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# Dynorphin A (1–13) Neurotoxicity *in Vitro:* Opioid and Non-Opioid Mechanisms in Mouse Spinal Cord Neurons

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**Dynorphin A is an endogenous opioid peptide that preferentially activates κ-opioid receptors and is antinociceptive at physiological concentrations. Levels of dynorphin A and a major metabolite, dynorphin A (1–13), increase significantly following spinal cord trauma and reportedly contribute to neurodegeneration associated with secondary injury. Interestingly, both κ-opioid and *N*-methyl-D-aspartate (NMDA) receptor antagonists can modulate dynorphin toxicity, suggesting that dynorphin is acting (directly or indirectly) through κ-opioid and/or NMDA receptor types. Despite these findings, few studies have systematically explored dynorphin toxicity at the cellular level in defined populations of neurons coexpressing κ-opioid and NMDA receptors. To address this question, we isolated populations of neurons enriched in both κ-opioid and NMDA receptors from embryonic mouse spinal cord and examined the effects of dynorphin A (1–13) on intracellular calcium concentration ($[Ca^{2+}]_i$) and neuronal survival *in vitro.* Time-lapse photography was used to repeatedly follow the same neurons before and during experimental treatments. At micromolar concentrations, dynorphin A (1–13) elevated $[Ca^{2+}]_i$ and caused a significant loss of neurons. The excitotoxic effects were prevented by MK-801 (Dizocilpine) (10 μM), 2-amino-5-phosphopentanoic acid (100 μM), or 7-chlorokynurenic acid (100 μM)—suggesting that dynorphin A (1–13) was acting (directly or indirectly) through NMDA receptors. In contrast, cotreatment with (−)-naloxone (3 μM), or the more selective κ-opioid receptor antagonist *nor*-binaltorphimine (3 μM), exacerbated dynorphin A (1–13)-induced neuronal loss; however, cell losses were not enhanced by the inactive stereoisomer (+)-naloxone (3 μM). Neuronal losses were not seen with exposure to the opioid antagonists alone (10 μM). Thus, opioid receptor blockade significantly increased toxicity, but only in the presence of excitotoxic levels of dynorphin. This provided indirect evidence that dynorphin also stimulates κ-opioid receptors and suggests that κ receptor activation may be moderately neuroprotective in the presence of an excitotoxic insult. Our findings suggest that dynorphin A (1–13) can have paradoxical effects on neuronal viability through both opioid and non-opioid (glutamatergic) receptor-mediated actions. Therefore, dynorphin A potentially modulates secondary neurodegeneration in the spinal cord through complex interactions involving multiple receptors and signaling pathways.** © 1999 Academic Press

***Key Words:*** **κ-opioid receptors, *N*-methyl-D-aspartate receptors; MK-801 (Dizocilpine); 2-amino-5-phosphopentanoic acid; 7-chlorokynurenic acid; *nor*-binaltorphimine; intracellular calcium; neurotoxicology; neuroprotection.**

## INTRODUCTION

Dynorphin A is an endogenous opioid neuropeptide and a major product of the preprodynorphin gene (14, 19). Although dynorphin and other related opioid peptides have a defined role as inhibitory neurotransmitters in pain reduction, there is growing evidence that exposure to high concentrations of dynorphin can induce hyperalgesia and allodynia and/or may contribute to neurodegeneration (21, 23, 27, 36, 56, 78, 79). Dynorphin A-derived peptides are significantly elevated following neurotrauma and sustained exposure to these peptides is neurotoxic (21, 23, 27, 56, 58, 78, 79).

In addition to their known actions as opioids, dynorphin A and dynorphin A (1–13) (a major bioactive product of dynorphin A) (19, 29) may impact secondary neuronal injury through non-opioid mechanisms (9–11, 18, 23, 45, 49, 56). Unlike other opioid peptides, opioid antagonists cannot block many of the effects of dynorphin. Intrathecal administration of low dosages of dynorphin A (1–13) (19, 29) causes opioid analgesia in tail-flick and hot-plate assays that can be prevented by opioid antagonists (45). In contrast, high dosages or sustained exposure to dynorphin A (1–13) causes lasting maladaptive changes to the spinal cord including hyperalgesia, allodynia, and even hindlimb paralysis (18, 21, 79). Because the deleterious effects of dynorphin can be prevented by MK-801, but are largely



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unaffected by opioid antagonists, these data suggest that opioid receptors mediate the antinociceptive actions, while *N*-methyl-D-aspartate (NMDA)–glutamatergic receptors mediate the negative effects (40, 79). It is important to note that motor impairment potentially confounds the measurement of pain in these studies (79). Findings that dynorphin activates NMDA receptors (NMDAR) directly, or indirectly by increasing excitatory amino acid levels (4, 20, 48, 49, 80), provide a rationale for the apparent non-opioid receptor-mediated aspects of dynorphin-induced hyperalgesia and allodynia, excitotoxicity, and exacerbated neural injury (9–11, 18, 20, 45, 49, 56, 79, 85).

To assess the role of dynorphin in neurodegeneration, we isolated neurons from the spinal cord of embryonic mice *in vitro* and examined the effects of dynorphin on intracellular calcium ($[Ca^{2+}]_i$) levels and neuronal survival. Importantly, these cultures were maintained under conditions that propagated enriched populations of κ-opioid and NMDAR1-expressing neurons. Evidence for functional κ-opioid and glutamate receptor phenotypes was confirmed immunocytochemically, pharmacologically, and by examining functional changes in $[Ca^{2+}]_i$. The findings show that at micromolar concentrations, dynorphin A (1–13) elevated $[Ca^{2+}]_i$ and caused a significant loss of neurons. These excitotoxic effects can be completely blocked by MK-801—suggesting that dynorphin A (1–13) was acting (directly or indirectly) through NMDA receptors. In contrast, the toxic effect of dynorphin was exacerbated by the opioid antagonist (−)-naloxone and by the selective κ receptor antagonist *nor*-binaltorphimine (*nor*-BNI), but not by (+)-naloxone, an inactive stereoisomer, providing indirect evidence that κ-opioid receptor activation is neuroprotective. These initial results suggest that dynorphin A (1–13) affects neuronal viability through both opioid and non-opioid mechanisms. The toxic effects of dynorphin are mediated by glutamatergic receptors and override the potential beneficial actions at opioid receptors.

## METHODS

Pregnant ICR mice (Harlan Sprague–Dawley, Indianapolis, IN) were anesthetized with ether and euthanized by cardiac puncture on gestational day (E) 14. Fetuses were removed aseptically by cesarean section and the spinal cords dissected. The spinal cords from E14 mice were considered sexually undifferentiated and bipotent (35).

Following removal of the meninges and dorsal root ganglia, spinal cords were minced into 1-$mm^3$ fragments and placed into DMEM containing 0.25% trypsin (Difco, 1:250, Detroit, MI) and 0.01% DNase for 15 min at 35°C. After gentle centrifugation (190*g*, 5 min), the supernatant was discarded and the tissue pellet was suspended in serum-containing basal media and triturated through a flame-polished, fine-bore Pasteur pipette. Cells were centrifuged (190*g*, 3 min) and resuspended in fresh growth media. Defined cell culture medium consisted of Neurobasal medium (Gibco/Life Technologies, Grand Island, NY) supplemented with B27 (2% v/v; Gibco/Life Technologies), L-glutamine (0.5 mM), and gentamicin (10 μg/ml). Spinal cord neurons were also initially supplemented with 25 μM L-glutamate on days 0–2 *in vitro* and 12.5 μM L-glutamate on days 3–4 *in vitro*. B27 is a medium supplement consisting of vitamins, hormones, essential fatty acids, and minimal glutathione (6) and is utilized at very low concentrations. The cell culture medium approximates physiological concentrations of $Mg^{2+}$ and glycine (1 μM), which are important for NMDA receptor function (42, 75). Cells were plated at low density ($20 \times 10^5$ cells/$cm^2$) unto poly-D-lysine-coated glass-bottom dishes (MatTek, Natick, MA) or on glass coverslips that were inserted into multiwell plastic chambers. The culture conditions favored the growth and survival of neurons from the dorsal spinal cord, but not the ventral cord. Muscle or muscle-derived trophic factors (35, 66, 72, 81), as well as other centrally derived trophic factors (65), essential for maintaining motoneurons, were not provided. Serum was not used because of the potentially confounding effects of the presence of endogenous opioids and excitatory amino acids (e.g., 93). Cell cultures were continuously exposed to dynorphin A (1–13) and/or NMDA agonists and/or antagonists and assessed following 0–96 h. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), except where noted. (−)-Naloxone and (+)-naloxone were obtained from E. I. du Pont Co. (Wilmington, DE), and 2-amino-5-phosphopentanoic acid (AP-5), 7-chlorokynurenic acid, and *nor*-BNI were obtained from Research Biochemicals International (Natick, MA).

