the development of opioid-induced increases in pain response.

Pain signaling propagates through the spinal cord by glutamatergic neurotransmission involving the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors. These receptors are located postsynaptically and extrasynaptically in the membrane of dorsal horn (DH) neurons and are activated by the presynaptic release of glutamate from nerve terminals of dorsal root ganglia cells depolarized by pain-provoking mechanical or chemical stimuli. Upon activation, NMDA receptors fa-
cilitate the influx of cations, such as Na⁺ and Ca²⁺, causing the DH neurons to depolarize and fire action potentials, resulting in a further release of glutamate and propagation of the pain signal from neuron to neuron through the spinal cord to the brain.

At many points in this pathway, the pain signal can be pathologically augmented. In particular, the enhancement of NMDA receptor function in DH neurons may underlie increased pain states in chronic pain and OIH. Many behavioral studies have reinforced a role for NMDA receptor antagonism in the prevention of OIH and tolerance. As well, in vitro study of spinal cord neurons showed NMDA current enhancement by 1 µM [D-Ala², N-Mcphe⁴, Gly⁵-ol]enkephalin (DAMGO) is mediated by increases in intracellular protein kinase C (PKC). This series of studies concluded that µ-opioid receptor activation resulted in NMDA receptor enhancement despite more recent recognition that 1 µM DAMGO also activates other opioid receptor subtypes, such as the δ-opioid receptor. It is unknown whether selective δ-opioid receptor activation induces an enhancement of NMDA receptor function.

In the spinal cord, µ- and δ-opioid receptors like NMDA receptors are also primarily located postsynaptically in some DH neurons, specifically excitatory interneurons. These subtypes of opioid receptors colocalize with NMDA receptors. This laboratory previously identified a subpopulation of DH neurons in which the coincident activation of opioid and NMDA receptors enhance the function of NMDA receptors.

Therefore, using electrophysiologic studies of NMDA receptor–mediated responses from cultured rat DH neurons that were untreated and treated with remifentanil, it was questioned (1) whether NMDA receptor function in spinal cord DH neurons is modulated acutely by remifentanil, (2) whether this effect is concentration and time dependent, and (3) whether the activation of δ-opioid receptors contributes to the remifentanil-induced enhancement of NMDA responses.

Materials and Methods

Experimental protocols were approved by the Animal Care Committee at the Lab Animal Services Department of the Hospital for Sick Children, Toronto, Ontario, Canada. All chemicals used in these experiments were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada) unless otherwise stated.

Cell Culture

Primary DH neuron cultures were prepared as previously described. In short, Timed-pregnant female Wistar rats (Charles River, Wilmington, MA) were killed by cervical dislocation after inhalational general anesthesia with halothane, and their fetuses (E18–19 days) were re-moved. The fetuses were decapitated, and their spinal cords were surgically extracted by an anterior approach and placed in recently thawed Hanks solution. After carefully stripping the meninges, each spinal cord was laid open by a longitudinal incision along the dorsal commissure down through the central canal. This allowed the dissection of the dorsal half of the cord by cutting along the lateral funiculus bilaterally. The dorsal halves of the spinal cords were pooled, minced, and incubated in Hanks solution containing 0.25% trypsin (Invitrogen Canada Inc., Burlington, Ontario, Canada) at 37°C for 45 min. Subsequently, the tissue was washed, mechanically triturated, and plated in Minimum Essential Medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 10% horse serum (Invitrogen), and 1 U/ml insulin (Novo Nordisk Canada Inc., Mississauga, Ontario, Canada). The tissue from seven spinal cords was used for primary culture of 10 collagen-coated 35-mm Nalgene dishes (Fisher Scientific Co., Ottawa, Ontario, Canada). After 6 days of incubation, the dishes were treated for 24 h with 13 µg/ml 5’-fluoro-2-deoxyuridine and 33 µg/ml uridine. Beyond 6 days of incubation, the culture media contained Minimum Essential Media with 10% horse serum only.

Because of developmental changes in the subunit composition of NMDA receptors that affect NMDA-evoked current amplitudes and desensitization, all electrophysiologic recordings were undertaken in the DH neurons after 14–21 days of incubation when mature complements of NMDA receptors are expressed.

