The Journal of Neuroscience, December 1990, 10(12): 3793–3800

Dynorphin A-(1-17) Induces Alterations in Free Fatty Acids, Excitatory Amino Acids, and Motor Function Through An Opiate-Receptor-Mediated Mechanism

Rohit Bakshi,[1] Amy H. Newman,[2] and Alan I. Faden[1]

[1]Center for Neural Injury, Department of Neurology, University of California, San Francisco, California 94121 and Neurology Service (127), Department of Veterans Affairs, San Francisco, California 94121, and [2]Department of Applied Biochemistry, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100

The endogenous opioid dynorphin A-(1-17) (Dyn A) has been implicated as a mediator of tissue damage after traumatic spinal cord injury (TSCI) and causes hindlimb paralysis when administered intrathecally. Motor impairment following intrathecal Dyn A is attenuated by antagonists of excitatory amino acids (EAAs); whether opioid receptors mediate such injury has been questioned. TSCI causes various biochemical changes associated with secondary tissue damage, including alterations in tissue amino acids, phospholipids, and fatty acids. Such changes reflect injury severity and correlate with motor dysfunction. The present studies examined whether dynorphin administration causes similar biochemical alterations and whether effects of Dyn A can be modified by treatment with opioid-receptor antagonists. At 24 hr after intrathecal Dyn A, there were significant declines in tissue levels of glutamate, aspartate, and glycine. Increases in total free fatty acids were found at 2 and 24 hr, reflecting changes in both saturated and unsaturated components, which were associated with significant decreases in tissue cholesterol and phospholipid phosphorus at the earlier time point. Each of these neurochemical changes, as well as corresponding motor deficits, were limited by pretreatment with the opioid antagonist nalmefene. In separate experiments, both nalmefene and the selective κ-opioid antagonist nor-binaltorphimine (nor-BNI) limited dynorphin-induced motor dysfunction; effects of nor-BNI were dose related, and those of nalmefene were stereospecific. Therefore, behavioral and neurochemical consequences of Dyn A administration are mediated in part through opiate receptors, most likely κ-receptors. These studies indicate that phospholipid hydrolysis and release of EAAs may contribute to dynorphin-induced tissue damage, suggesting for the first time a potential linkage among opioid, excitotoxin, and membrane lipid mechanisms of secondary injury after neurotrauma.

Received Mar. 12, 1990; revised June 21, 1990; accepted July 24, 1990.

This work was supported by NIH Grant RO1 NS23422. We wish to thank Florence Cheng, Brendan Chan, and Vicky Cardenas for technical assistance and Dr. Steven Graham for reviewing this work. We adhered to the principles enumerated in the *Guide for the Care and Use of Laboratory Animals,* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council [DHEW Pub. No. (NIH) 85-23, 1985]. R.B. is a recipient of an Alpha Omega Alpha Research Scholarship to Medical Students.

Correspondence should be addressed to Alan I. Faden, M.D., Neurology Service (127), Department of Veterans Affairs, 4150 Clement Street, San Francisco, CA 94121.

Phamacological studies with opioid-receptor antagonists support the concept that endogenous opioids contribute to the pathophysiology of tissue damage after CNS trauma (Faden et al., 1981; Flamm et al., 1982; Hayes et al., 1983; Arias, 1985; Inoue, 1986; McIntosh et al., 1987). Dynorphin A (Dyn A), a 17-amino acid opioid peptide thought to be an endogenous ligand for the κ-opiate receptor (Chavkin and Goldstein, 1981; Yoshimura et al., 1982), has been implicated as a secondary injury factor after spinal cord (Faden et al., 1985) and brain trauma (McIntosh et al., 1987). Following traumatic spinal cord injury (TSCI), levels of dynorphinlike immunoreactivity increase in proportion to the degree of trauma (Faden et al., 1985), whereas treatment with dynorphin antiserum or κ-selective opiate-receptor antagonists improve neurological recovery (Faden, 1990).