Spinal cord neurons were characterized both morphologically and immunocytochemically by using rabbit antibodies directed against human neuronal ubiquitin (PGP 9.5; Ultraclone, Cambridge, UK; 1:1200 dilution) (91), MAP-2 (AP20 clone; 1:1000 dilution of their stock) (84), and neurofilaments. Neurofilaments were detected using $IgG_1$ monoclonal antibodies directed against neurofilaments (MU073; Biogenex Labs., San Ramon, CA; 1:150 dilution of their stock) (MAP-2 immunoreactive neurons are shown). Neurons were further characterized as to phenotype using rabbit anti-KOR1 polyclonal antibodies against the κ-opioid receptor (1) (1:2500 dilution) and $IgG_{2a}$ monoclonal antibodies against the NMDAR1 receptor (Chemicon International, Inc., Temecula, CA; 0.5 μg/ml working dilution) (68). Cultures were incubated in diluted primary antibodies at 4°C on an orbital shaker (40–60 rpm) for 24 h. Appropriate secondary biotinylated antibodies conjugated to avidin-peroxidase were used

as directed (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) to detect primary antibodies. Nickel-intensified diaminobenzidine (DAB) consisting of 2.5% nickel ammonium sulfate, 0.35% DAB, and 0.012% $H_2O_2$ in 0.1 M sodium acetate (pH 6.0) was used as a substrate for peroxidase. Goat anti-rabbit IgG second antibodies conjugated to Cy2 at 1:250 dilution (Amersham) were used to detect KOR1 antibodies, while donkey anti-mouse $IgG_2$ antibodies conjugated to Cy3 at 1:250 dilution were used to detect NMDAR1 antibodies by immunofluorescence. Fluorescent cells were mounted in Prolong Antifade (Molecular Probes, Eugene, OR). Preabsorbed controls (when possible) and controls lacking primary antiserum were included to ensure specificity of the reaction, as well as to confirm the absence of cross-reactivity in the dual-labeling studies.

$[Ca^{2+}]_i$ was measured in individual cells by ratiometric fluorescence imaging using fura-2, as previously described (32). Briefly, cells were loaded with 10 μM fura-2/AM (Molecular Probes) for 45 min at 35–37°C in growth medium containing 25 mM Hepes and 3% DMSO. Cells were rinsed and incubated for 30 min in growth medium to permit complete hydrolysis of the fura-2/AM. An MCID M4 imaging system (Imaging Research, Inc., St. Catharines, Ontario) was used to acquire and process the images. $[Ca^{2+}]_i$ was determined by exciting fura-2 at 340 and 380 nm wavelengths and analyzing the ratio of emitted fluorescence intensity at 590 nm. About 25–35 neurons were sampled in each culture and mean $[Ca^{2+}]_i$ levels were determined from at least three cultures. Each culture consisted of independent samples of cells from separate mice.

Neuronal viability was assessed by repeatedly photographing the same neurons at 24-h intervals. Glass coverslips were lightly scored with a diamond marker, before being placed into 12-well plates, to aid in finding the same field of neurons (neurons were plated on the side opposite to the score). Neurons were photographed using a Nikon Diaphot inverted microscope with phase-contrast optics and 20× objective and counted as previously described (52). Neuron viability was additionally assessed using a commercially available kit (Live/Dead Cytotoxicity Kit; Molecular Probes) as previously described (64). Cultures were rinsed three times with Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL) and incubated for 40 min at 34–35°C in DPBS containing ethidium homodimer and calcein-AM as previously described (64). Ethidium binds DNA in dead neurons and emits red fluorescence, while calcein-AM is converted by esterase within living cells and imparts a green fluorescence.

The effects of pharmacological treatments on mean $[Ca^{2+}]_i$ and neuronal viability (using the Live/Dead assay) were assessed statistically using one-way ANOVA. The effects of pharmacological treatments on neuronal survival were assessed repetitively in the same neurons using a two-way repeated-measures ANOVA design (treatment versus time), and the data are presented as the percentage of change in cell number compared to pretreatment values. When significant treatment effects were observed using ANOVA, post hoc comparisons of individual group differences were performed using Duncan's test. Statistical analyses were performed using Statistica (StatSoft, Tulsa, OK). Data are presented as the means ± the standard errors of the mean (SEM).

## RESULTS

At 7–10 days in culture, large numbers of small, multipolar cells were present that displayed a neuronal phenotype and possessed MAP-2 immunoreactivity (Figs. 1A and 1B). Further characterization of this morphologically distinct population of multipolar neurons indicated that 79.8 ± 4.3% of these neurons displayed κ (KOR1), while 92.6 ± 4.1% possessed NMDAR1 immunoreactivity (Figs. 1B, 1C, and 2A–2F). Double-labeling studies demonstrated that 71.0 ± 0.8% of the same neurons possessed both κ-opioid and NMDAR1 receptor immunofluorescence (Figs. 2A–2F). Subsequent characterizations of dynorphin toxicity were conducted in this distinct neuronal population.

Dynorphin A (1–13) exposure (33 μM) caused a significant loss in neuronal viability at 4 h (Figs. 2G–2I) with a visible destruction in neuronal morphology seen at 16 h (not shown). The loss in viability seen with dynorphin was similar to that observed with NMDA exposure and was prevented by concurrent treatment with MK-801 (Fig. 2I).

Exposure to dynorphin A (1–13) caused acute increases in $[Ca^{2+}]_i$ in individual neurons similar to increases seen with acute NMDA treatment (Fig. 3A). When $[Ca^{2+}]_i$ was measured in neuronal populations at 2 h following continuous exposure, dynorphin caused significant increases in mean $[Ca^{2+}]_i$ that were concentration dependent (Fig. 3B) and prevented by coadministrating MK-801, but only partially attenuated by concurrent treatment with (−)-naloxone (Fig. 3C). Significant increases in mean $[Ca^{2+}]_i$ occurred following exposure to ≥10 μM concentrations of dynorphin A (1–13) at 2 h. Mean $[Ca^{2+}]_i$ in untreated neurons was 162 ± 16 nM.