Electrophysiologic Recording

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 MΩ. 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.).

Tonic-firing, small-sized DH neurons with capacitance less than 22 pF were previously shown to have an increased likelihood for the coexpression of NMDA and opioid receptors by demonstrating enhanced NMDA-evoked current amplitude after chronic morphine treatment. Therefore, only DH neurons with these electrophysiologic properties were used in this study.

Determination of Firing Pattern

Recording electrodes were filled with intracellular solution consisting of 140 mM KCl, 10 mM HEPES, 2 mM MgCl₂, 10 mM EGTA, and 4 mM MgATP. This solution was...
buffered to a pH of 7.4 using KOH, and the osmolality was adjusted to 290–300 mOsm. Culture media from dishes of DH neurons were gently replaced with an extracellular recording solution containing 140 mM NaCl, 1.3 mM CaCl2, 5.4 mM KCl, 25 mM HEPES, and 33 mM glucose, buffered to a pH of 7.4 with NaOH and adjusted to an osmolality of 320–325 mOsm.

Cultured DH neurons were initially patched and voltage clamped at a holding potential of −60 mV. Stability was determined by a small and unchanging leak current between 0 and −100 pA. The head stage was then changed to the current clamp configuration, and the firing pattern, in response to a previously described current injection protocol, was determined for each neuron. Briefly, the resting membrane potential was recorded, and firing patterns were determined in response to hyperpolarizing and depolarizing current injections (50 pA steps from −200 to +350 pA) at 500-ms intervals. Only tonic-firing, rather than phasic-firing, neurons that maintained a small leak current and looked healthy under light microscopy were further studied under voltage clamp.

**Determination of Neuronal Size**

To determine the size of neurons, membrane capacitance was estimated from the area under the capacitance transient evoked by a hyperpolarizing 10-mV step. Only cultured DH neurons with tonic firing patterns and membrane capacitances less than 22 pF were retained for further study.

The hyperpolarizing step was made approximately 150 ms before each recording of an NMDA-evoked current. All current amplitudes were normalized to cell membrane capacitance to ensure the uniformity of comparisons, which was based on current density, irrespective of cell size.

**NMDA Current Recordings with or without Remifentanil**

At a holding potential of −60 mV, the selected DH neurons were perfused continuously with the extracellular solution containing 3 μM glycine and 0.3 μM tetrodotoxin. This solution was delivered via a three-barrel capillary tube system with each barrel attached to a 7-ml reservoir, the height of which was adjusted to deliver solutions at a rate of 1 ml/min. Rapid exchange of solutions (τ = approximately 3 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 a 1-s application of NMDA at a saturating concentration of 1 mM. NMDA-evoked currents were recorded at 15-min intervals with 2 min between applications before, during, and after remifentanil (Ultiva®; Abbott Laboratories Ltd., Montreal, Quebec, Canada) coperfusion at 0, 1, 2, 4, 6, 8, and 10 nM concentrations using the protocol shown in figure 1. Therefore, each recording interval was approximately 20 min in duration, and there were a total of five to six recording intervals per experiment after baseline NMDA current acquisition.

The vehicle of the pharmaceutical preparation of remifentanil (Ultiva®) contains 3 mM glycine per 50 μM remifentanil. At this concentration but not at 10 μM, glycine directly gates NMDA receptors in rat spinal cord slice recordings, where neurons are studied in the presence of ambient glutamate. The remifentanil concentrations (1, 2, 4, 6, 8, and 10 nM) applied in these experiments are similar to the plasma levels in human subjects given an infusion of remifentanil intravenously for analgesia. At this concentration, the vehicle contains only 0.06, 0.12, 0.24, 0.36, 0.48, and 0.60 μM glycine, respectively. These concentrations of glycine are unlikely to potentiate NMDA receptor function through direct activation, coactivation, or receptor trafficking. Extracellular solutions used routinely in our laboratory and most others for NMDA receptor current recordings contain 3 μM glycine and do not enhance peak current amplitudes during the recording session (1.5–2 h).

Therefore, the remifentanil, other agonist/antagonist, and control perfusion solutions were adjusted to contain uniformly 3 μM glycine for all experiments performed.

