

# Activation of $\mu$ - and $\delta$ -Opioid Receptors Causes Presynaptic Inhibition of Glutamatergic Excitation in Neocortical Neurons

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**Background:** The mechanism underlying the depressant effect of opioids on neuronal activity within the neocortex is still not clear. Three modes of action have been suggested: (1) inhibition by activation of postsynaptic potassium channels, (2) interaction with postsynaptic glutamate receptors, and (3) presynaptic inhibition of glutamate release. To address this issue, the authors investigated the effects of  $\mu$ - and  $\delta$ -receptor agonists on excitatory postsynaptic currents (EPSCs) and on membrane properties of neocortical neurons.

**Methods:** Intracellular recordings were performed in rat brain slices. Stimulus-evoked EPSCs mediated by different glutamate receptor subtypes were pharmacologically isolated, and opioids were applied by addition to the bathing medium. Possible postsynaptic interactions between glutamate and opioid receptors were investigated using microiontophoretic application of glutamate on neurons functionally isolated from presynaptic input.

**Results:**  $\delta$ -Receptor activation by D-Ala<sup>2</sup>-D-Leu<sup>5</sup>-enkephalin (DADLE) reduced the amplitudes of EPSCs by maximum 60% in a naltrindole-reversible manner (EC<sub>50</sub>: 6–15 nM). In 30–40% of the neurons investigated, higher concentrations (0.1–1  $\mu$ M) of DADLE activated small outward currents. The  $\mu$ -receptor selective agonist D-Ala<sup>2</sup>-N-MePhe<sup>5</sup>-Gly<sup>5</sup>-ol-enkephalin (0.1–1  $\mu$ M) depressed the amplitudes of EPSCs by maximum 30% without changes in postsynaptic membrane properties. In the absence of synaptic transmission, inward currents induced by microiontophoretic application of glutamate were not affected by DADLE.

**Conclusions:** Activation of  $\mu$ - and  $\delta$ -opioid receptors depresses glutamatergic excitatory transmission evoked in neocortical neurons by presynaptic inhibition. A weak activation of a postsynaptic potassium conductance becomes evident only at high agonist concentrations. There is no evidence for a postsynaptic interaction between glutamate and opioid receptors. (Key words: EPSC; glutamate; neocortex.)

DURING general anesthesia, opioids are used as supplements to produce analgesia and to reduce the concentrations of volatile anesthetics necessary to induce sufficient anesthesia.<sup>1</sup> At higher doses, opiates and, in particular,  $\mu$ -receptor agonists, lead to unconsciousness associated with slow  $\delta$  waves.<sup>2</sup> These electroencephalogram alterations suggest a major involvement of the thalamocortical system in the generation of opioid effects on higher central nervous system functions.

From electrophysiologic studies *in vivo* it is known

that opioids depress both spontaneous and stimulus-evoked action potential discharge of neocortical neurons.<sup>3–5</sup> However, the mechanisms underlying this inhibition are still not clear. Intracellular recordings from neocortical neurons *in vitro* consistently demonstrate an opioid-induced reduction in the amplitudes of stimulus-evoked excitatory postsynaptic potentials (EPSPs).<sup>6</sup> This depression of EPSPs has been explained, in part, by a  $\delta$ -opioid receptor-mediated presynaptic inhibition of glutamate release.<sup>7</sup> In addition, a  $\delta$ -receptor-activated increase in a postsynaptic potassium conductance has been described.<sup>7</sup> Because  $\delta$ -receptors are located predominantly on the dendrites of neocortical pyramidal cells,<sup>8</sup> such a conductance increase might shunt EPSPs at their dendritic site of generation.<sup>7</sup> However, another study on neocortical neurons<sup>6</sup> reported on a direct interaction between postsynaptically located glutamate receptors and opioid receptors. Similar interactions have been described in neurons of the nucleus accumbens.

Because the mechanisms underlying the depressant effect of opioids on the excitability of neocortical neurons are still not clear, we reinvestigated the actions of opioids on excitatory transmission in rat neocortical neurons.

## Materials and Methods

The experiments were performed on slices prepared from the neocortex of adult Wistar rats (body weight 100–180 g) of either sex. The experiments were approved by the local animal care committee. The method of slice preparation was similar to that described previously.<sup>10</sup> In brief, the rats were deeply anesthetized with isoflurane (Abbott GmbH, Wiesbaden, Germany) and decapitated. The brains were removed and stored for 1–2 min in ice-cold artificial cerebrospinal fluid (ACSF). After chilling the brain, the hemispheres were separated and trimmed to obtain coronal slices of the somatosensory cortex. The slices (400  $\mu$ m) were cut using a vibratome (Leica VT 1000E; Leica Instruments GmbH, Nussloch, Germany) and stored in ACSF at room temperature. After at least 1 h of incubation, individual slices were transferred to the recording chamber (volume: 1.5 ml), fixed by two nylon meshes, and kept submerged by perfusion (flow rate: 5 ml/min) with ACSF. The ACSF consisted of 125 mM NaCl, 3 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, and 10 mM D-glucose. The ACSF was saturated with carbogen (95% O<sub>2</sub> and 5% CO<sub>2</sub>), resulting in a pH of 7.4 at a recording temperature of 31°C.

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A concentric bipolar stimulation electrode (tip diameter: 100  $\mu\text{m}$ ) positioned in layer IV of the neocortex was used to evoke postsynaptic responses. Stimulus currents (10–1,000  $\mu\text{A}$ ) were supplied by an isolation unit (Isoflex; A.M.P.I., Jerusalem, Israel) that was triggered by a central timer device (Master 8, A.M.P.I.). The stimulus duration was set to 50  $\mu\text{s}$ , and synaptic responses were elicited at a frequency of 0.05 Hz. During the experiments in which pharmacologically isolated  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazol-propionate (AMPA)-EPSCs were investigated, the frequency was increased to 0.14–0.2 Hz to suppress polysynaptic activity.<sup>11</sup>

Intracellular recording electrodes were pulled from thick-walled borosilicate capillaries (Clark Electromedical Instruments, Reading, United Kingdom) using a P-87 micropipette puller (Sutter Instruments, San Rafael, CA). Electrodes were filled with either 3 M KCl or 2 M K-acetate (containing 0.3 M KCl). The electrode resistances ranged between 70 and 130 M $\Omega$ . Intracellular current injections and single-electrode voltage-clamp recordings were performed using the switched mode of a single-electrode voltage-clamp amplifier (SEC-10L; npi, Tamm, Germany) at a switching frequency of 22–25 kHz (duty cycle: 25%). During voltage-clamp measurements, the headstage output of the amplifier was continuously monitored on a separate oscilloscope.<sup>11</sup>

After amplification, the signals were displayed on an oscilloscope and stored on a personal computer by means of a laboratory interface (CED 1401; Cambridge Electronic Devices, Cambridge, United Kingdom). Membrane potentials and holding currents were continuously recorded using a Yt-chart recorder (RS 3400; Gould, Cleveland, OH).

All drugs, except glutamate, were applied by addition to the bathing solution. Drugs used were as follows: the  $\gamma$ -aminobutyric acid-A (GABA<sub>A</sub>) receptor antagonist bicuculline methiodide, the GABA<sub>B</sub> receptor antagonist CGP 35348, the AMPA receptor antagonist 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), the *N*-methyl-D-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonovaleric acid (D-2-APV), the  $\delta$ -receptor-preferring agonist D-Ala<sup>2</sup>-D-Leu<sup>5</sup>-enkephalin (DADLE), the selective  $\delta$ -receptor agonist H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE), the selective  $\mu$ -receptor agonist D-Ala<sup>2</sup>-N-MePhe<sup>5</sup>-Gly<sup>5</sup>-ol-enkephalin (DAMGO), the selective  $\mu$ -receptor agonist fentanyl citrate, the broad-spectrum opioid receptor antagonists naloxone and naltrexone, the selective  $\delta$ -receptor antagonist naltrindole, and the sodium channel blocker tetrodotoxin (TTX). All drugs were obtained from Biotrend (Köln, Germany). After a control period of 20–30 min, the drugs were applied for 10–15 min. The development of the drug effects were monitored by measurements taken at 5-min intervals. The opioid effects were usually attained at steady state within 5–7 min.