Continuous exposure to dynorphin A (1–13) (100 μM) caused a significant loss of neurons over time (Figs. 4 and 5). The toxic effects of dynorphin were selective and could be prevented by coadministering the NMDA receptor antagonist MK-801 (10 μM) (Dyn/MK801), but were not prevented by the opioid antagonist (−)-naloxone (3 μM). At an equimolar concentration (100

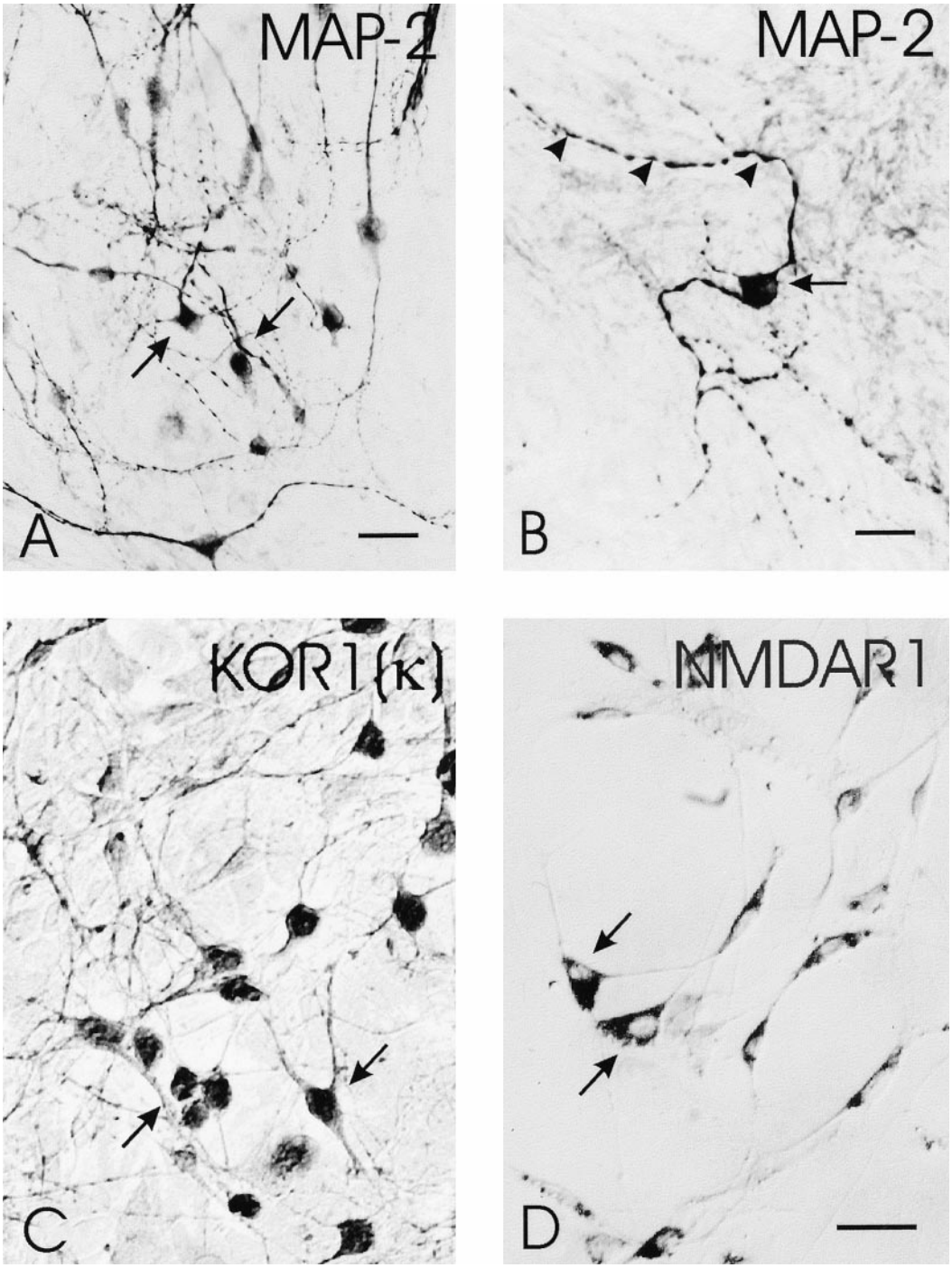



**FIG. 1.** Bright-field photomicrographs of cells from spinal cord cultures at 7 days *in vitro*. A majority of the cells in our cultures were small, multipolar neurons and displayed MAP-2 (arrows) (A,B), κ-opioid receptor (KOR1) (arrows) (C), and NMDAR1 (arrows) (D) immunoreactivity. The arrowheads in B indicate MAP-2-immunoreactive neurites. Scale bar in A, 20 μm; B, 10 μm; and D, 15 μm; C and D are the same magnification.

μM), dynorphin A (1–13) was less toxic than NMDA (Fig. 5). Interestingly, exposure to the opioid antagonist (−)-naloxone (3 μM) exacerbated the toxic effect of dynorphin ($P < 0.05$; Dyn/(−)Nal vs dynorphin) (Fig. 5A). The enhanced toxicity of (−)-naloxone was selective and mediated through specific opioid receptors, because the inactive stereoisomer, (+)-naloxone (3 μM), had no effect on dynorphin toxicity (Dyn/(+)Nal). Importantly, neither (−)-naloxone (3 μM) nor MK-801 (10 μM) alone affected neuron survival (Fig. 5).