**N-Methyl-D-aspartate-evoked maximal current amplitudes were measured using pCLAMP (Axon Instruments), and the data were plotted using GraphPad Prism (Graph Pad, San Diego, CA). Only DH neurons surviving beyond 67 min of electrophysiologic recording were retained for analysis.**

The μ- and δ-opioid receptor inhibitors 100 nM naloxone and 1 nM naltrindole, respectively, were perfuned in the presence or absence of 4 nM remifentanil to determine the site of action of remifentanil for inducing NMDA enhancement. Agonist experiments using selective δ-opioid receptors agonists [D-Pen2, D-Pen5]-enkephalin (DPDPE) at 4, 12, and 30 nM and deltorphin II at 4, 10, and 30 nM were performed under the same recording conditions as remifentanil. At these concentrations of DPDPE and deltorphin II, the agonist is selective for δ-opioid receptor activation and is inactive at the μ-opioid receptor.
Statistical Analyses

All results were reported as mean ± SEM. Data were analyzed using SigmaStat 3.11 (SYSTAT Software Inc., San Jose, CA). The mean NMDA peak and steady state amplitudes, measured in control and various concentrations of remifentanil-treated neurons, were compared using a two-way analysis of variance, unless otherwise stated. A Tukey posttest was used to compare all treatment groups with the naive control group. Significance was reported at $P < 0.05$.

Results

Acute and Prolonged NMDA Receptor Enhancement in Relatively Low-dose Remifentanil-treated DH Neurons

Many recent studies indicate that opioids increase NMDA receptor activity in DH neurons of the spinal cord.12–15 However, no studies have demonstrated that the rapidity by which an opioid, such as remifentanil, increases NMDA receptor function directly correlates with its ability to induce acute hyperalgesia and tolerance in a clinical setting. Therefore, NMDA receptor responses in DH neurons before, during, and after a 60-min perfusion of remifentanil were investigated. In particular, the time course for the development of NMDA receptor response enhancement and the concentrations of remifentanil that caused this increase were noted.

Remifentanil Concentration Dependence of Remifentanil-induced Enhanced NMDA Responses

N-Methyl-D-aspartate–evoked recordings in the subset of small, tonic-firing DH neurons showed a significant increase in peak current amplitude of NMDA responses after 60 min of 4 nM remifentanil perfusion (fig. 2). These enhanced NMDA responses persisted during remifentanil washout. NMDA-evoked recordings from remifentanil-treated neurons were compared with responses from a similar subset of DH neurons grown in sister cultures not exposed to remifentanil (naive controls), which revealed a progressive decline in the amplitude (fig. 2B) or “run-down” of the peak current known to occur under these recording conditions.31 In contrast to a previous study using a recombinant expression system,32 there was no evidence of direct NMDA receptor gating by remifentanil in this study.

The NMDA peak current densities were normalized to the baseline values recorded during the first 10 min of recording. Baseline NMDA peak current densities were not significantly different between the naive control and 1, 2, 4, 6, 8, and 10 nM remifentanil-treated neurons (53.16 ± 7.84, 55.18 ± 9.22, 60.95 ± 3.20, 52.35 ± 5.24, 43.43 ± 4.17, 49.98 ± 3.12, and 54.58 ± 8.17 pA/pF, respectively; one-way analysis of variance, $P = 0.55$).

Remifentanil at 4, 6, and 8 nM caused increases in NMDA currents of 37.30% (n = 19), 30.19% (n = 14) ($P < 0.001$, two-way analysis of variance with Tukey posttest), and 23.52% (n = 12) ($P = 0.025$, two-way analysis of variance with Tukey posttest), respectively.

Time Dependence of Remifentanil-induced Enhanced NMDA Responses

Because repetitive activations of NMDA receptors may hasten cellular demise,33 exposure to NMDA was limited by reducing the number of applications to three per 20-min interval of remifentanil or control extracellular solution perfusion. This enabled us to record from the DH neurons for at least 120 min. The increases in NMDA peak current amplitude to 4 nM remifentanil are significant as early as 36 min after the start of remifentanil perfusion (fig. 2B). In two cells exposed to 4 nM remifentanil at 60 min of perfusion (fig. 2B). In contrast, no significant effect was observed at any of the other concentrations of remifentanil (figs. 3A and B).