Consistent with its putative pathophysiologic role, Dyn A and related fragments, administered intrathecally, cause chronic paralysis (Faden and Jacobs, 1984; Stevens and Yaksh, 1986), loss of the tail-flick reflex (Herman and Goldstein, 1985), neuroanatomical damage (Caudle and Isaac, 1987; Long et al., 1988), and a decrease in local spinal cord blood flow (Long et al., 1987; Thornhill et al., 1989). Effects of dynorphin on the tail-flick reflex or motor function are prevented by treatment with NMDA antagonists (Caudle and Isaac, 1988; Long et al., 1989a; Bakshi and Faden, 1990a,b), suggesting that dynorphin-induced neurological dysfunction involves the release of excitatory amino acids (EAAs). However, the role of opiate receptors has been more controversial. Some groups have shown that opiate-receptor antagonists attenuate Dyn A-induced paralysis (Przewlocki et al., 1983; Spampinato and Candeletti, 1985; Faden, 1990), whereas others have not (Herman and Goldstein, 1985; Stevens and Yaksh, 1986; Long et al., 1988, 1989b). In addition, nonopioid fragments of Dyn A, including Dyn A-(2-17) and Dyn A-(3-13), also cause paralysis, though with markedly less potency than Dyn A-(1-17) (Faden and Jacobs, 1984; Stevens and Yaksh, 1986). A recent report critically reviews this controversy and provides experimental evidence that both opioid and nonopioid mechanisms play a role in Dyn A-induced paralysis (Faden, 1990).

TSCI causes increased tissue levels of free fatty acids (FFAs; Demediuk et al., 1985; Faden et al., 1987) and decreased tissue levels of EAAs (Demediuk et al., 1989). Release of FFAs after trauma reflects phospholipid hydrolysis and may contribute to subsequent tissue damage either through direct toxic effects (Chan et al., 1983) or through the actions of such metabolic products as thromboxanes (Hsu et al., 1985). Early release of EAAs into

the extracellular space after spinal cord trauma (Panter et al., 1990) is believed to lead to subsequent loss of total tissue levels (Demediuk et al., 1989). Pharmacological studies indicate that these EAA changes contribute to delayed tissue damage after spinal cord trauma (Faden and Simon, 1988) or brain trauma (Hayes et al., 1988; Faden et al., 1989). The present experiments were intended to explore whether changes in FFAs and EAAs in Dyn A-induced injury parallel those after traumatic injury and whether such changes are opioid-receptor mediated.

Materials and Methods

Intrathecal infusion model. Male Sprague–Dawley rats, weighing 300–350 gm, were anesthetized with sodium pentobarbital (70 mg/kg, i.p.). An intrathecal line was implanted to the eighth thoracic vertebral level using a modification of the method of Yaksh and Rudy (1976), as previously detailed (Bakshi and Faden, 1990a). Briefly, polyethylene tubing (PE-10) was implanted into the subarachnoid space through the atlanto-occipital membrane and passed to T8. The catheter was then secured below the skin and the wound sutured. Animals were allowed 24 hr to recover, at which time those showing motor deficits were removed from the experiment. Agents were infused through the intrathecal catheter in a vehicle of 20 μl physiologic saline.

Study 1. Animals were randomly assigned to pretreatment with either nalmefene (8 nmol; Key Pharmaceuticals, Miami, FL) or vehicle in a volume of 10 μl. Fifteen minutes later, Dyn A-(1-17) (24 nmol; Peninsula Laboratories, Belmont, CA) or equivolume vehicle was administered. Nalmefene and Dyn A-(1-17) doses were based on previous studies (Bakshi and Faden, 1990a) and pilot data. At 2 and 24 hr after infusions, animals were reanesthetized (70 mg/kg sodium pentobarbital, i.p.) and ventilated; after laminectomy, the T7–T9 spinal cord region was frozen *in situ* with liquid nitrogen and the animal killed.