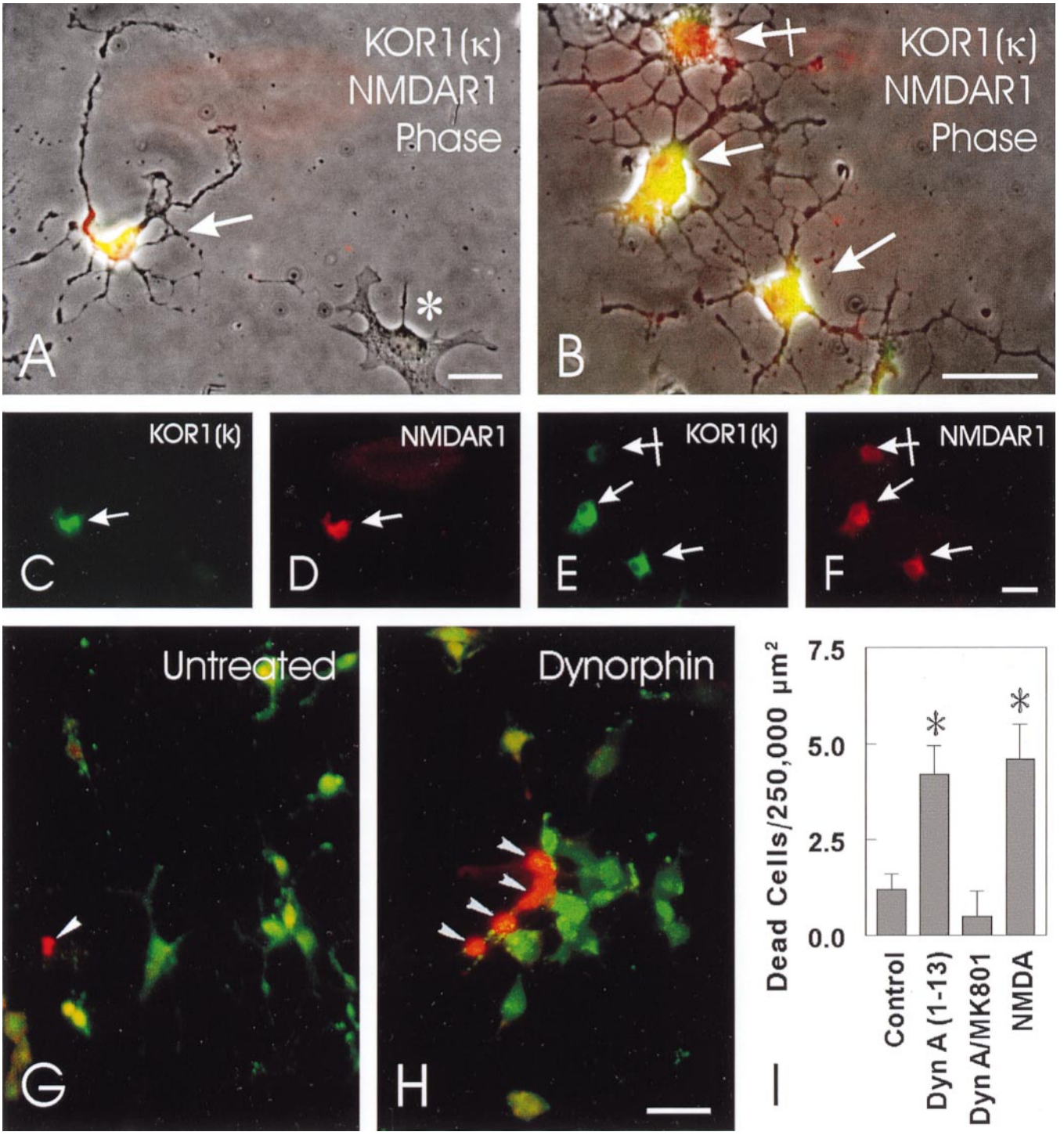



**FIG. 2.** (A–F) Photomicrographs of κ-opioid- and NMDAR1-immunofluorescent spinal cord neurons at 7 days *in vitro* (A–F). Many neurons displayed κ (KOR1)-opioid (Cy2) (C,E) and/or NMDAR1 (Cy3) (D,F) receptor immunoreactivity. 71.0 ± 0.8% of the same neurons were immunopositive for both receptors, as indicated by the arrows, while some apparently NMDAR-positive cells failed to display κ receptor immunofluorescence above background (hatched arrow; asterisk shows an unlabeled astrocyte). (G–I) Effect of dynorphin A (1–13) and/or NMDA on neuronal viability. Fluorescent photomicrographs show living (green calcein fluorescence) and nonviable cells (red ethidium fluorescence, arrowheads) in untreated (G) and dynorphin-exposed (H) neurons. Dynorphin exposure (33 μM) caused a significant loss in neuronal viability at 4 h. The initial loss in viability seen with dynorphin was similar to that observed with NMDA (100 μM) exposure and was prevented by concurrent treatment with MK-801 (10 μM) (I). *$P < 0.05$ vs untreated controls (Duncan's test); $n = 5$ experiments. Scale bar in A, 20 μm; B, F, and H, 15 μm; C–F and G–H are the same magnifications.

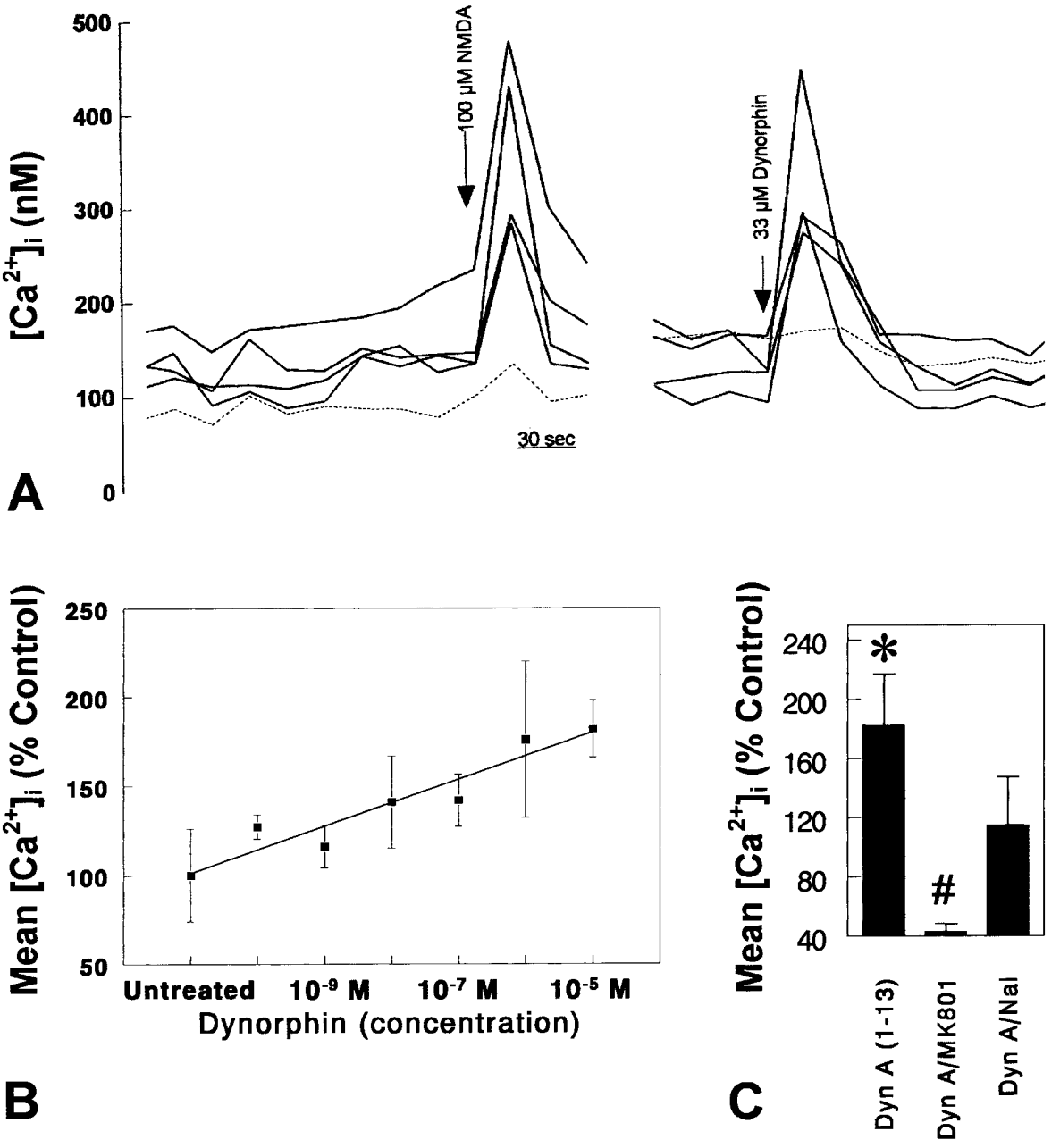
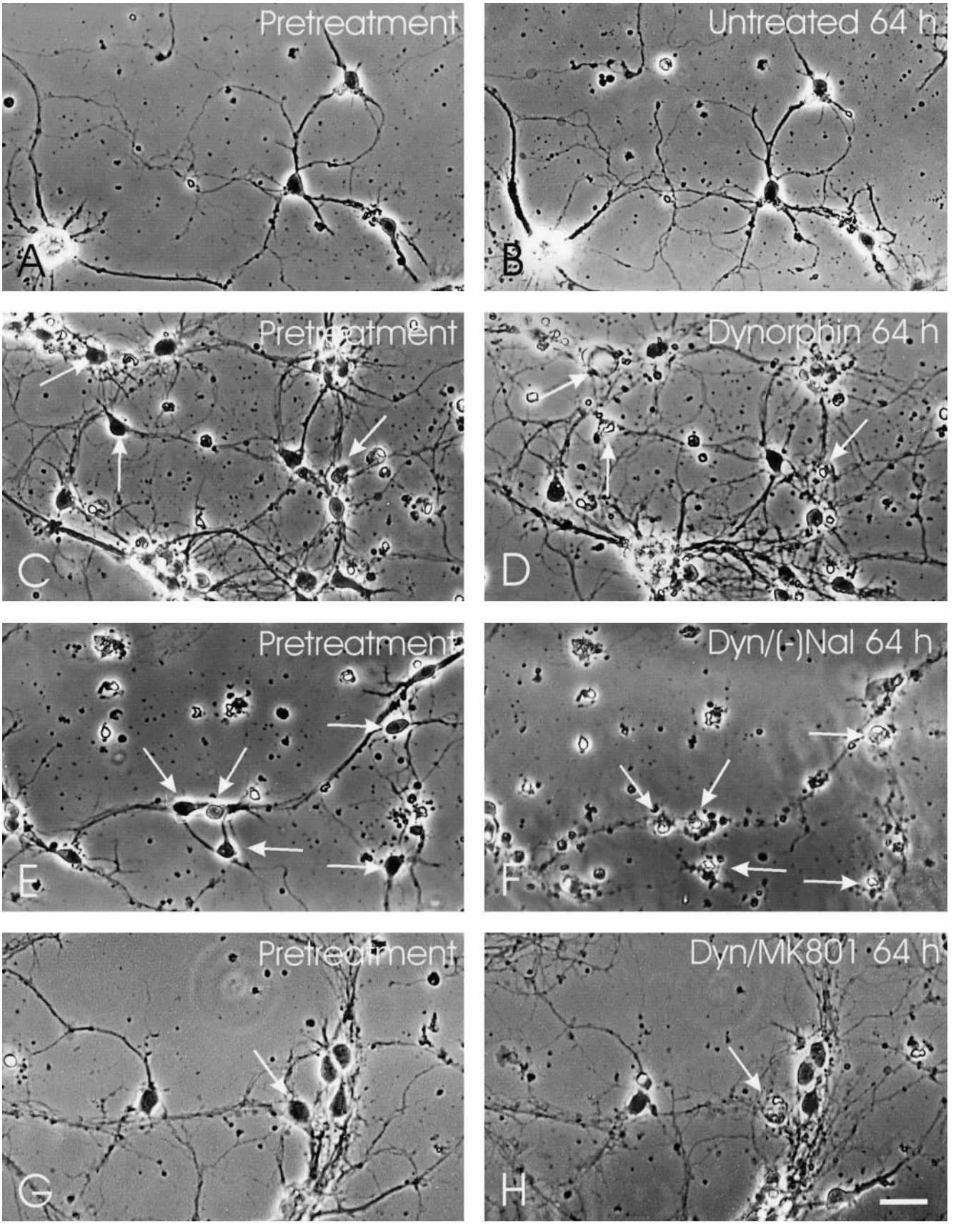