Glutamate was applied by microiontophoresis<sup>12</sup> using a high-voltage iontophoresis unit (HVCS-03C; npi). Ion-

tophoresis pipettes were pulled from double barreled  $\theta$  capillaries (Clark Electromedical Instruments, Reading, United Kingdom). One barrel was filled with sodium glutamate (1 M, pH 8.0), the other barrel contained NaCl (1 M). At total tip diameters of 1–2  $\mu\text{m}$ , the pipette resistances ranged between 120 and 200 M $\Omega$ . Iontophoretic currents (60–400 nA for 1 s) used to apply glutamate were compensated for by currents of equal amplitude but opposite polarity ejected *via* the NaCl barrel. Leakage of glutamate from the barrels during the interval between two applications was controlled by application of retaining currents (5–10 nA). After impalement of a neuron, the iontophoresis pipette was positioned on a virtual vertical line between the recording electrode and the pial surface of the slice with a distance of 100–150  $\mu\text{m}$  to the electrode. At this position, glutamate was applied predominantly to the dendrites of the neurons. Positioning of the micropipettes was performed by means of a piezo-driven micromanipulator and the pipette location was adjusted to obtain the largest possible response amplitude with a minimum of iontophoretic current.

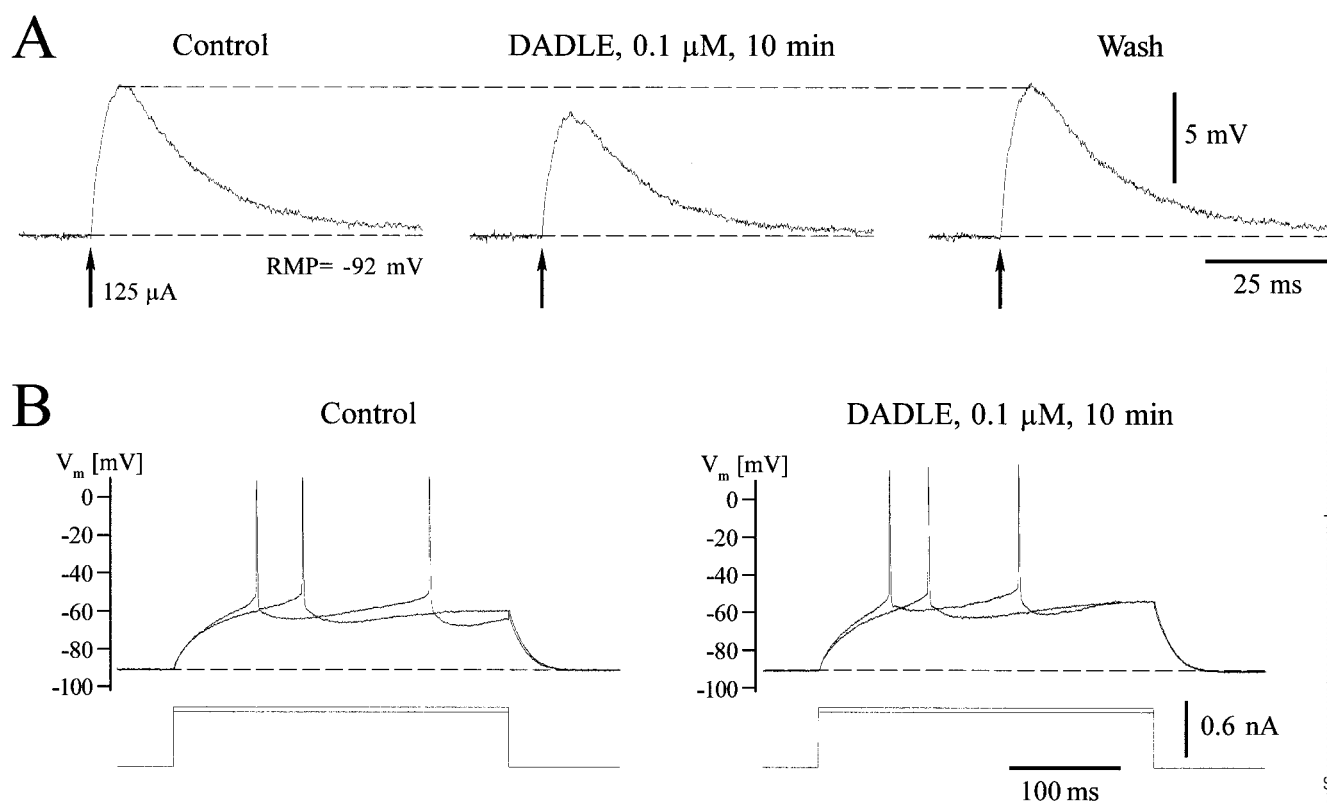
The data obtained from intracellular recordings were analyzed offline using the AutesW software (H. Zuckermann, Max-Planck Institute, Martinsried, Germany). Curve fitting, EC<sub>50</sub> determinations, and statistical tests were made using Graph Pad Prism 2.0 (San Diego, CA).

#### Statistical Analysis

If not stated differently, values are expressed as mean  $\pm$  SD. For comparison of measurements performed in different cells, values obtained in a single experiment were normalized with respect to the corresponding control values (100%) and expressed as relative change (in percent). To test for the statistical difference of such a relative change (in relation to 100%), one-sample *t* test was applied. Because data were normalized, statistical comparisons of the mean values of two experimental groups were performed using the nonparametric Mann-Whitney test. The significance level was set to  $P < 0.5$ .

## Results

Intracellular recordings were obtained from 165 neurons (mean resting membrane potential:  $-82.3 \pm 3.5$  mV; mean input resistance:  $35.0 \pm 10.2$  M $\Omega$ ) located in layer II–III of the rat somatosensory cortex. Intracellular injection of depolarizing square wave current pulses with suprathreshold strengths elicited trains of action potentials displaying frequency adaptation. Thus, according to their discharge properties, all neurons recorded could be classified as so-called regular spiking neurons.<sup>13</sup> In line with detailed analyses performed previously in our laboratory,<sup>10</sup> we can assume that the neuron population investigated in the present study consisted predominantly of pyramidal cells.