Study 2. Animals were randomly assigned to pretreatment with (−)- or (+)nalmefene [16 nmol; synthesized by A. H. Newman (Rice and Newman, 1986)], nor-binaltorphimine (nor-BNI; 20, 35, 50 nmol; gift of P. S. Portoghese and A. E. Takemori), or vehicle. Fifteen minutes later, Dyn A-(1-17) (24 nmol; Peninsula Laboratories, Belmont, CA) was administered.

Amino acid analysis. Free amino acids were extracted, derivatized, separated, and quantitated using a modification of the method of Allison et al. (1984), as previously described (Demediuk et al., 1989). Spinal cord samples (20–40 mg each) were sonicated in 850 μl 0.4 M perchloric acid and 1.0 mM EDTA, containing α-aminobutyric acid as an internal standard. The homogenate was centrifuged for 1.0 min in a Beckman Microfuge, then neutralized with 400 μl 1.0 M potassium bicarbonate and recentrifuged for 90 sec. The supernatant was removed for amino acid analysis, and the pellet was saved for protein determination using the method of Bradford (1976). Electrochemically active derivatives of the extracted amino acids were made by mixing 20 μl of the extract with 100 μl derivatizing solution (2 ml methanol, 27 mg *o*-phthalaldehyde, 20 μl *tert*-butylthiol, and 4.5 ml 100 mM borax; pH, 10.0). The solution was allowed to react for 2 min, and 20 μl was subjected to high-performance liquid chromatography (HPLC) separation and quantitation with electrochemical detection. Integrated peak areas were used to quantitate individual amino acids. Linearity of detector response was checked with pure standards of each measured amino acid.

Free fatty acids (FFAs). Changes in FFAs were measured, as previously described (Lemke et al., 1990). In brief, total lipids were extracted from brain tissues by homogenizing samples in 2 ml hexane:2-propanol (3:2, vol/vol; Hara and Radin, 1978; Saunders and Horrocks, 1984). The homogenate was filtered and washed twice with 2 ml of the extracting solvent; the combined filtrate was evaporated under a stream of N_2, and the lipids were redissolved in 2 ml chloroform. The lipids were then separated into fractions of 1.0-gm Sil-LC (Sigma, St. Louis, MO) silicic acid columns. Elution was with 100 ml each of chloroform (neutral lipids) and methanol (polar lipids). Solvents were removed on a rotary evaporator, and each fraction was redissolved in 100 μl hexane: 2-propanol (3:2). A 10-μl aliquot was removed from the polar liquid fraction for colorimetric determination of total lipid phosphorus as detailed by Rouser et al. (1969). The remaining particulate matter from the homogenization was used for estimation of protein as above.

Total FFAs were separated by thin-layer chromatography (TLC) with petroleum ether : diethyl ether : acetic acid (110:90:4, by vol) as the developing solvent. Spots on TLC were visualized under UV after spraying with a solution of 2-*p*-toluidinyl napthalene-6-sulfonate (Jones et al., 1982). Lipid bands were scraped from the plates prior to analysis. Cholesterol, in the presence of silica gel, was quantitated by the method of Bowman and Wolf (1962). FFAs were extracted from the silica gel using chloroform : methanol (2:1, vol/vol). Silica gel was removed by filtering through 0.2-μm nylon filters into conical centrifuge tubes. A heptadecanoate internal standard was added, and the filtrate was evaporated under N_2. Fatty acid methyl esters (FAMEs) were prepared using a modification of the method of Allen et al. (1984). To each centrifuge tube, 20 μl 0.5 M NaOH, 80 μl *N,N*-dimethyl acetamide, and 40 μl methyl iodide was added, with vortexing after every addition. The reaction mixtures were then heated at 65°C for 10 min and allowed to cool. Ninety microliters pyridine was added with vortexing, and the tubes were again heated at 65°C for 10 min. After cooling, 0.8 ml 0.1 M phosphoric acid equilibrated with ethylene chloride was added, followed by 25 μl ethylene chloride. The tubes were vortexed for 30 sec and centrifuged at 500 × *g* for 2 min. Two microliters of the ethylene chloride lower phase was removed with a Hamilton syringe and injected into a Perkin-Elmer Sigma 300 gas chromatograph for separation and quantitation of FAMEs. A Supelco (Bellefonte, PA) prepacked 5% DEGS-PS (Supelcoport 100/200 mesh) column was used with N_2 as the carrier gas for all gas–liquid chromatography experiments. The initial column temperature was 180°C and was linearly increased at a rate of 4°C/min. The injector and flame-ionization detector temperatures were set at 250°C. Detector linearity was checked with known standards. Chromatograms were recorded, and peak areas were calculated using the chromatographic data station listed above.