**FIG. 3.** Dynorphin A (1–13) increases intracellular calcium concentration ($[Ca^{2+}]_i$) in spinal cord neurons (A–C). Dynorphin A (1–13) caused acute increases in $[Ca^{2+}]_i$ in individual neurons that were similar to those from treatment with NMDA (A). In populations of spinal cord neurons, continuous exposure for 2 h in dynorphin A (1–13) caused significant concentration-dependent increases in mean $[Ca^{2+}]_i$ (B) (mean ± SEM from $n = 4$ experiments). Dynorphin A (1–13)-induced (Dyn A; 33 μM) elevations in $[Ca^{2+}]_i$ were blocked by the NMDA antagonist MK-801 (10 μM) and attenuated by (−)-naloxone (Nal; 100 μM) (C) (means ± SEM from $n = 5$ experiments). $^*P < 0.05$ vs dynorphin plus MK-801 or untreated neurons, $^{\#}P < 0.05$ vs dynorphin or dynorphin plus (−)-naloxone-treated neurons (Duncan's test).

Coadministering the selective κ-opioid receptor antagonist, *nor*-binaltorphimine (3 μM), significantly accelerated dynorphin-induced (10 μM) neuronal losses at 48 h (Fig. 6B), compared to 10 μM dynorphin treatment alone or pretreatment numbers ($^{\#}P < 0.05$) (Fig. 6B). Coadministering (−)-naloxone (3 μM) + dynorphin (10 μM) tended to reduce the number of neurons at 24 h compared to dynorphin alone, although

**FIG. 4.** Bright-field photomicrographs of neurons before (A,C,E,G) and after treatment with dynorphin A (1–13) and opioid or NMDA antagonists (B, D, F, H). Dynorphin A (1–13) (100 μM) caused neuronal losses at 64 h (D), compared to untreated controls which showed no significant neuronal loss (B). Dynorphin toxicity was prevented by coadministering MK-801 (10 μM) (H), while cotreating with (−)-naloxone (3 μM) enhanced dynorphin toxicity (F). The arrows show examples of degenerating neurons.


Pretreatment
A
Untreated 64 h
B
Pretreatment
C
Dynorphin 64 h
D
Pretreatment
E
Dyn/(-)Nal 64 h
F
Pretreatment
G
Dyn/MK801 64 h
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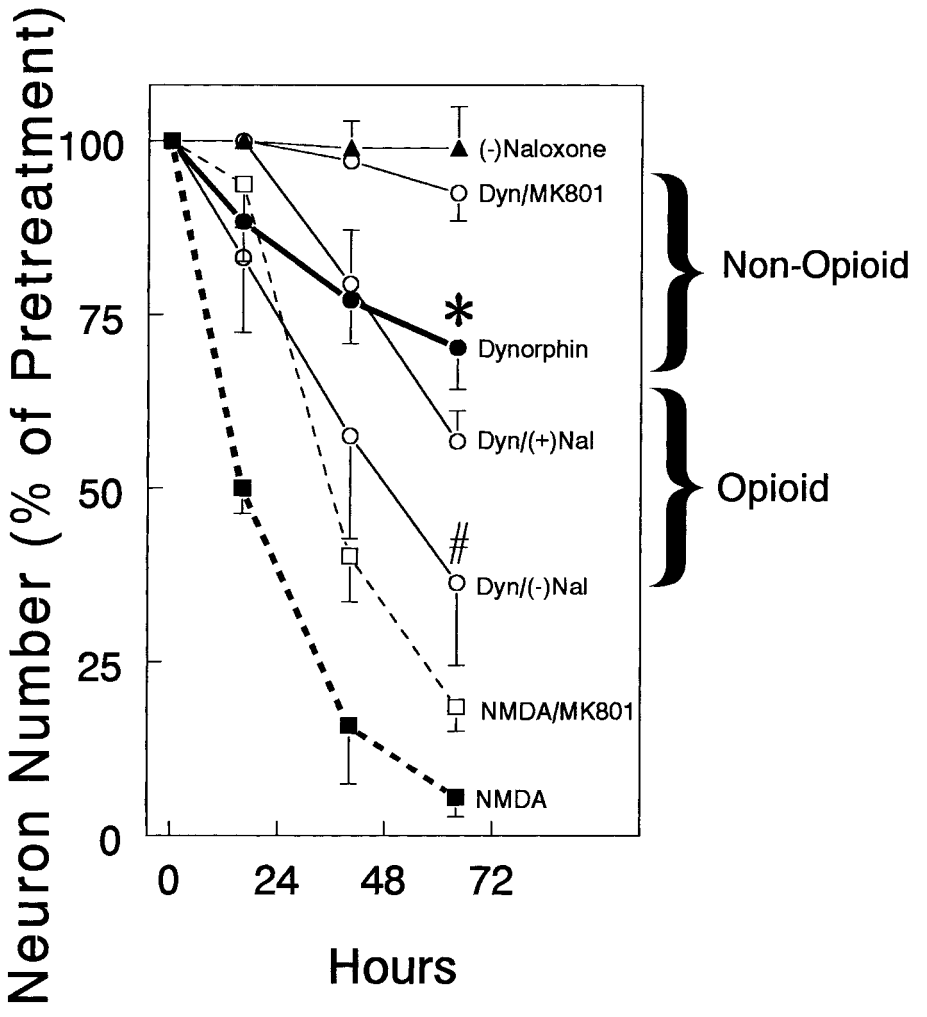


**FIG. 5.** Effect of dynorphin A (1–13) (100 μM) exposure on spinal cord neuronal numbers. Dynorphin caused a significant loss of neurons ($^{*}P < 0.05$ vs untreated controls) that was prevented by coadministering the non-opioid NMDA antagonist MK-801 (10 μM) (Dyn/MK801). In contrast, the opioid antagonist, (−)-naloxone (3 μM), markedly exacerbated dynorphin-induced neuronal losses (Dyn/(−)Nal) ($^{\#}P < 0.05$ vs non-NMDA-treated groups), while the inactive stereoisomer, (+)-naloxone (3 μM), did not affect dynorphin toxicity (Dyn/(+)Nal) (A). $^{*}P < 0.05$ vs pretreatment values (Duncan's post hoc test); $n = 5$ experiments.

this effect was not significant ($P = 0.069$). Higher (10 μM) concentrations of *nor*-BNI or (−)-naloxone did not affect neuronal viability, suggesting that the heightened toxicity of combining dynorphin with naloxone or *nor*-BNI did not result from the nonspecific effect combining two agents each at threshold concentrations for toxicity (Figs. 6A and 6B). Dynorphin A (1–13) caused significant concentration-dependent decreases in the number of neurons at 24 or 48 h following exposure (Figs. 6C and 6D). Dynorphin-induced (100 μM) neuronal losses were significantly attenuated by coadministering AP-5 (100 μM) or 7-chlorokynurenic acid (100 μM) (Figs. 6C and 6D).