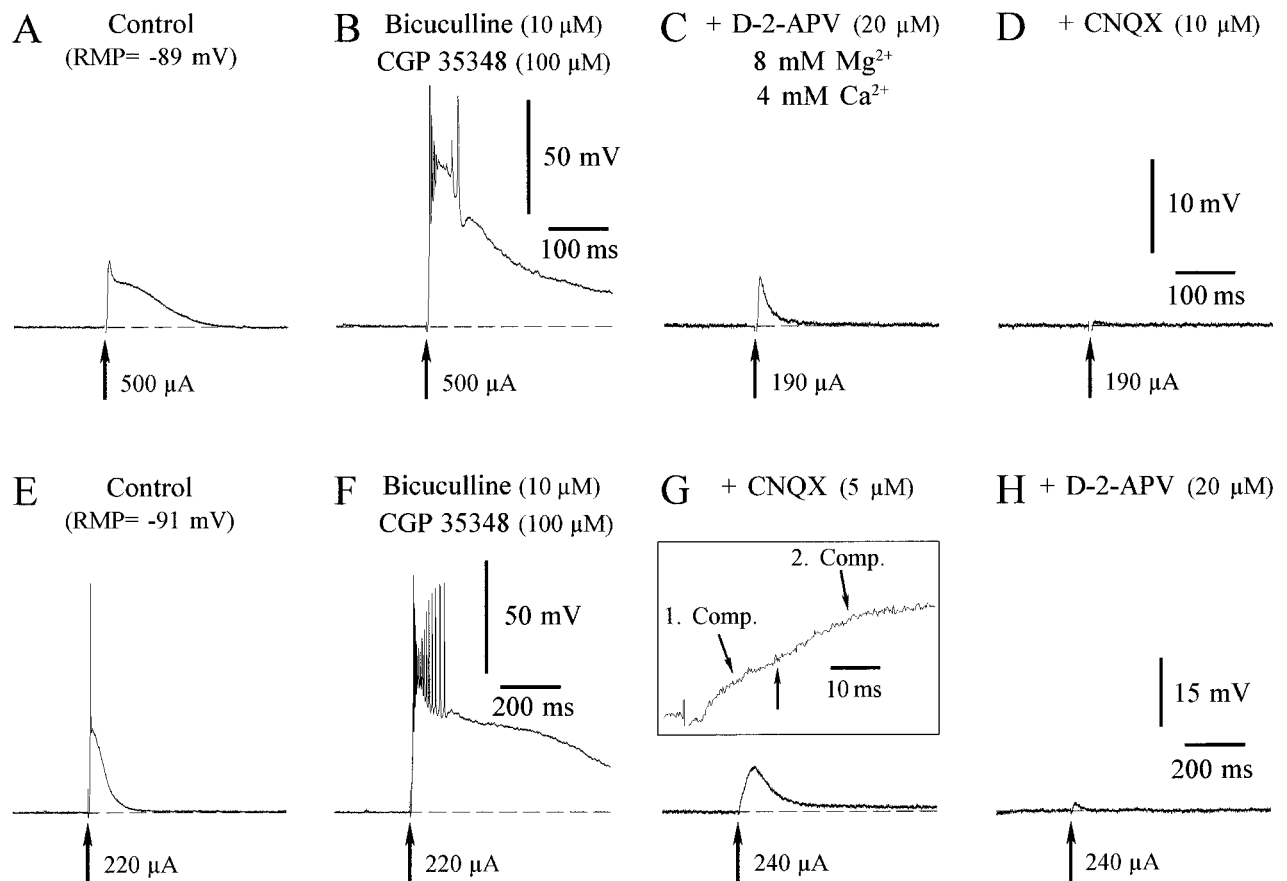


**Fig. 1.** Action of DADLE on stimulus-evoked EPSPs and on current pulse-induced action potential discharge in rat neocortical neurons. **(A)** Electrical stimulation of neocortical layer IV evoked an e-EPSP in an intracellularly recorded neuron of layer II-III (control). Bath application of DADLE led to a reversible (wash) reduction in the EPSP amplitude. Each trace represents the average of five consecutive single recordings. In this and in the following figures, the time of stimulation is indicated by an arrow. **(B)** Responses of the same cell to depolarizing current injection before and during DADLE application. During control conditions, the amplitudes of the injected currents (lower traces) were adjusted to the strength necessary to evoke one action potential (upper traces, threshold intensity, 0.55 nA) and to just suprathreshold levels (0.63 nA). The corresponding traces are displayed superimposed. DADLE did not affect membrane potential, threshold intensity, and action potential discharge pattern evoked by suprathreshold currents.

Figure 1A (control) shows an intracellularly recorded AMPA receptor-mediated early EPSP (e-EPSP)<sup>11</sup> evoked by low-intensity stimulation of neocortical layer IV. Addition of DADLE (0.1  $\mu\text{M}$ ) to the bathing solution led to a reversible reduction in the EPSP amplitude by 21%. Resting membrane potential, neuronal input resistance, and the action potential discharge pattern induced by depolarizing current pulses (fig. 1B) were not affected by the peptide. Similar observations were made in all neurons recorded during current-clamp conditions ( $n = 25$ , mean reduction in EPSP amplitude in the presence of 0.1  $\mu\text{M}$  DADLE:  $26.0 \pm 4.7\%$ ). In addition, the electrotonic properties of the neurons (*i.e.*, membrane time constants  $\tau_0$  and  $\tau_1$ , electrotonic length  $L^{10}$ ) remained unchanged in the presence of DADLE at all concentrations tested (0.1–1  $\mu\text{M}$ ,  $n = 8$ ).

Detailed investigations of the synaptic response pattern of rat neocortical layer II-III neurons demonstrated a considerable overlap of different synaptic components.<sup>11,12,14</sup> Depending on the stimulus intensity, AMPA and NMDA receptor-mediated EPSPs as well as GABA<sub>A</sub> and GABA<sub>B</sub> receptor-mediated IPSPs can be observed. These postsynaptic potentials are generated *via* mono-

synaptic or polysynaptic pathways. Thus, to investigate the effects of opioid peptides on excitatory synaptic transmission, we isolated the different synaptic components using selective receptor antagonists. Figure 2 depicts the experimental procedure. A stimulus with an intensity of 500  $\mu\text{A}$  evoked a subthreshold synaptic response (fig. 2A) consisting of EPSPs and a depolarizing IPSP.<sup>11,14</sup> Bath application of the GABA<sub>A</sub> receptor antagonist bicuculline together with the GABA<sub>B</sub> receptor antagonist CGP 35348 led to a complete block of GABAergic inhibition resulting in a stimulus-evoked paroxysmal depolarization (fig. 2B). To isolate AMPA-EPSPs, the NMDA receptor antagonist D-2-APV was added to the ACSF. During these conditions, the stimulation induced a short-latency EPSP followed by a polysynaptic EPSP that displayed all-or-nothing burst properties (data not shown). Such bursts are difficult to control during voltage-clamp conditions. Therefore, they were blocked by increasing the extracellular  $\text{Mg}^{2+}$  concentration to 8 mM and the extracellular  $\text{Ca}^{2+}$  concentration to 4 mM.<sup>15</sup> In addition, we reduced the stimulus intensity and enhanced the stimulus frequency from 0.05 Hz to 0.14–0.2 Hz.<sup>11</sup> Now, the stimuli evoked EPSPs with short latencies and



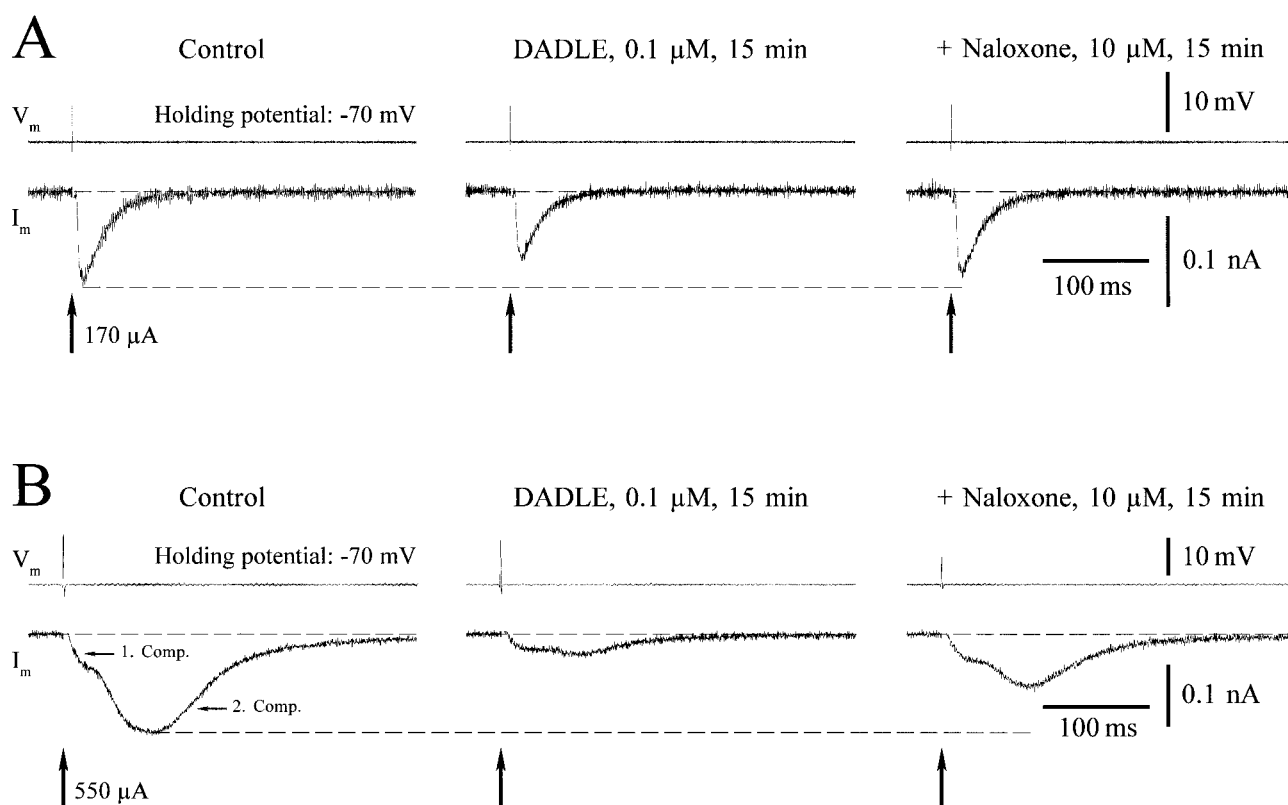
**Fig. 2.** Pharmacologic isolation of AMPA receptor-mediated (A–D) and NMDA receptor-mediated (E–H) components of evoked postsynaptic potentials in rat neocortical neurons. (A–D) The stimulus induced a subthreshold EPSP followed by a depolarizing IPSP (A). After inhibition of GABA<sub>A</sub> and GABA<sub>B</sub> receptors by bicuculline and CGP 35348, respectively, the stimulus evoked a paroxysmal depolarization (B). On an increase in the extracellular concentration of divalent cations and addition of the NMDA receptor antagonist D-2-APV to the bathing solution, the neuron responded to electrical stimulation (at lower intensity) with an EPSP (C) that could be blocked by the AMPA receptor antagonist CNQX (D). (E–H) During control conditions, the stimulus elicited a suprathreshold EPSP followed by a depolarizing IPSP (E). Application of bicuculline and CGP 35348 caused the induction of a paroxysmal depolarization (F). On addition of CNQX and a slight increase in stimulus intensity, the stimulus evoked an EPSP consisting of two components (G, the inset shows the rising phase of the EPSP on an expanded time scale). This EPSP could be blocked by application of D-2-APV (H). Calibration in (B) corresponds to (A) (similarly that in D to C, F to E, and H to G).