Neurologic assessment. At 60 min and 24 hr after infusions, surviving animals were blindly scored for neurological function, including scores of motor function and ability to maintain position on an inclined plane. Motor function was graded on an 8-point ordinal grading scale, as follows: 0, no hindlimb movement; 1, minimal movement; 2, full flexion but cannot support weight; 3, supports weight but unable to walk; 4, walks with severe spasticity and ataxia; 5, walks with moderate spasticity and ataxia; 6, walks with mild spasticity and ataxia; 7, normal. Neuroscores were also categorized and compared by functional ability in terms of walkers (4–7) or nonwalkers (0–3). For inclined plane evaluation, animals were placed horizontally on the incline in 2 orientations: left-facing and right-facing, as previously detailed (Rivlin and Tator, 1977). The maximum angle was noted and subtracted from scores generated just prior to infusion of compounds. For each time point, a composite angle-board score was calculated by averaging the right-facing and left-facing angle change.

Statistical analysis. Changes in FFAs, cholesterol, phospholipid phosphorus, amino acids, and angle-board measurements were evaluated using analysis of variance (ANOVA) followed by individual post hoc comparisons (Fisher's), survival rates and walkers versus nonwalkers by Fisher's exact probability test, and neuroscores by a Kruskal–Wallis ANOVA followed by Mann–Whitney *U* tests. In all cases, a p value less than 0.05 was considered statistically significant.

Results

Study 1

Free fatty acids (FFAs)

Dyn A caused significant increases in total FFAs, at both 2 and 24 hr after infusion (Fig. 1). At 2 hr, changes were comprised of significant increases in the unsaturated species 18:1, 18:3, and 22:6 and the saturated species 16:0 and 18:0 (Fig. 2) and of modest increases in 20:5 (not shown). At 24 hr, total changes reflected significant increases in the unsaturates 18:1, 18:3, and 20:5 and the saturates 16:0 and 18:0 (Fig. 3) and modest increases in 20:4, 22:4, and 22:6 (not shown). Pretreatment with nalmefene significantly attenuated the increases in total FFAs at 2 and 24 hr, as well as the individual increases in 16:0, 18:0, 18:1, 18:3, 22:6 (Figs. 1–3), and 22:4 (not shown).

Cholesterol and phospholipid phosphorus

At 2 hr postinfusion, Dyn A caused significant declines in cholesterol and phospholipid phosphorus (as compared to vehicle controls), which partially resolved by 24 hr. The declines were significantly attenuated by nalmefene (Fig. 4).