## DISCUSSION

The results support the hypothesis that dynorphin affects the viability of spinal cord neurons through actions at both κ-opioid and NMDA receptors. Our findings indicate that the effects of dynorphin A (1–13) at the cellular level are complex. At nanomolar concentrations, dynorphin is known to activate κ-opioid receptors (51, 82, 92). The results of the present study additionally suggest that κ-receptor activation may impart a neuroprotective effect in the presence of an excitotoxic insult—based on circumstantial evidence that κ-opioid receptor blockade intensifies dynorphin toxicity. Alternatively, at micromolar concentrations dynorphin is excitotoxic. The toxic effects are accompanied by NMDA receptor activation, functional increases in $[Ca^{2+}]_i$, and subsequent neuronal death that can be prevented by cotreating with MK-801, AP-5, or 7-chlorokynurenic acid. Although not all investigators have found dynorphin to be toxic to spinal cord neurons *in vitro* (50), we assessed the effects of dynorphin in a phenotypically defined subset of neurons that coex-

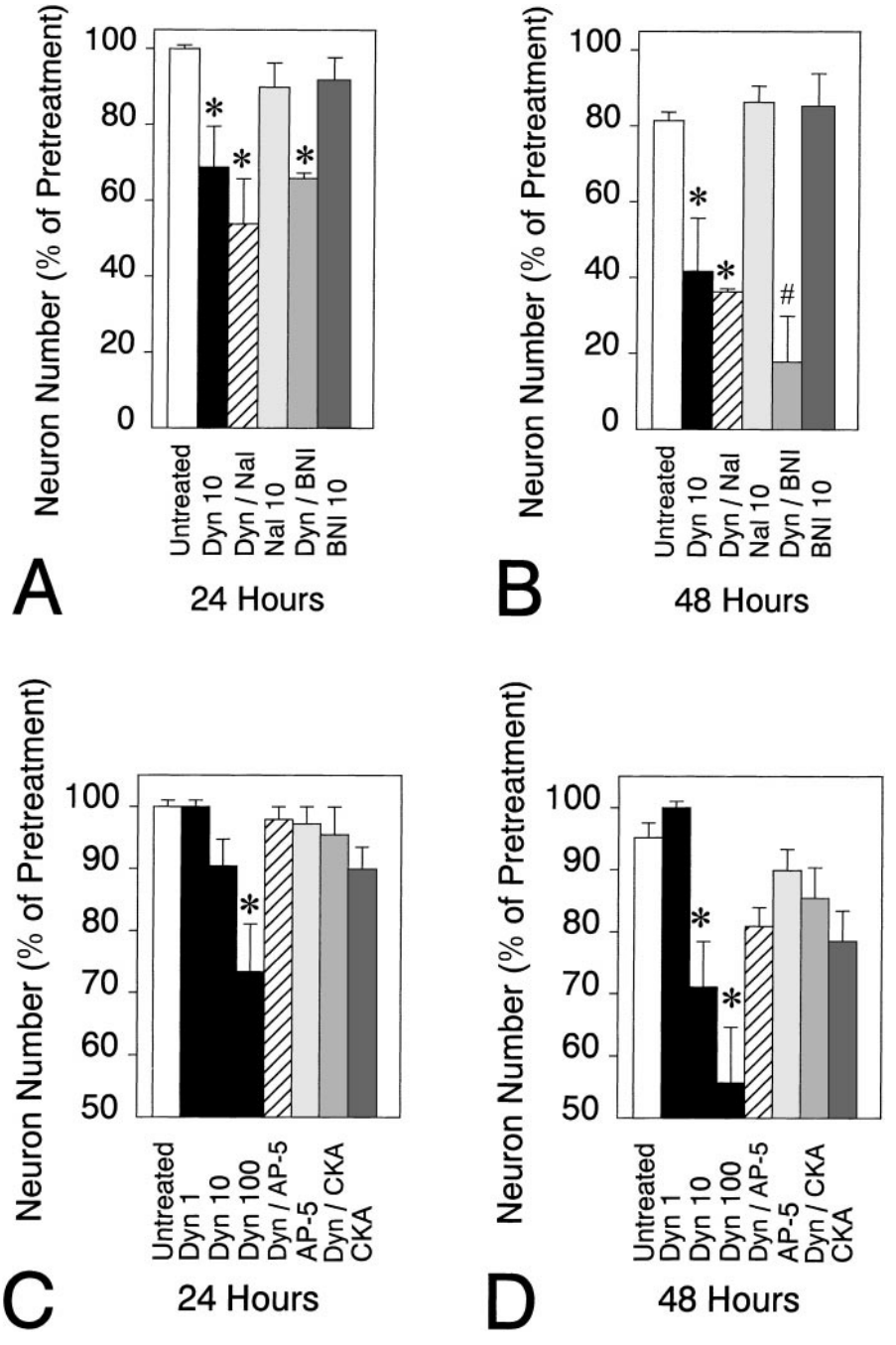


**FIG. 6.** Effects of opioid or NMDA receptor antagonists on dynorphin A (1–13) neurotoxicity (A–D). (A, B) Dynorphin (10 μM) exposure significantly reduced the number of neurons at 24 (A) or 48 h (B) following exposure. Coadministering the selective κ-opioid receptor antagonist, *nor*-BNI (3 μM) (Dyn/BNI), further exacerbated the dynorphin-induced (10 μM) neuronal losses at 48 h (B), compared to dynorphin (10 μM) alone or pretreatment values ($^{\#}P < 0.05$) (note the difference in *y*-axis scale, A–B vs C–D). Coadministering the less selective κ antagonist, (−)-naloxone (3 μM), and dynorphin (10 μM) (Dyn/Nal) tended to reduce the number of neurons compared to dynorphin alone, although the effect was not significant. Higher (10 μM) concentrations of *nor*-BNI (BNI 10) or (−)-naloxone (Nal 10) alone did not affect neuronal viability (Duncan's test); $n = 3$ experiments. (C,D) Dynorphin A (1–13) caused significant concentration-dependent decreases in neuron numbers at 24 (C) or 48 h (D), and 2-amino-5-phosphopentanoic acid (100 μM) (AP-5) or 7-chlorokynurenic acid (100 μM) (CKA) markedly attenuated dynorphin (100 μM) neurotoxicity (C and D). Note at threshold-toxic concentrations (10 μM), dynorphin displayed variable effects at 24 h. $^{*}P < 0.05$ vs pretreatment values; 1 μM dynorphin (Dyn 1), 10 μM dynorphin (Dyn 10), 100 μM dynorphin (Dyn 100).

pressed κ-opioid and NMDA receptors using a sensitive, repeated-measures design. Whether dynorphin directly affects NMDA receptors cannot be determined from the results of the present study, and both direct and indirect (via release of glutamate) mechanisms have been proposed by other investigators. Thus, dynorphin potentially serves independent functions through actions at κ-opioid and NMDA receptors (Fig. 7).

Excitotoxicity refers to a mechanism of neuronal injury involving excessive activation of particular glutamate receptors, which often accompanies metabolic compromise (12, 13, 52, 54, 76). The disruption of $[Ca^{2+}]_i$ homeostasis is an intrinsic feature in excitotoxicity, and $Ca^{2+}$ entry through NMDA receptor ion channels is a key destabilizing event (13, 53, 67). Findings that dynorphin A (1–13) elevates $[Ca^{2+}]_i$ and causes a subsequent loss of neuronal viability suggest that dynorphin is excitotoxic. Moreover, the ability of MK-801 to attenuate dynorphin-induced losses in $Ca^{2+}$ homeostasis and in neuronal numbers, and failure of opioid antagonists to prevent such changes, indicates that the excitotoxic effects are mediated through a glutamatergic mechanism.