Fig. 2. N-Methyl-D-aspartate (NMDA)–evoked current responses in control and 4 nM remifentanil–treated dorsal horn neurons. (A) Representative current traces in response to 1 mM NMDA recorded from a selected dorsal horn neuron before, during, and after treatment with 4 nM remifentanil (Remi). (B) Peak NMDA-evoked current amplitude per unit cell capacitance normalized to baseline responses recorded immediately before the start of the remifentanil infusion at concentrations of 4 nM (Remi 4 nM; black closed circles), 6 nM (Remi 6 nM; gray closed circles), and 8 nM (Remi 8 nM; black open circles) compared with naive controls (gray open circles). Significant increases in NMDA peak current amplitude occurred with remifentanil exposure at these concentrations. Mean differences from naive controls (n = 9) were 37.30% (n = 19) ($P < 0.001$, two-way analysis of variance with Tukey posttest), 30.19% (n = 14) ($P < 0.001$, two-way analysis of variance with Tukey posttest), and 23.52% (n = 12) ($P = 0.025$, two-way analysis of variance with Tukey posttest), respectively.
Remifentanil concentration and time dependence of the enhanced NMDA responses in vitro that correlate with and may underlie remifentanil-induced hyperalgesia.

**NMDA Receptor Enhancement in Remifentanil-Treated DH Neurons Inhibited by μ- and δ-Opioid Receptor Inhibitors**

Activation of μ- and δ-opioid receptors, but not κ-opioid receptors, has been implicated in the development of opioid-induced hyperalgesia, possibly due to their effect on certain intracellular G proteins. The ability of the μ-opioid antagonist naloxone at 100 nM or the δ-opioid antagonist naltrindole at 1 nM to attenuate the remifentanil-induced enhancement of NMDA responses was tested by coperoxusion of these antagonists with remifentanil. Naloxone at 100 nM significantly attenuated the increase in NMDA peak current during perfusion and washout of 4 nM remifentanil (fig. 4). Control recordings demonstrated that, in the absence of remifentanil, naloxone does not significantly influence NMDA currents (fig. 4). Similarly, selective μ-opioid receptor inhibition by 1 nM naloxonazine abolished the NMDA response enhancement to 4 nM remifentanil (data not shown; fig. 4 on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org). Selective δ-opioid inhibition by 1 nM naltrindole also completely attenuated the increases in NMDA peak current due to 4 nM remifentanil, and even at a higher concentration, 1 μM naltrindole did not alter NMDA current in the absence of remifentanil (fig. 5). Therefore, μ- and δ-opioid receptor inhibition independently attenuated the remifentanil-induced increases in NMDA responses, which suggest that these opioid re-
ceptor subtypes are activating a common or converging intracellular pathway leading to enhanced NMDA receptor function. Consequently, eliminating the activity of just one of these opioid receptor subtypes is sufficient to completely abolish downstream signaling to NMDA receptors.

**NMDA Receptor Enhancement Induced by the Selective δ-Opioid Agonist, DPDPDE, Displays Similar Concentration Responses as Remifentanil**

To test whether δ-opioid receptor activation alone is sufficient to induce an enhancement of NMDA current, recordings were undertaken using selective δ-opioid agonists DPDPDE at 4, 12, and 30 nM and deltorphin II at 4, 10, and 30 nM in place of remifentanil. Binding studies in perfused dorsal horn neurons revealed that 4 nM DPDPDE did not enhance NMDA-evoked currents compared with naive controls (\(P < 0.001\), two-way analysis of variance with Tukey posttest). DPDPDE showed a similar concentration–response relation as that observed with remifentanil (figs. 2 and 3). The mean increase in NMDA-evoked current after 12 nM DPDPDE treatment was significantly greater than with 4 nM remifentanil (Remi 4 nM; black closed circles) and 30 nM remifentanil (Remi 4 nM; black open circles) (\(‡P < 0.001\), two-way analysis of variance with Tukey posttest). DPDPDE = [D-Pen², D-Pen⁵]enkephalin.