small amplitudes (fig. 2C) that could be completely blocked by the AMPA receptor antagonist CNQX (fig. 2D). To pharmacologically isolate NMDA-EPSPs, GABA<sub>A</sub> and GABA<sub>B</sub> receptors were inhibited (figs. 2E and 2F) and CNQX was applied to the bathing solution (fig. 2G). In most cases, the unmasked NMDA-EPSPs consisted of two components (fig. 2G, inset) that could be inhibited by the NMDA receptor antagonist D-2-APV (fig. 2H).

To investigate the effects of opioids on pharmacologically isolated AMPA and NMDA receptor-mediated synaptic responses, the membrane potentials of the neurons were voltage clamped to a holding potential of -70 mV, and the respective excitatory synaptic currents (EPSCs) were evoked by electrical stimulation. Figure 3 shows the actions of DADLE on an AMPA-EPSC (fig. 3A) and on an NMDA-EPSC (fig. 3B). The peptide (0.1  $\mu$ M) reduced the amplitude of the AMPA-EPSC by 31%. This effect could be antagonized by naloxone (fig. 3A). Similar to the NMDA-EPSC, the NMDA-EPSC consisted of two

components (fig. 3B, control). DADLE (0.1  $\mu$ M) decreased the amplitude of the first component by 55% and that of the second component by 81%. Again, these actions of DADLE could be antagonized by naloxone (fig. 3B). The antagonistic action of naloxone (10  $\mu$ M) on the depressant effect of DADLE (0.1  $\mu$ M) on EPSC amplitude was observed in all neurons tested (n = 6).

To determine the concentration dependency of DADLE's action on the different EPSCs, voltage-clamp measurements similar to those described were performed in 30 neurons (AMPA-EPSC: 15 neurons, NMDA-EPSC: 15 neurons). In each cell, three to seven different concentrations of DADLE ranging from 1 nM to 1  $\mu$ M were tested. The amplitudes of the EPSCs were determined and normalized with respect to the control amplitude measured before application of the peptide. Figure 4 shows diagrams in which the relative reduction of the amplitudes was plotted as a function of the DADLE concentration. The data points could be fitted by sig-

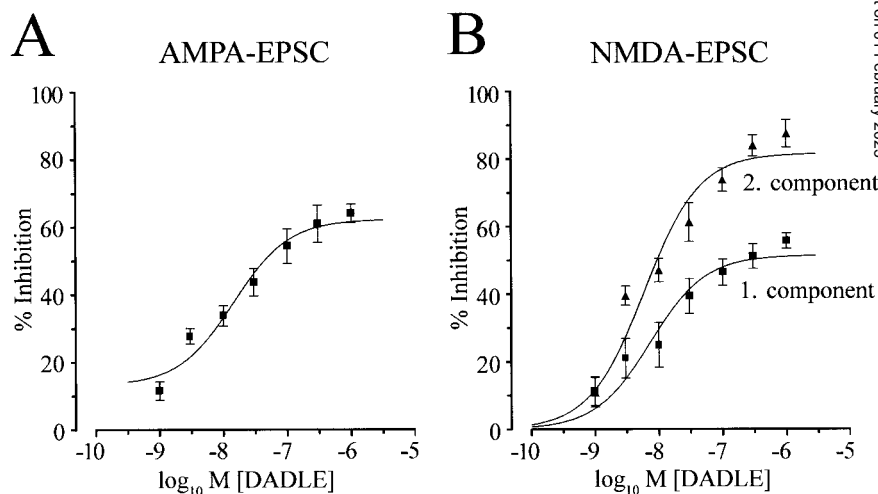


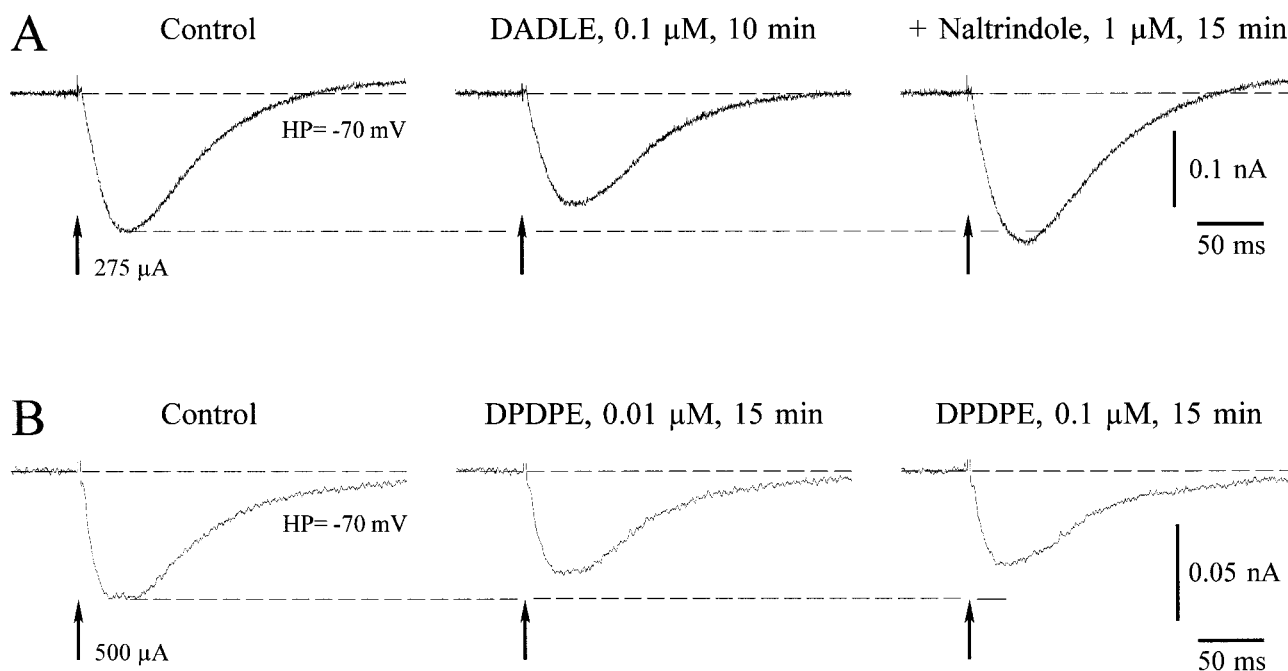
**Fig. 3.** Action of DADLE on the amplitudes of AMPA receptor-mediated (*A*) and NMDA receptor-mediated EPSCs (*B*). (*A*) Single electrode voltage-clamp recordings of evoked AMPA-EPSCs in the absence (control) and presence of DADLE and DADLE plus naloxone. The holding potential was set to  $-70$  mV. DADLE reduced the amplitude of the EPSC by 31%. This inhibition was reversed by naloxone. Upper traces: voltage; lower traces: current. Each trace represents the average of three consecutive single recordings. Recording conditions similar to (*A*). Note the two components of the EPSC. DADLE reduced the amplitude of the first EPSC component by 55% and that of the second EPSC component by 81%. This inhibition was also naloxone reversible.