The neurotoxic effects of dynorphin appear to be mediated predominately through NMDA receptors. This contrasts with the known actions of dynorphin as a preferential endogenous κ-opioid receptor agonist (8), with some dynorphin-derived peptide fragments cross-reacting at alternative μ- and/or δ-opioid receptors at high concentrations (19, 28). However, our experiments indicate that the toxic effects of dynorphin are not mediated by κ-opioid receptors, because opioid antagonists fail to block dynorphin toxicity and because high concentrations of selective κ agonists, such as U69,593 or U50,488H, which have no intrinsic affinity for NMDA receptors, fail to mimic the toxic effects of dynorphin (in preparation). In contrast, the present findings suggest that dynorphin is killing neurons through a glutamatergic mechanism. Equivalent concentrations of dynorphin mimicked NMDA-induced increases in $[Ca^{2+}]_i$ and cell death, and dynorphin toxicity was blocked by MK-801, AP-5, or 7-chlorokynurenic acid. Despite similarities to NMDA, equimolar concentrations of dynorphin A (1–13) were less toxic. Without knowing how dynorphin interacts with the NMDA receptor complex, it is not possible to speculate whether the diminished response results from pharmacokinetic differences at the glutamate site or dynorphin acting as a coagonist at glycine or polyamine sites (7, 41, 95). In addition, dynorphin A (1–13) may not be the peptide fragment doing the damage. Importantly, we have found other fragments of dynorphin A, such as dynorphin A (1–17), to be at least 10-fold significantly more toxic than dynorphin A (1–13) in our *in vitro* model (in preparation). Although our experimental model did not permit us to discern whether dynorphin is acting directly or indirectly (via opioid-induced release of glutamate), the quantities of glutamate released in our low-density cultures are likely to be rapidly diluted in the surrounding medium. Moreover, dynorphin has been shown to have the opposite effect and to inhibit glutamate release by hippocampal mossy fiber synaptosomes (25). Finally, it is noteworthy that (−)-naloxone treatment reduced dynorphin A (1–13)-induced increases in $[Ca^{2+}]_i$ at 2 h (Fig. 3C). We anticipated that (−)-naloxone would increase dynorphin A (1–13)-induced loss of $[Ca^{2+}]_i$ homeostasis, since (−)-naloxone and/or *nor*-BNI typically enhanced dynorphin-induced neurotoxicity after about 24 h following exposure. It was assumed that the exaggerated neuronal losses would coincide with an exaggerated loss in $Ca^{2+}$ homeostasis. A potential explanation is that the attenuation of dynorphin-induced increases in $[Ca^{2+}]_i$ by (−)-naloxone is short-lived, and $[Ca^{2+}]_i$ levels need to be sampled at multiple times to accurately describe the $Ca^{2+}$ response.

Dynorphin has long been suspected of having dual actions (46, 48, 50, 87–89). As mentioned, there has been consistent evidence that, in addition to its role as an opioid, dynorphin has potent non-opioid actions that are mediated by glutamatergic receptors (21, 23, 46, 48, 50, 56, 79, 87–89). Glutamate antagonists attenuate the deleterious effects of dynorphin in experimental models of hyperalgesia and/or spinal cord injury, and dynorphin fragments lacking the amino-terminal tyrosine necessary for opioid activity remain toxic (21, 23, 27, 44, 46, 48, 50, 56, 78, 79) and have a longer half-life *in vivo* and *in vitro* (94). The dichotomous actions at κ-opioid and NMDA receptors may be intrinsic features of the dynorphin A peptide itself (87) or dynorphin may induce glutamate release and/or accumulation during injury (50, 62). More recently, Huang and co-workers have provided strong evidence that dynorphin per se directly interacts with NMDA receptors in isolated single-channel and whole-cell patch-clamp studies (9, 10, 44). Porreca and others have provided further evidence that dynorphin A-derived peptides can directly interact with NMDA receptors in isolated membrane preparations and in HEK 293 cells transfected with NMDAR1/NMDAR2A subunit complexes (71). As mentioned, it has also been proposed that dynorphin may act at the glycine and/or polyamine sites on the NMDA receptor complex (7, 41, 95). Dynorphin A (1–13) can increase NMDA-activated currents despite very low extracellular glycine concentrations, and antagonists of the NMDA receptor glycine site can attenuate the effects (41, 95). Collectively, the above evidence and our present findings suggest that dynorphin may be excitotoxic through a direct action on NMDA receptors.

Our finding that κ-opioid receptor antagonists exacerbated neuronal losses provides indirect evidence that κ-opioid receptor stimulation may be neuroprotective. This idea has been proposed before (26, 38, 73, 77, 83,