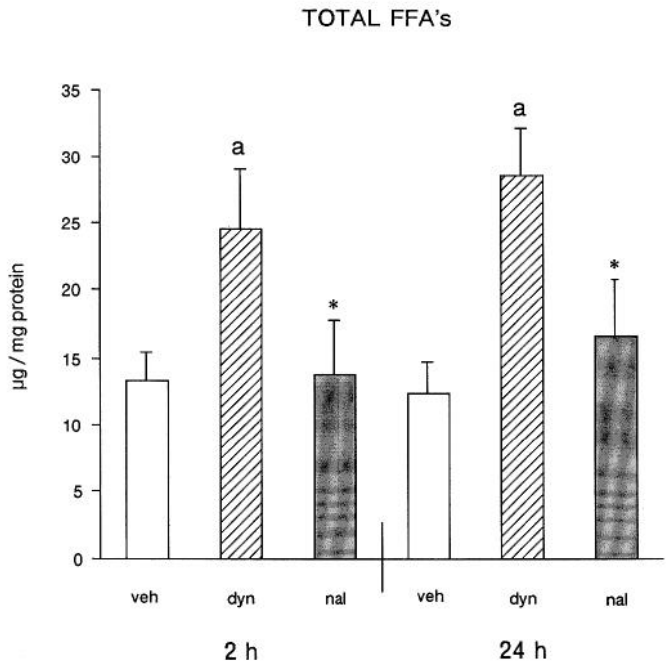

Figure 1. Changes in spinal cord FFAs (expressed as µg/mg protein) at infusion site after intrathecal administration of vehicle [saline + saline (*veh*)], saline + 24 nmol Dyn A (*dyn*), or 8 nmol nalmefene + Dyn A (*nal*) at 2 and 24 hr postinfusion. Values are expressed as means ± SEM (n = 5–7 per group). *a*, different from veh ($p < 0.05$); *, different from dyn ($p < 0.05$).

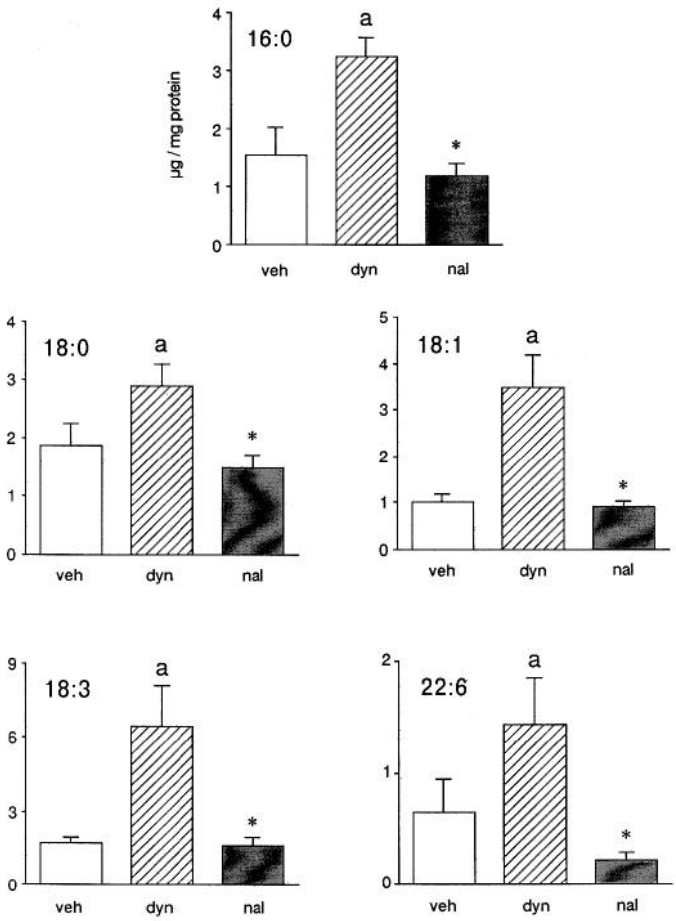

Figure 2. Significant changes in individual FFAs as in Figure 1 at 2 hr postinfusion. *a*, different from veh ($p < 0.05$); *, different from dyn ($p < 0.05$).