moid functions, and from the resulting curves, the median effective concentration ( $EC_{50}$ ) of DADLE was obtained. As seen in figure 4A, the AMPA-EPSCs were inhibited by maximum  $63.8 \pm 5.6\%$  at a DADLE concentration of  $1 \mu\text{M}$  ( $n = 4$ ). Higher concentrations ( $3 \mu\text{M}$ ) exerted no additional effects ( $n = 4$ ). The  $EC_{50}$  value of DADLE's action on the AMPA-EPSC was found to be  $14.5$  nM. DADLE reduced the two components of the

NMDA-EPSC with different efficacy (fig. 4B). The first component of the NMDA-EPSC amplitude was depressed by maximum  $55.8 \pm 4.6\%$  ( $1 \mu\text{M}$  DADLE,  $n = 5$ ). At the same concentration, the second component was found to be reduced by  $87.5 \pm 6.9\%$  ( $n = 4$ ). The  $EC_{50}$  values of DADLE's action on the NMDA-EPSC were  $7.4$  nM (first component) and  $6.0$  nM (second component), respectively.

**Fig. 4.** Concentration-response curves of the effects of DADLE on AMPA receptor-mediated and NMDA receptor-mediated EPSCs. (*A*) Semilogarithmic plot of the relative inhibition of the AMPA-EPSC amplitudes as a function of the DADLE concentration. (*B*) Semilogarithmic plot of the relative inhibition of the two components of the NMDA-EPSC amplitudes as a function of the DADLE concentration. In both plots, the data points could be fitted by sigmoid functions. Data are expressed as mean  $\pm$  SEM.





**Fig. 5.** Actions of selective  $\delta$ -receptor agonists and antagonists. (A) The  $\delta$ -receptor-selective antagonist naltrindole reversed the depressant action of DADLE on the amplitude of stimulus-evoked NMDA-EPSCs. (B) The  $\delta$ -receptor-selective agonist DPDPE induced a concentration-dependent reduction in the amplitudes of evoked NMDA-EPSCs. Each trace represents the average of five consecutive single recordings performed at an interval of 30 s.

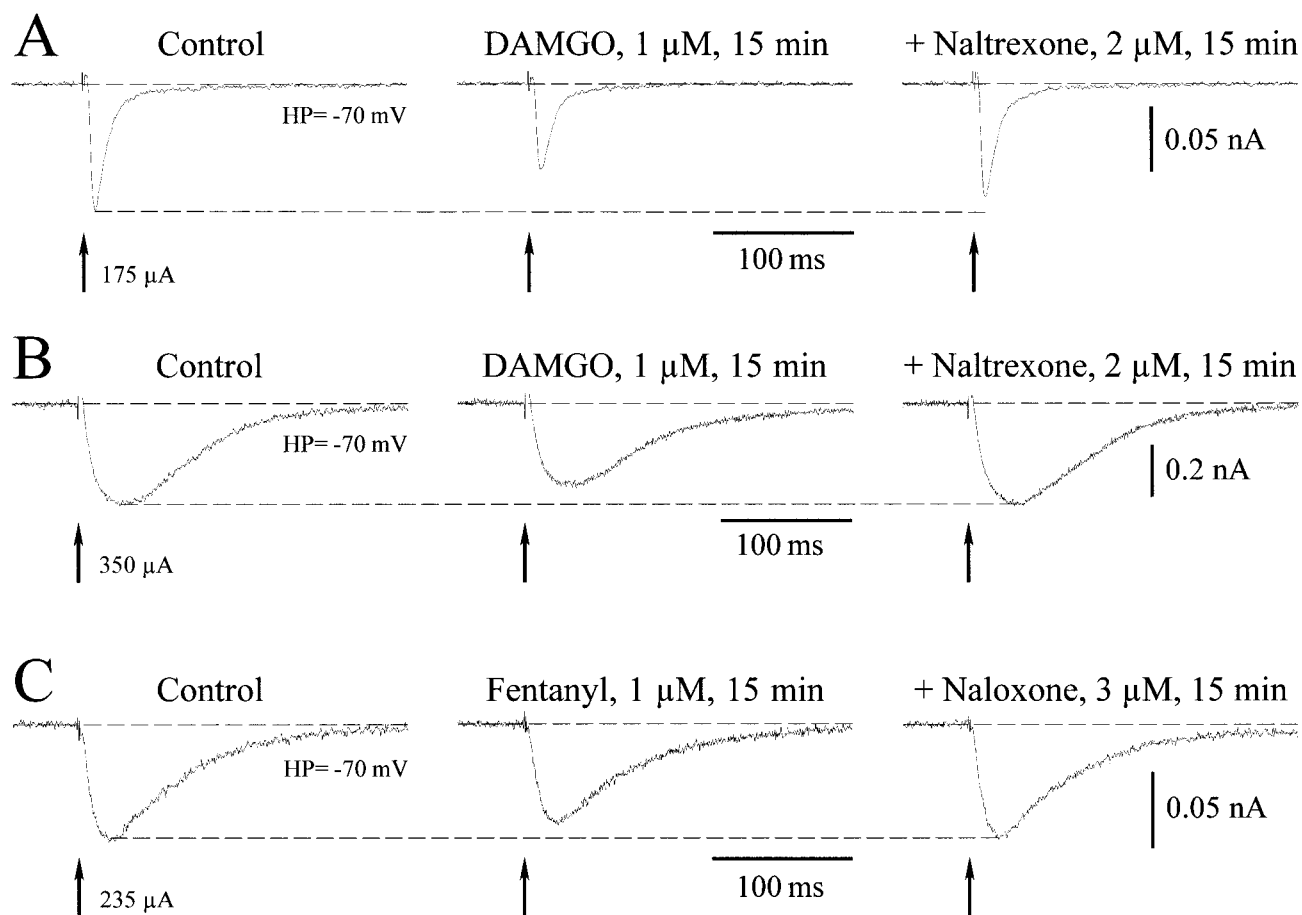
DADLE is a  $\delta$ -receptor-preferring agonist.<sup>16</sup> However, it has been demonstrated that this peptide can also interact with the  $\mu$ -receptor.<sup>17,18</sup> To determine the contribution of  $\delta$ - and  $\mu$ -receptor activation to the DADLE-induced effects, we examined the actions of selective opioid receptor agonists and antagonists. In all neurons tested ( $n = 4$ ), the depressant effect of DADLE (0.1  $\mu$ M) on the amplitude of the NMDA-EPSC (first and second components) could be reversed by the selective  $\delta$ -receptor antagonist naltrindole (fig. 5A). During the given experimental conditions, the required concentrations of naltrindole ranged between 0.1 and 3  $\mu$ M. In a second set of experiments ( $n = 8$  neurons), the effects of the selective  $\delta$ -receptor agonist DPDPE (0.001–1  $\mu$ M) on the NMDA-EPSC amplitudes were investigated. DPDPE concentration-dependently decreased the EPSC amplitudes (fig. 5B). The maximum effect (reduction by  $22.5 \pm 4.0\%$ ,  $n = 4$ ) was achieved at a concentration of 0.1  $\mu$ M.

In four of seven neurons investigated, the selective  $\mu$ -receptor agonist DAMGO (1  $\mu$ M) reduced the amplitude of the AMPA-EPSC by  $27.0 \pm 5.6\%$  (fig. 6A). This effect could be antagonized by naltrexone (2  $\mu$ M,  $n = 4$ ; fig. 6A). Increasing the peptide concentration to 3  $\mu$ M did not produce a significantly larger depression of the EPSC amplitude ( $n = 3$ ). The minimum effective concentration was found to be 0.3  $\mu$ M. The efficacy of DAMGO on NMDA-EPSCs was similar to that on AMPA-EPSCs. DAMGO (1  $\mu$ M) reduced the amplitude of NMDA-EPSCs (first component) by  $26.5 \pm 9.2\%$  (fig. 6B). This effect was observed in five of six neurons tested. The action of DAMGO on NMDA-EPSCs could be

blocked by naltrexone (2  $\mu$ M,  $n = 4$ ; fig. 6B) and, similar to the AMPA-EPSCs, an increase in the peptide concentration did not result in a significantly larger depression of the EPSC amplitude. The very narrow range of DAMGO concentrations (0.3–1  $\mu$ M) producing minimum and maximum effects, respectively, did not allow the reliable measurement of concentration-response curves and the determination of  $EC_{50}$  values. In four neurons the effects of the clinically relevant  $\mu$ -receptor agonist fentanyl (1  $\mu$ M) on the NMDA-EPSC amplitude were analyzed. However, in only one neuron was a naloxone-reversible depression of the EPSC amplitude by 13.1% observed (fig. 6C).