**NMDA Receptor Enhancement by the Selective δ-Opioid Agonist Deltorphin II Inhibits Remifentanil Effects**

It was postulated that the enhancement of NMDA receptor current after 4 nM remifentanil exposure is due to the specific interaction between NMDA receptors and remifentanil. A primary effect of remifentanil is the inhibition of the 𝛼₂-adrenoceptor on presynaptic terminals, which reduces neurotransmitter release and downstream signaling to NMDA receptors. Consequently, eliminating the activity of just one of these opioid receptor subtypes is sufficient to completely ablate downstream signaling to NMDA receptors.

**Figures**

Fig. 5. N-Methyl-D-aspartate (NMDA)–evoked peak current amplitude per unit cell capacitance normalized to baseline responses during and after 4 nM remifentanil without (Remi 4 nM; black closed circles) or with (Remi 4 nM + Naltr 1 nM; black closed inverted triangles) copresentation of 1 nM naltrindole. Data from recordings undertaken in control dorsal horn neurons perfused with 1 μM naltrindole alone are also shown (gray open inverted circles). There are no significant differences in the peak amplitude of the NMDA-evoked current responses with 1 μM naltrindole added to control and 1 nM naltrindole to remifentanil-treated neurons when compared with naive controls (gray open circles). Only 4 nM remifentanil-treated neurons demonstrated an increase in NMDA-evoked peak current compared with naive controls (\(P < 0.001\), two-way analysis of variance with Tukey posttest).

Fig. 6. N-Methyl-D-aspartate (NMDA)–evoked peak current amplitude per unit cell capacitance normalized to baseline responses during and after 4, 12, and 30 nM DPDPDE treatments in dorsal horn neurons. There was a significant increase in NMDA-evoked current after 12 nM DPDPDE treatment (gray closed triangles) (mean increase of 73.59%; \(†P < 0.001\), two-way analysis of variance with Tukey posttest) from naive controls that was not observed for 4 nM DPDPDE (black closed triangles) and 30 nM DPDPDE (black open triangles). Hence, DPDPDE showed a similar concentration–response relation as that observed with remifentanil (figs. 2 and 3). The mean increase in NMDA-evoked current after 12 nM DPDPDE treatment was significantly greater than with 4 nM remifentanil (Remi 4 nM; black closed circles) (\(‡P < 0.001\), two-way analysis of variance with Tukey posttest). DPDPDE = [D-Pen², D-Pen⁵]enkephalin.

Fig. 7. N-Methyl-D-aspartate (NMDA)–evoked peak current amplitude per unit cell capacitance normalized to baseline responses during and after 4 nM deltorphin II (black closed squares), 10 nM deltorphin II (gray closed squares), and 30 nM deltorphin II (black open squares) treatments in dorsal horn neurons. There were significant increases in NMDA-evoked current after 4 nM deltorphin II (mean increase of 26.12%, \(n = 4\)) and 10 nM deltorphin II treatments (mean increase of 38.64, \(n = 7\), but not after 30 nM deltorphin II treatment, compared with naive controls (gray open circles) (\(†P < 0.05\) and \(‡P < 0.05\), respectively, two-way analysis of variance with Tukey posttest). The increases of NMDA current with 10 nM deltorphin II were of similar magnitude as with 4 nM remifentanil (Remi 4 nM; black closed circles) compared with naive controls.

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to the activation of δ-opioid receptors via a similar pathway as deltorphin II (10 nM). The selected DH neurons were exposed to 10 nM deltorphin II for 40 min to induce NMDA-evoked current enhancement (mean increase of 38.64%; fig. 7) that was similar to or greater than with 4 nM remifentanil alone (mean increase of 37.30%; fig. 2B). This 10 nM deltorphin II exposure was then followed by the addition of 4 nM remifentanil to the 10 nM deltorphin II perfusion solution for a further 40 min in order to assess the ability of remifentanil to further enhance the NMDA responses. Because of the greater enhancement of NMDA current with 12 nM DPDPE (mean increase of 73.59%; fig. 6), 10 nM deltorphin II was unlikely to have achieved a ceiling effect on NMDA current enhancement. Therefore, if 10 nM deltorphin II and 4 nM remifentanil caused an augmentation of NMDA responses by different mechanisms, there would be an additive effect, whereas if their mechanisms of enhancement were convergent through a common pathway, one agonist effect would occlude the other. There were no further NMDA response increases with the addition of remifentanil (P = 0.103, paired t test; figs. 8A and B), suggesting that remifentanil and deltorphin II affected NMDA current through a common pathway.