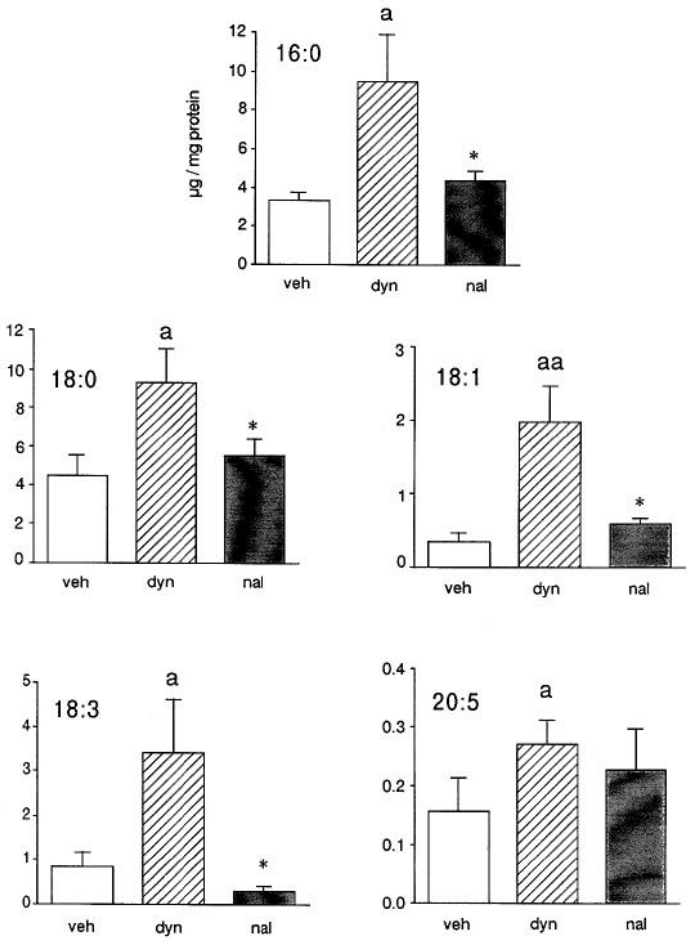

Figure 3. Significant changes in individual FFAs as in Figure 1 at 24 hr postinfusion. *a*, different from veh ($p < 0.05$); *aa*, different from veh ($p < 0.005$); *, different from dyn ($p < 0.05$).

Amino acids

Twenty-four hr after infusion, Dyn A-(1-17) caused significant decreases in total tissue levels of aspartate, glutamate, and glycine (Fig. 5), but only modest decreases in serine, GABA, glutamine, alanine, and taurine (data not shown). The changes in aspartate, glutamate, and glycine were limited by pretreatment with nalmefene (Fig. 5).

Neurologic outcome

In these same rats, nalmefene treatment significantly improved Dyn A-induced mortality and paralysis (measured by neuroscore and angle-board score) as compared to vehicle-pretreated controls (each $p < 0.05$; Table 1).

Study 2

Effects of nalmefene, nor-BNI, or vehicle alone

Infusion of saline, (+)- or (−)nalmefene, or 20 nmol nor-BNI had no detectable effect on hindlimb function; however, 35 and 50 nmol nor-BNI given alone caused mild motor deficits that resolved by 10 min (35 nmol) or 30 min (50 nmol; data not shown). These transient effects are similar to observations made with competitive NMDA antagonists (Caudle and Isaac, 1988; Bakshi and Faden, 1990a).