During the experiments in which the effects of the peptides on EPSCs were determined, both the holding current necessary to clamp the neurons' membrane potential to  $-70$  mV and the input conductance were continuously monitored. The values obtained in the presence of DADLE were normalized with respect to the control measurements. Within the whole concentration range tested (0.001–1  $\mu$ M), the resulting mean values for both parameters were statistically not different from controls (*i.e.*, from 100%;  $P > 0.1$ ;  $n = 30$ ). Similarly, neither DAMGO ( $n = 13$ , 0.3–3  $\mu$ M) nor DPDPE ( $n = 8$ , 0.001–1  $\mu$ M) affected holding current and input conductance.

At higher DADLE concentrations (0.1–1  $\mu$ M), the mean value of the holding current (usually an applied outward current) displayed a statistically nonsignificant tendency to decrease. This reduction was caused by a peptide-induced activation of an intrinsic outward current in a

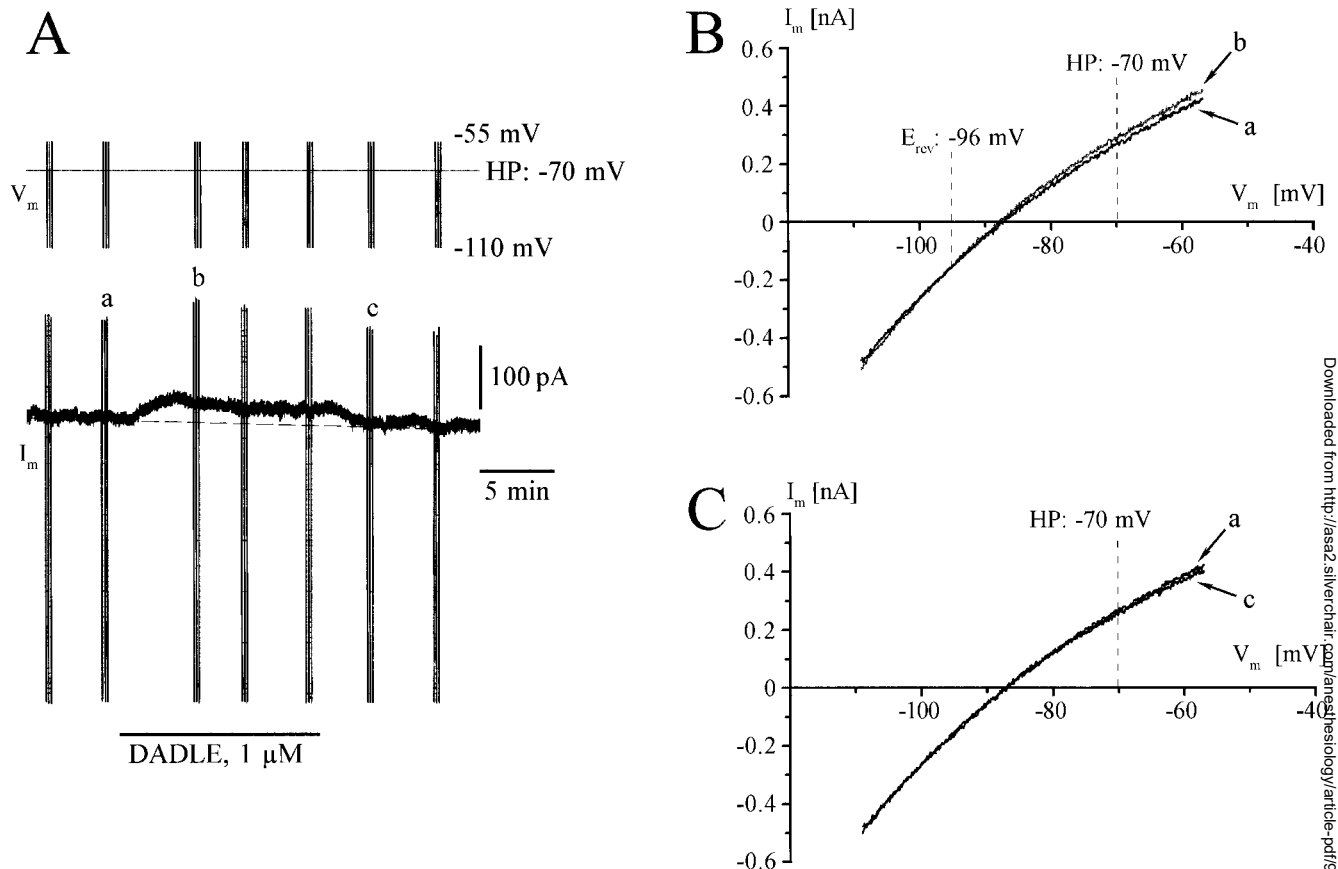


**Fig. 6.** Action of DAMGO on AMPA receptor-mediated (A) and NMDA receptor-mediated EPSCs (B) and effects of fentanyl on NMDA receptor-mediated EPSCs (C). (A) Voltage-clamp recordings of AMPA-EPSCs in the absence (control) and presence of DAMGO and DAMGO plus naltrexone. DAMGO reduced the amplitude of the EPSC by 33%. This inhibition was reversed by naltrexone (2  $\mu\text{M}$ ). (B) Voltage-clamp recordings of NMDA-EPSCs in the absence (control) and presence of DAMGO and DAMGO plus naltrexone. DAMGO reduced the amplitude of the EPSC by 21%. This inhibition was also naltrexone reversible. (C) Voltage-clamp recordings of NMDA-EPSCs in the absence (control) and presence of fentanyl and fentanyl plus naloxone. Fentanyl reduced the amplitude of the EPSC by 13%. This inhibition was also naloxone reversible. Each trace represents the average of five consecutive single recordings.

few neurons. To study the ability of DADLE to generate outward currents by activating postsynaptic conductances, we functionally isolated the neurons from presynaptic input using TTX. During these conditions, voltage-current curves (VI curves) were determined before, during, and after bath application of DADLE using either the voltage step technique (data not shown) or the voltage ramp technique (fig. 7). With the step method, the holding potential was set to  $-70$  mV, and the neuron's membrane potential was successively clamped to different values using voltage steps (300 ms, membrane potential range analyzed:  $-110$  to  $-55$  mV). The amplitudes of the corresponding membrane currents were measured and plotted as a function of the membrane potential. In other experiments, voltage ramps (2 s in duration) were applied starting from  $-110$  mV and ending at  $-55$  mV. The resulting current responses were plotted as a function of the voltage (figs. 7B and 7C). The VI curves obtained with these two methods were very similar. In 6 of 11 neurons tested, DADLE (1  $\mu\text{M}$ ) induced

an outward current (fig. 7A) with maximum amplitude ranging between 30 and 40 pA. These currents were found to decline by 10–15 pA during drug application. In the presence of the peptide, the slopes of the VI curves became slightly steeper (fig. 7B), indicating a small increase in membrane conductance. The VI curves obtained during control conditions and those determined in the presence of DADLE intercepted at membrane potentials between  $-95$  mV and  $-100$  mV (fig. 7B), suggesting that the DADLE-induced outward current was caused by the activation of a potassium conductance.<sup>19</sup> The effect of the peptide on holding current and VI curve was reversible on washout of the compound (fig. 7C). At lower DADLE concentrations, we rarely observed detectable changes in holding currents and VI curves (0.1  $\mu\text{M}$ : in one of six neurons; 0.3  $\mu\text{M}$ : in one of seven neurons).