**Discussion**

In the current study, it was demonstrated that exposure to 4, 6, and 8 nM remifentanil (fig. 2B), but not 1, 2, and 10 nM (figs. 3A and B), for 36 min caused a significant increase in NMDA-evoked peak current lasting throughout the 60-min drug application and 40-min washout periods. This enhancement of NMDA responses was attenuated by the application of either a μ- or a δ-opioid antagonist (figs. 4 and 5, respectively), suggesting that the concurrent activation of μ- and δ-opioid receptors by remifentanil is required to increase NMDA-evoked current. However, exposure to selective δ-opioid agonists DPDPE and deltorphin II alone were able to induce increases in NMDA receptor responses (figs. 6 and 7, respectively) with similar rank order potency as their binding affinity to the δ-opioid receptor. As well, DPDPE and deltorphin II caused similar bell-shaped concentration-dependent changes in NMDA responses as remifentanil (fig. 6). Deltorphin II at 10 nM occluded further NMDA current increases induced by 4 nM remifentanil (fig. 8). These results suggest that remifentanil causes the enhancement of NMDA responses through activation of δ-opioid receptors and their associated intracellular pathways.

Two previous studies of remifentanil effects on NMDA receptor function showed direct gating and enhancement of NMDA receptor current. One group of investigators studied the effects of a very high concentration of remifentanil (50 μM) in recombinant human NR1-

**Fig. 8.** (A) N-Methyl-D-aspartate (NMDA)–evoked peak current amplitude per unit cell capacitance normalized to baseline responses during and after 10 nM deltorphin II for 30 min followed by 10 nM deltorphin II with 4 nM remifentanil for the following 30 min (Delt 10 then Delt 10 + Remi; gray closed circles). Remifentanil at 4 nM alone (Remi 4 nM; black closed circles), 10 nM deltorphin II alone (black closed squares), and 10 nM deltorphin II followed by the addition of 4 nM remifentanil (gray closed squares) enhanced NMDA current compared with naive controls (gray open circles) (\(P < 0.05\), \(\dagger P < 0.05\), and \(\ddagger P < 0.05\), respectively, two-way analysis of variance with Tukey posttest). (B) Bar graph representation of NMDA-evoked peak current normalized to cell capacitance and baseline after 30 min of 10 nM deltorphin II perfusion (Delt 10 nM; black bar) and after the subsequent 30 min of 10 nM deltorphin II with 4 nM remifentanil (Delt 10 nM + Remi 4 nM; gray bar). There was no further enhancement of the NMDA responses with the addition of 4 nM remifentanil after 10 nM deltorphin II pretreatment (not significant [NS], \(P = 0.103\), paired t test).

NR2A-containing NMDA receptors. It showed that remifentanil directly gated NMDA receptors; however, these results were probably due to the different functional properties of recombinant NMDA receptors concurrent with the incidental administration of a high concentration of 3 mM glycine present in the remifentanil formulation. A second group of investigators showed that 10 μM remifentanil together with 10 μM glycine did not directly activate but potentiated NMDA 10 μM-evoked current recorded in spinal cord slice. This concentration of remifentanil is also relatively high and not likely clinically relevant. In contrast, the current study demonstrated that even at concentrations as low as 4 nM remifentanil, there was a significant increase in 1 mM NMDA-evoked current. A saturating concentration of NMDA was used to simulate a barrage of surgical nociceptive stimuli resulting in a high quantal release of NMDA-evoked current.
RAPID NMDA PLASTICITY AFTER REMIFENTANIL EXPOSURE

Glutamate onto postsynaptic DH neurons. This may account for the differences in remifentanil concentration–response between this and the other two previous studies, in that a common intracellular pathway activated by NMDA and opioid receptor activity may have become saturated or an inhibitory pathway initiated by relatively high concentrations of one of the agonists. Therefore, the concentration–response relationship for remifentanil-induced enhancement of NMDA receptor function may depend on the activity of NMDA receptors. The possible cooperativity between opioid and NMDA receptors requires further investigation.