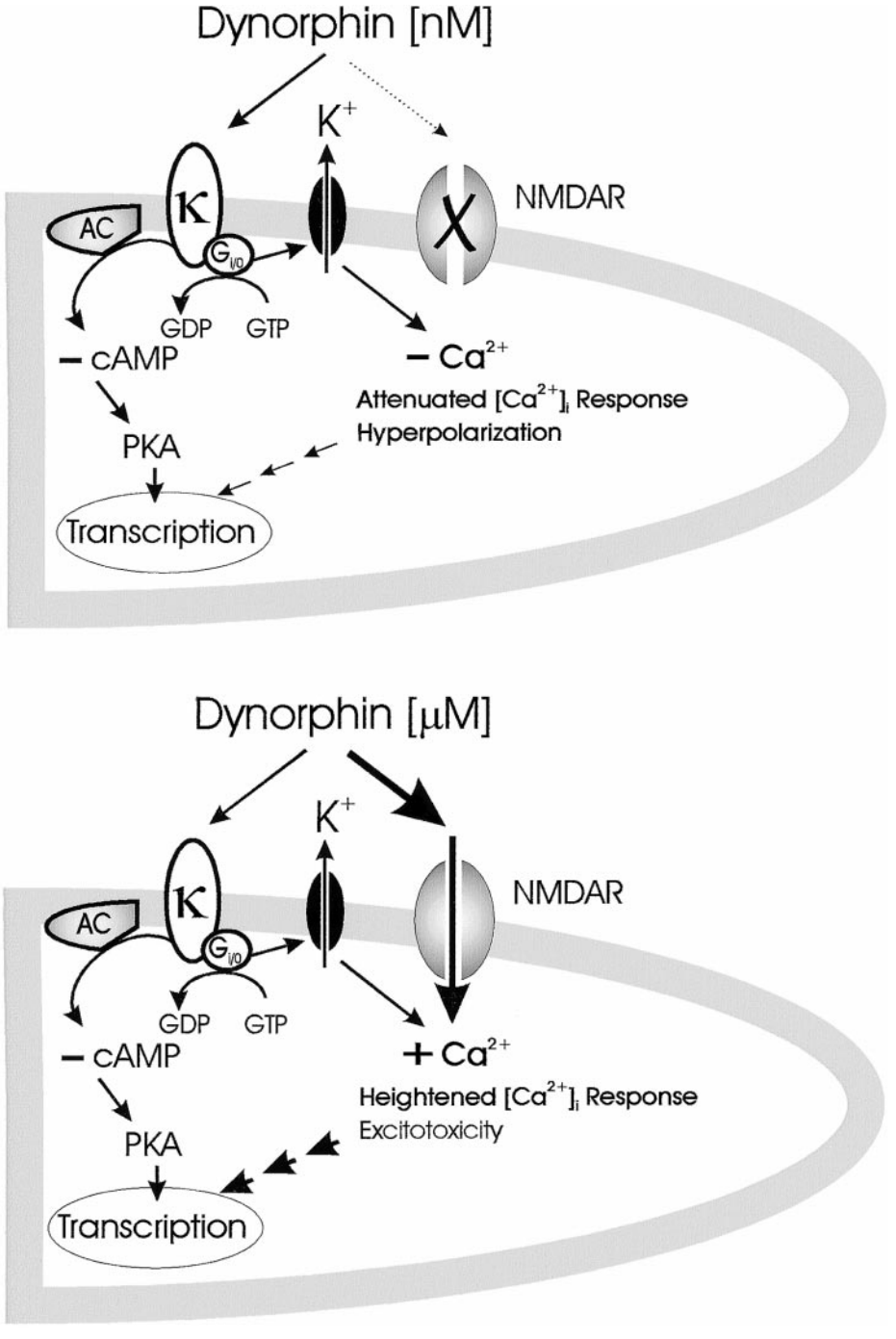


**FIG. 7.** Hypothetical model for dynorphin action. At nanomolar concentrations, dynorphin A typically acts as an inhibitory opioid peptide and preferentially activates κ-opioid receptors (κ) (37, 51, 82, 92). κ-Opioid receptors, via coupling through specific G proteins ($G_{i/o}$), typically inhibit adenylate cyclase (AC) and open potassium channels ($K^+$), which reduces cyclic AMP (cAMP) levels and hyperpolarizes the neuron. This may reduce free intracellular calcium levels ($[Ca^{2+}]_i$) and buffer $Ca^{2+}$ transients. During neural injury, dynorphin A-derived peptides accumulate and concentrations within the confined extracellular space of the CNS increase (57, 79). At micromolar concentrations, dynorphin additionally activates NMDA receptors (NMDAR), causing $Ca^{2+}$ influx. Sustained exposure to dynorphin A (1–13) causes excitotoxic injury that is accompanied by NMDA receptor stimulation, an overriding loss of $[Ca^{2+}]_i$ homeostasis, and neuronal death. Key: protein kinase A (PKA), guanosine triphosphate (GTP), and guanosine diphosphate (GDP).

86), but has not been well defined at the cellular level. Although the mechanisms underlying the putative neuroprotective effects of κ receptor activation are uncertain, several possible explanations are proposed. The activation of postsynaptic κ-opioid receptors typically inhibits the electrical activity of neurons by closing voltage-dependent N-type $Ca^{2+}$ channels (51, 82, 92). Hyperpolarizing the neuronal membrane is likely to reduce $[Ca^{2+}]_i$ and increase the excitotoxic threshold (31). Selective κ receptor agonists (e.g., U50,488H) attenuate both the release of glutamate and increases in $[Ca^{2+}]_i$ in synaptosomes in response to $K^+$-induced membrane depolarization (24, 25). Although κ receptor-mediated reductions in $[Ca^{2+}]_i$ may result from decreased activity of voltage-dependent $Ca^{2+}$ channels, κ stimulation may also increase $Ca^{2+}$ efflux (63) and/or modulate the organization of NMDA-induced oscillations in $[Ca^{2+}]_i$ (31). In addition, dynorphin A has been shown to hyperpolarize membrane potential by decreasing voltage-dependent $K^+$ currents ($K_M$ currents) (61). Dynorphin A similarly reduces the sensitivity of dorsal horn neurons to excitatory amino acids through modulation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptors (43). Chen and Huang have shown that some of the effects of dynorphin on NMDA receptor channel opening can be partially or completely blocked by opioid antagonists (9–11). In addition to a direct action on NMDA receptors, dynorphin acting via κ-opioid receptors may also affect AMPA receptor function through heterologous interactions (43). It is important to emphasize that, in all the above experiments, κ agonist actions were prevented or significantly attenuated by κ-opioid antagonists, indicating that these effects are mediated by opioid receptors. Finally, Bakalkin and co-workers have presented preliminary evidence that prodynorphin peptide precursors can enter the cell nucleus and directly affect cell viability by activating the Yin-Yang 1 protooncogene and p53 tumor suppressor protein (2, 3). Although the effects in the nucleus are seemingly distant from effects of dynorphin at plasma membrane receptors, collectively these findings suggest that dynorphin regulates cellular function at many levels. Irrespective of whether κ receptor activation increases the excitotoxic threshold by hyperpolarizing the membrane and/or by stabilizing $[Ca^{2+}]_i$, the effects are potentially beneficial during an excitotoxic insult and may underlie the neuroprotective role of κ receptors.

It is uncertain how precisely the dynorphin concentrations used our *in vitro* studies mimic concentrations during injury *in vivo*. Concentrations of dynorphin A in homogenized tissue and/or determined via release or microdialysis ($<$1 nM) are admittedly less than those used in the present study (39); however, many issues must be considered before comparing concentrations *in vivo* and *in vitro*. Dynorphin levels can increase significantly with experimental inflammation or injury (15, 22, 57, 69, 74). As part of our ongoing studies on dynorphin A and spinal cord injury, we find that dynorphin A (1–13) is 10- to 100-fold less toxic than other dynorphin A peptide fragments, such as dynorphin A (1–17) (in preparation). Metabolites of dynorphin [e.g., dynorphin A (2–13)], which retain NMDAR activity but lack κ-opioid receptor activity, may be more stabile than dynorphin A (1–17) (94). Assuming dynorphin diffuses in a gradient from an injured cell, the levels within the vicinity of that cell will be greater. Also, because dynorphin is released into the confined extracellular space within CNS tissue, its concentrations within the extracellular compartment are likely to be greater than in homogenates. Moreover, only transient exposure to NMDA is required to set off toxic cascades (12, 50), and a similar "hit and run" phenomenon may be operative for dynorphin. Last, our *in vitro* findings are in agreement with the toxic effects of dynorphin seen *in vivo* (50).

Can the neuroprotective effects mediated by κ-opioid receptors in spinal cord neurons be generalized to other neurons in the spinal cord or brain? Interestingly, current evidence indicates that depending on the cell type and opioid dosage, opioids can have paradoxical—neuroprotective or neurodegenerative—effects (30, 59, 60). Morphine or Met-enkephalin can inhibit neuronal cell death in the avian ciliary ganglion (59, 60). In a cell line transfected with μ-opioid receptors, the μ agonist DAMGO ([D-Ala$^2$,*N*-Me-Phe$^4$,Gly$^5$-ol]-enkephalin) activates Akt-induced neuroprotection (70). Alternatively, the μ agonist morphiceptin amplifies staurosporine or wortmannin-induced apoptosis in neurons cultured from the cerebral hemispheres of embryonic chicks (30). In other studies, opioids similarly enhance cell losses, but only if apoptosis is induced by other factors (17). Together, the above findings suggest that the effects of opioids on cell death are variable and cell-type specific. A greater understanding of opioid signaling is needed before we can predict *a priori* how opioids will act in a particular neuronal type [see (34)]. For example, some of the diversity of opioid actions in part results from an apparent promiscuity in coupling between individual opioid receptor types and particular G-protein-coupled intracellular effector pathways (16, 34, 37, 90). In the cerebellum, rather than being neuroprotective, high concentrations of morphine are neurotoxic to Purkinje cells through an action at μ-opioid receptors (33). Last, any potentially neuroprotective effects of κ receptor activation appear to be offset by the more deleterious systemic pathophysiological actions of opioids. Opioid antagonists have been shown to be beneficial in some experimental models of traumatic brain injury in rats (47, 55–57, 86). Presumably, blocking the otherwise negative consequences of systemic

opioids on metabolism, cerebrovascular, and/or neuroendocrine functions results in a positive outcome. However, despite findings that κ-opioid agonists may be neuroprotective during excitotoxic insult, the mechanisms by which κ receptor activation protects neurons are not understood and need be further assessed at the cell, molecular, and systems levels. The results of this and other studies underscore the potential importance of dynorphin A in the pathophysiology of neurodegenerative diseases and in secondary injury following neurotrauma.

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