The depressant effect of DADLE on glutamatergic EPSCs might be explained by a direct postsynaptic interaction between opioid receptors and glutamate recep-



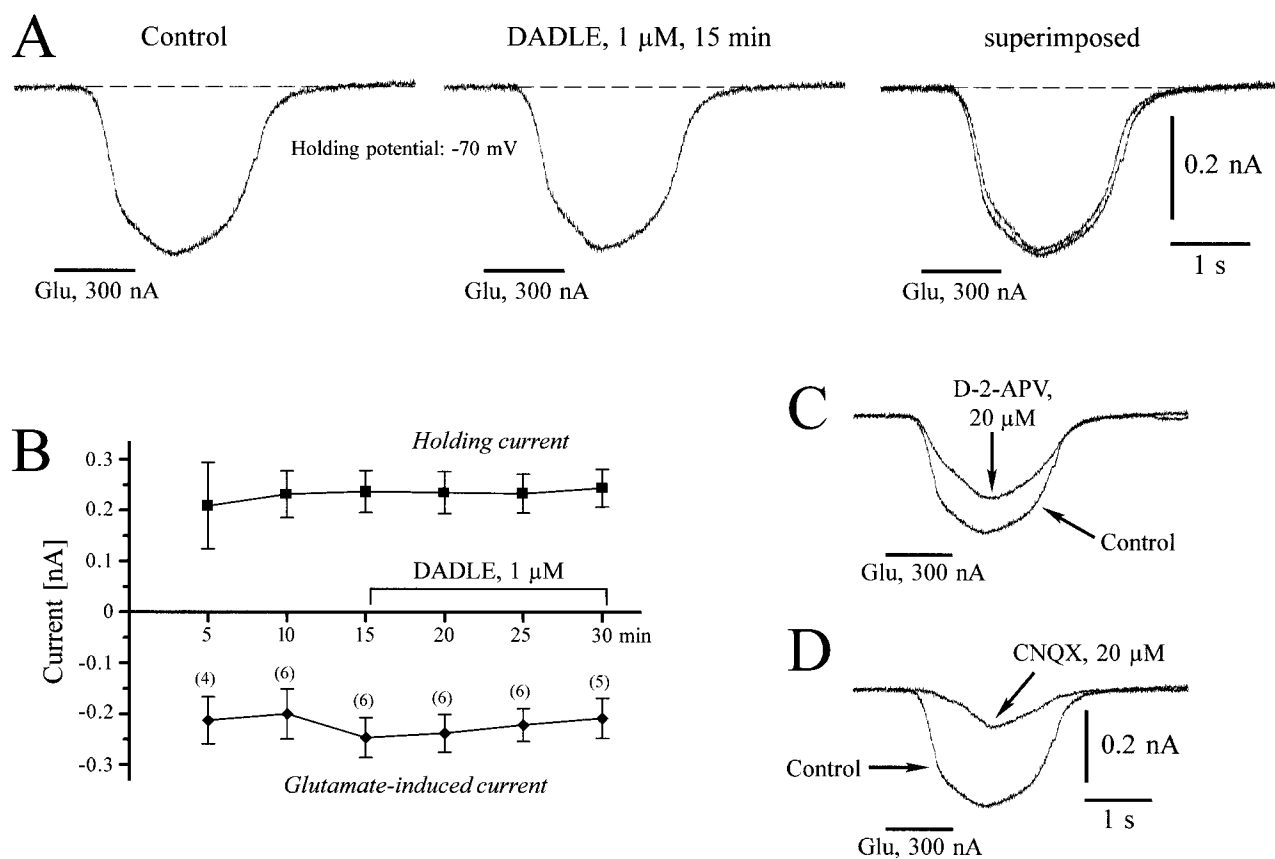
**Fig. 7.** In 30–40% of all neurons tested, high concentrations of DADLE activated an outward current and slightly increased the slope of the voltage-current curve. (A) Chart recording of the holding current (lower trace) before, during, and after application of DADLE (15 min, application indicated by bar). The neuron was functionally isolated from presynaptic input by TTX (1.2  $\mu$ M). Holding potential was set at  $-70$  mV. The upward and downward deflections are caused by voltage ramps ( $-110$  to  $-55$  mV, 2 s) applied to determine VI curves. DADLE induced an outward current with an initial amplitude of 38 pA that declined to 27 pA during ongoing application. (B and C) VI curves derived from voltage ramp measurements before (a), during (b), and after (c) application of DADLE. The curve determined in the presence of DADLE intersected that obtained during control conditions at a membrane potential of  $-96$  mV (B). The effect of DADLE was reversible on washout (C).

tors.<sup>6,9</sup> To investigate this possibility, we functionally isolated the neurons from presynaptic input using TTX and determined the effect of DADLE on inward currents induced by microiontophoretically applied glutamate. The iontophoresis pipettes were positioned into an area corresponding to that of the dendritic tree of the neurons (see Materials and Methods). During voltage-clamp conditions, glutamate was administered by means of negative iontophoresis currents (60–400 nA, 1 s). The neurons responded to glutamate application with inward currents (fig. 8) in a dose-dependent manner (data not shown). These currents were reduced by both NMDA (fig. 8C) and AMPA (fig. 8D) receptor antagonists, indicating the involvement of both receptor subtypes in their generation. After a control period of 15 min, DADLE (1  $\mu$ M) was applied for 15 min (fig. 8A). In six of six neurons investigated, the peptide did not affect the glutamate-induced inward currents or the holding current necessary to clamp the membrane potential to  $-70$  mV (fig. 8B).

## Discussion

In layer II–III neurons of the rat somatosensory cortex the predominant effect of  $\mu$ - and  $\delta$ -opioid receptor activation was a depression of excitatory synaptic transmission mediated *via* the AMPA or NMDA subtype of the glutamate receptor. This depression could be reversed by application of opioid receptor antagonists, indicating the involvement of these receptors in this inhibition. The efficacy of the  $\delta$ -receptor-preferring agonist DADLE to inhibit glutamatergic synaptic transmission was approximately 100-fold larger than that of the  $\mu$ -receptor selective agonist DAMGO. The maximum effect of DAMGO on EPSC amplitudes (reduction by 25–30%) was observed at a concentration of 1  $\mu$ M. Similar reductions in EPSC amplitudes were obtained at DADLE concentrations of approximately 0.01  $\mu$ M, and further decreases in the amplitudes (to a maximum of approximately 60%) were achieved after increases in the DADLE concentration. DADLE is known to activate  $\mu$ -receptors to some degree.<sup>16–18</sup> However, because maximum activation of





**Fig. 8.** DADLE did not affect the amplitude of membrane currents induced by glutamate applied microiontophoretically in the presence of TTX. (A) Membrane inward currents induced by glutamate application (1 s, bars indicate application period) in the absence (control) and presence of DADLE. Recordings were taken in the presence of TTX (0.6–1.2 μM). The membrane potential was clamped to -70 mV. DADLE did not affect the amplitude of the glutamate-induced currents (see superimposed recordings, right). (B) Summary of similar experiments performed in different neurons (n = 6). Absolute values of the holding current (upper) and the amplitudes of the glutamate-induced current (lower) were plotted as a function of recording time. In each neuron DADLE was applied for 15 min after a control period of 15 min. The opioid peptide did not affect the holding current and the amplitude of the glutamate-induced inward current. (C and D) Glutamate-induced inward current consisted of both an NMDA and an AMPA receptor-mediated component. This was demonstrated by the application of D-2-APV (C) and CNQX (D), respectively. Calibration in (D) corresponds also to recordings depicted in (C).

μ-receptors by DAMGO could hardly mimic the quantitative effect of DADLE, we conclude that the action of DADLE on EPSC amplitudes involved predominantly the activation of δ-receptors. This conclusion is substantiated by our observations that the effect of DADLE on EPSC amplitudes could be blocked by the δ-receptor selective antagonist naltrindole and that the δ-receptor selective agonist DPDPE mimicked the action of DADLE.