In this study, the remifentanil-induced enhancement of NMDA-evoked peak current amplitude was shown to be concentration dependent and occurred at clinically relevant concentrations. Plasma concentrations achieved by remifentanil intravenous infusion at 0.05–0.3 μg · kg⁻¹ · min⁻¹ are between 1.5 and 8 ng/ml (4.14–22.08 nm). Therefore, 4- to 8-nM concentrations of remifentanil in the spinal fluid would be found with venous plasma concentrations of 5.41–10.81 nm (calculated based on previously determined cerebrospinal venous compartmentalization of remifentanil). Therefore, spinal fluid concentrations of remifentanil required for the enhancement of NMDA receptor responses are achieved with intravenous infusions of relatively low doses of remifentanil ranging from 0.1 to 0.2 μg · kg⁻¹ · min⁻¹. Thus, the effects of remifentanil on NMDA receptor-mediated responses observed at concentrations of 4, 6, and 8 nm are clinically relevant.

The concentration–response for remifentanil enhancement of NMDA current is unusual in that effects are only observed at 4, 6, and 8 nm but not at lower or higher concentrations. A similar concentration–response relation was observed with the selective δ-opioid agonists DPDPE and deltorphin II. With DPDPE, 4 and 30 nm did not result in NMDA current increases, but the intermediate concentration 12 nm enhanced NMDA peak current by 73.59% (twofold more than 4 nm remifentanil). With deltorphin II, 4 nm and 10 nm resulted in enhancement of NMDA peak current by 26.12% and 38.64%, respectively (P = 0.023 and P = 0.002), whereas 30 nm did not increase NMDA responses. The authors are unaware of any studies where concentration–responses have been studied for remifentanil in this context and, therefore, view this as a novel observation that is likely attributable to the activation of δ-opioid receptors. Presumably, at lower concentrations of remifentanil, the threshold for enhancement of NMDA receptor function has not been reached. However, currently, the failure of higher remifentanil, DPDPE, and deltorphin II concentrations to enhance NMDA responses is unexplained. It is possible that higher opioid agonist concentrations are more likely to increase (1) δ-opioid receptor desensitization, (2) recruitment of dephosphorylating enzymes that counter the effects of δ-opioid receptor activation; or (3) activation of a competing pathway, e.g., through other associated G-protein subtypes. As mentioned previously, the coactivation of NMDA receptors may also contribute to this possibly by activating a common second messenger protein (e.g., PKC) as opioid receptors.

The time-dependent enhancement of NMDA current by remifentanil is relevant to the acute development of OIH or tolerance. This correlation of in vitro and in vivo effects suggests that the remifentanil-induced changes are due to the recruitment of intracellular processes that operate on relatively rapid time scales, within 36 min. These processes may include NMDA receptor phosphorylation, e.g., by PKC, or transport of sub-synaptic NMDA receptors to the cell surface. Opioid receptor phosphorylation, arrestin binding, and uncoupling of G-protein activation resulting in receptor desensitization have been shown to occur within 10 min in some instances. Others, such as gene induction, superactivation of adenyl cyclase, and accumulation of cyclic adenosine monophosphate, may play less of a role. Although the duration of electrophysiologic experiments reported here was limited by the occurrence of cellular dysfunction or demise after approximately 120 min, the persistence of NMDA response enhancement above control levels for the duration of the recording interval was demonstrated. The enhancement of NMDA-evoked current was maintained even after 40 min of remifentanil washout, suggesting a lasting effect of possibly hours, correlating with the clinical experience.

Analgesic effects of endogenous and exogenous opioids, such as remifentanil, are due to the activation of pertussis toxin (PTX)-sensitive G_{i/o} protein–coupled opioid receptors that are classified into μ, κ, and δ subtypes. All three types are expressed in the brain and spinal cord and are expressed on the cell surface in pairs as homodimers or heterodimers. Activation of μ- or δ-opioid receptors, but not κ-opioid receptors, have been implicated in the development of OIH, possibly because of their effect on a common subset of intracellular G proteins. Activation of μ-opioid receptors by morphine increases the expression of δ-opioid receptors on cellular surfaces, thus increasing the probability for these subtypes to interact. In addition, the pharmacologic inhibition and genetic attenuation of δ-opioid receptors has been shown to enhance μ-opioid receptor spinal antinociception and eliminate morphine tolerance development in animals. The demonstration that increases in NMDA current result from δ-opioid receptor activation in a subpopulation of cultured DH neurons suggests that δ-opioid receptors play a role in the development of acute OIH and tolerance by this mechanism. Therefore, δ-opioid receptor inhibition may play a role in attenuating acute remifentanil tolerance, as well as in eliminating chronic morphine tolerance.