The high DAMGO concentrations necessary to elicit its depressant effect on excitatory transmission and the relative ineffectiveness of the μ-receptor agonist fentanyl suggest a low density of functional μ-receptors in layer II–III of the rat somatosensory cortex. However, in 9 of 13 neurons tested, DAMGO exerted a naltrexone-reversible depressant effect on EPSC amplitudes without detectable activation of a postsynaptic conductance. Thus, regular spiking neurons (presumably pyramidal cells) of this cortex area are not devoid of μ-receptors involved in the modulation of glutamatergic excitatory transmission. This finding is contradictory to a report on the action of

DAMGO on EPSPs recorded from layer V neurons of the rat anterior cingulate cortex.<sup>7</sup> In this study, μ-receptor activation induced hyperpolarizations in nonpyramidal neurons but did not affect evoked EPSPs recorded from pyramidal cells. This discrepancy might be explained by differences in the μ-receptor density in different areas of the cerebral cortex or by a cell type-specific distribution of these receptors.<sup>20</sup>

We used the single-electrode voltage-clamp technique to study the effects of opioids on AMPA- and NMDA receptor-mediated synaptic currents at a defined holding potential. The rationale behind this approach was to reveal possible different opioid actions on EPSCs mediated *via* different glutamate receptor subtypes and to exclude secondary effects of rectifying membrane conductances that might distort potential measurements.<sup>11</sup> We did not observe any qualitative or quantitative differences in the actions of DADLE on the AMPA-EPSC and on the first component of the NMDA-EPSC, respectively. Similarly, DAMGO depressed the amplitudes of AMPA-

and NMDA-EPSCs to the same extent. This suggests an influence of the opioid peptides on mechanisms involved in the generation of both types of EPSCs, for example, an influence on presynaptic glutamate release. The second, presumably polysynaptic component of the NMDA-EPSC was depressed by DADLE to a maximum of approximately 80%. This apparently higher efficiency of DADLE might be explained by the summation of the peptide's effects on the presynaptic terminals contributing to the polysynaptic response.

In addition to its depressant effects on EPSCs, DADLE, but not DAMGO, induced outward currents in approximately one third of all neurons tested. These currents were observed only at peptide concentrations that exerted the maximum reduction in the EPSC (0.1–1  $\mu\text{M}$ ). Furthermore, the amplitudes of the outward currents were rather small (maximum 40 pA) and faded during application of the peptide. Determinations of VI curves revealed a slight peptide-induced increase in slope conductance. Together with an estimation of the reversal potential (–95 to –100 mV), these data indicate that the small outward currents observed during application of DADLE resulted from the activation of a potassium conductance.

In a number of different brain areas, it has been shown that  $\delta$ -opioid receptor agonists hyperpolarize the membrane potential of neurons by activating a potassium conductance<sup>21–24</sup> that belongs to the family of inwardly rectifying currents.<sup>25,26</sup> Our study, as well as that of Tanaka and North,<sup>7</sup> revealed that only a fraction of neocortical neurons (30–40%) respond to  $\delta$ -receptor activation with the generation of outward currents. Ultrastructural analyses of the distribution of  $\delta$ -receptors on neocortical pyramidal neurons showed that these receptors are located predominantly on synaptic terminals that are in close contact to the dendrites of these cells.<sup>8</sup> Furthermore,  $\delta$ -receptors are constituents of the postsynaptic plasma membrane of pyramidal cell dendrites.<sup>8</sup> Voltage-clamp measurements in pyramidal neurons endowed with a complex dendritic tree are associated with considerable space-clamp errors.<sup>11,27</sup> Therefore, one might argue that the “lack” of a DADLE-induced outward current in the majority of neurons investigated resulted from poor space-clamp conditions, because a postsynaptic membrane current activated by the agonist at remote dendritic sites could not have been detected by somatic recordings. However, the attenuation of membrane voltages originating from remote dendritic sites is frequency dependent. Voltage changes induced by currents with fast kinetics are much more attenuated than those caused by slow currents or direct currents.<sup>27</sup> Thus, by clamping the neurons' membrane potential to –70 mV using long-lasting intracellular injections of direct currents, we should have been able to reliably resolve dendritic postsynaptic membrane currents induced by long-lasting (15-min) applications of DADLE. Therefore, on

the basis of this argument and in line with the study of Tanaka and North,<sup>7</sup> we conclude that only a fraction of neocortical neurons (30–40%) express functional postsynaptic  $\delta$ -receptors that are able to generate significant and detectable outward currents. In these neurons, the opioid-induced depression of EPSP-EPSC amplitudes might be explained by a dendritic shunt of these synaptic responses. However, to induce outward currents, it was necessary to apply DADLE at high concentrations. Half maximal depression of EPSC amplitudes occurred already at concentrations of 6–15 nM. We cannot exclude that shunting of EPSPs-EPSCs contributes to the depressant effect of DADLE elicited in a fraction of neurons by application of high concentrations. However, such a mechanism certainly cannot account for the DADLE-induced reductions of the EPSC that were observed at low peptide concentrations in all neurons investigated.

Because significant postsynaptic effects of the opioid peptides could be excluded, their depressant action on EPSC amplitudes might be explained either by a presynaptic inhibition of glutamate release or by a postsynaptic interaction between opioid receptors and glutamate receptors. Several investigators proposed such a postsynaptic interaction between the  $\mu$ -receptor and the NMDA receptor.<sup>9,28,29</sup> However, because, in our study, DADLE did not affect the inward currents induced by microiontophoretically applied glutamate in neurons functionally isolated from presynaptic input, a direct postsynaptic effect of DADLE on AMPA and NMDA receptors could be excluded.

Therefore, by analogy with studies performed in a variety of central nervous system neurons,<sup>30–35</sup> we conclude that activation of  $\mu$ - and  $\delta$ -opioid receptors depresses glutamatergic excitatory synaptic transmission in rat neocortical neurons almost exclusively by a presynaptic inhibition of glutamate release. This effect is probably mediated by an inhibition of presynaptic calcium channels.<sup>36,37</sup> Although we consider the opioid-activated postsynaptic membrane conductance to be of minor impact for the observed depressant effect on excitatory transmission, we must assume that this opioid-gated conductance serves a specific function that remains to be determined. The dendritic location of postsynaptic  $\delta$ -receptors and their association with asymmetric (*i.e.*, excitatory) synaptic junctions<sup>8</sup> suggest that these receptors might be involved in the local fine-tuning of the residual EPSP.

All types of opioid receptors ( $\mu$ ,  $\delta$ , and  $\kappa$ ), except the orphan receptor, have been shown to be involved in the generation of supraspinal analgesia.<sup>38</sup> However, “in most instances, the activation of  $\mu$ -receptors may explain the analgesic activity observed.”<sup>38</sup> We found a comparatively weak depressant action of the  $\mu$ -receptor selective agonists DAMGO and fentanyl on glutamatergic synaptic transmission in neocortical neurons. In another study on

the neocortex,<sup>7</sup> it has been reported that DAMGO inhibited nonpyramidal neurons, most probably inhibitory interneurons, by inducing potassium-dependent hyperpolarizations. Such an effect would result, similar to the hippocampus, in a disinhibition of neocortical activity. However, *in vivo* experiments demonstrated a powerful inhibition of single cell activity within the neocortex induced by  $\mu$ -receptor agonists.<sup>3,4</sup> Thus, the cellular functions of  $\mu$ -receptors in neocortical neurons remain to be determined.

Opioids used as supplements during general anesthesia are almost exclusively  $\mu$ -receptor-selective agonists (e.g., fentanyl) that act predominately on the lower part of the "nociceptive axis" to produce analgesia.<sup>38</sup> Higher concentrations of these drugs induce anesthesia associated with the occurrence of slow  $\delta$  waves during electroencephalogram recordings.<sup>2</sup> To our knowledge, no studies exist concerning the anesthetic potency of selective  $\delta$ -receptor agonists. From the cellular effects known so far, one might speculate that  $\delta$ -receptor agonists are equally potent to reduce consciousness as compared with  $\mu$ -receptor agonists. At least, they might potentiate the effects of volatile anesthetics. In a recent study,<sup>39</sup> we showed that the predominant effect of enflurane on neocortical neurons is a reduction in glutamatergic excitatory synaptic transmission, probably mediated *via* a presynaptic inhibition of glutamate release.<sup>40</sup> Thus, enflurane and  $\delta$ -receptor agonists act at the same target, suggesting the possibility of a mutual potentiation of their efficiency to reduce excitability of neocortical neurons.

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