The observation that 1 μM DAMGO activation of μ-opioid receptors caused an enhancement of NMDA recep-
tor current in DH neurons was initially made by Chen et al.16,17 DAMGO at 1 μm is also active at δ-opioid receptors,18 and the current study showed that the selective activation of δ-opioid receptors enhanced NMDA receptor function. Chen et al. also demonstrated that the consequent increase in intracellular PKC from opioid receptor activation caused a reduction in the physiologic Mg2+ block of the NMDA receptor, thereby increasing NMDA receptor current amplitude. The mechanism of NMDA receptor enhancement by δ-opioid receptor activation is not known but may also involve the increase in PKC activity. However, due to the lack of extracellular Mg2+ in these experiments, it is unlikely that the mechanism of PKC action on the NMDA receptor is the reduction of the physiologic Mg2+ block. Therefore, it is unknown which G protein–dependent/independent pathways are initiated after δ-opioid receptor activation to bring about increases in NMDA receptor function.

There is some evidence that both μ- and δ-opioid receptors play a role in the development of OIH and tolerance.16–18,34,35,46 These two subtypes of opioid receptors colocalize in the superficial dorsal layers of the spinal cord47 and exist as homodimers or heterodimers.48,49 Heterodimers formed by μ- and δ-opioid receptors have been found in native spinal cord neuronal tissue50 and have unique properties.49,51,52 These unique properties include their selectivity for the δ-agonist deltorphin II (albeit at a lower binding affinity than to δ/δ receptors51) rather than DPDPE, and coupling to PTX-insensitive Gαδ proteins of the Gαδ subtype.51,52 Homodimers of μ- and δ-opioid receptors activate Gαδ protein subtypes that are PTX sensitive,51 but in a minority of neurons, they activate cholether toxin–sensitive Gα proteins, which presumably mediate the cellular processes that can also underlie hyperalgesia.52 Preliminary unpublished data from this laboratory are inconclusive for determining whether μ/δ-heterodimeric opioid receptors coupled to Gαδ proteins or μ-μ- or δ/δ-homodimeric opioid receptors coupled to Gαδ proteins are responsible for the observed increases in NMDA responses after remifentanil exposure. A 24-h 1-μg/ml PTX (inhibiting Gαδ) but not Gαγ proteins) or 5-μg/ml cholether toxin A + B (inhibiting Gαδ proteins) pretreatment of the DH neurons before electrophysiologic recording led to the enhancement of NMDA receptor current in the absence of remifentanil or an opioid agonist (data not shown; figs. 2 and 3 on the ANESTHESIOLOGY Web site at http://www.anesthesia.org). However, the NMDA receptor up-regulation by PTX correlated directly with animal hyperalgesia and allodynia after intrathecal PTX injection.24,55 Unfortunately, the G-protein subtype that is activated and ultimately leads to NMDA receptor up-regulation remains unknown.

Therefore, our experimental model presents a cellular mechanism involving the rapid and prolonged up-regulation of NMDA receptor function by remifentanil which may contribute to the clinical development of remifentanil hyperalgesia and tolerance. The concentration dependence, time course, and δ-opioid receptor involvement in the remifentanil-induced NMDA response enhancement have been shown in these experiments. These results support the validity of this model in which NMDA receptor function enhancement in DH neurons corresponds well with the development of pathologic pain. Furthermore, opioid-induced pathologic pain as shown by increased NMDA receptor responses to remifentanil can develop rapidly and run a protracted course even at clinical concentrations. Therefore, this model can be used in future studies to define the mechanism for δ-opioid receptor-induced increases in NMDA receptor function. More importantly, results of this study support a role for δ-opioid receptor inhibition in the elimination of rapid NMDA receptor enhancement and the attenuation of acute OIH and tolerance development.

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