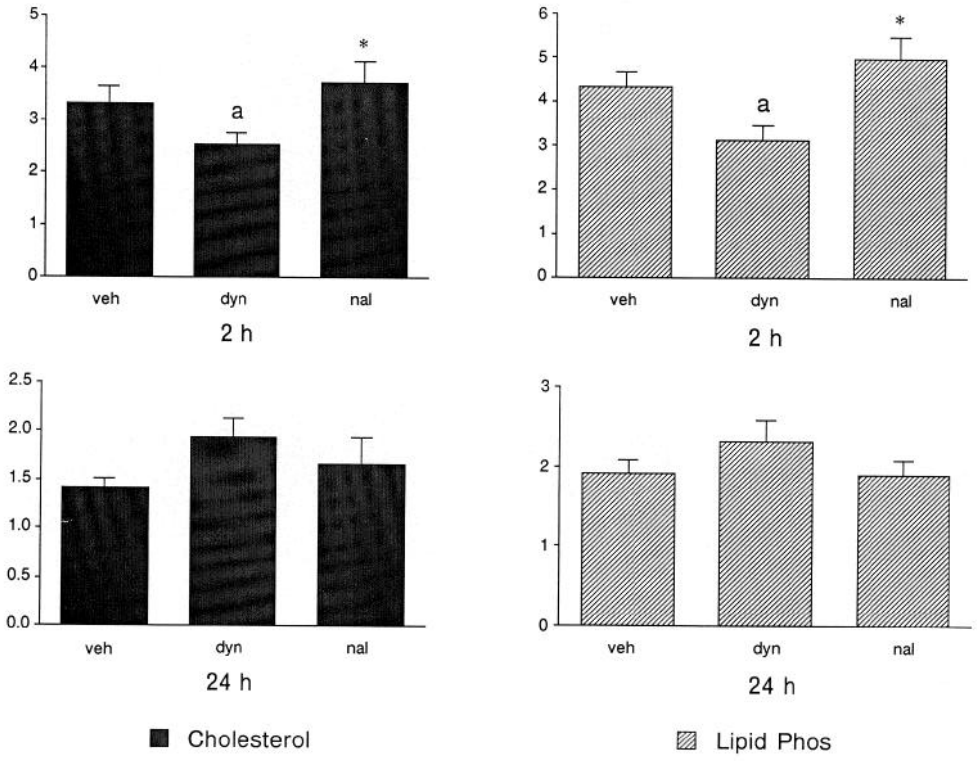

Figure 4. Corresponding changes in spinal cord cholesterol and total phospholipid phosphorus (*lipid phos*) of animals from Figures 1–3 at the infusion site after intrathecal administration of vehicle [saline + saline (*veh*)], saline + Dyn A (*dyn*), or nalmefene + Dyn A (*nal*) at 2 and 24 hr postinfusion. Each mean and SEM is expressed as μmol/mg protein. *a,* different from veh ($p < 0.05$); *, different from dyn ($p < 0.05$).

Blockade of Dyn A-induced effects

Intrathecal infusion of 24 nmol Dyn A produced flaccid hindlimb weakness by 5 min, resulting in either severe (28%) or moderate (22%) paralysis or death (50%) by 24 hr (Figs. 6, 7). Nalmefene or nor-BNI limited these effects: each compound significantly improved hindlimb function measured by neuroscore (Fig. 6), walking ability (Fig. 7, middle panel), and angle-board score (Fig. 7, lower panel), as compared to vehicle-pretreated controls. Effects of nalmefene were stereospecific: (−)nalmefene, but not (+)nalmefene, showed significant protection. Actions of nor-BNI were dose related: 20 and 35 nmol significantly improved neuroscores at both 60 min and 24 hr, whereas the 50-nmol dose did not (Fig. 6); the 2 lower doses also significantly improved other behavioral measures of recovery (Fig. 7). Additionally, nalmefene (stereospecifically) and nor-BNI (35 and 50 nmol) improved survival rate (Fig. 7, upper panel).

Discussion

Dyn A caused sustained increases in FFAs, as well as early declines in tissue phospholipid and cholesterol content. These changes likely reflect membrane lipid hydrolysis, which has been well described after TSCI (Demediuk et al., 1985; Faden et al., 1987). Phospholipid decomposition results in release of FFAs (Rehncrona et al., 1982) and is found after a variety of insults, including ischemia (Yoshida et al., 1982), hypoxia (Gardiner et

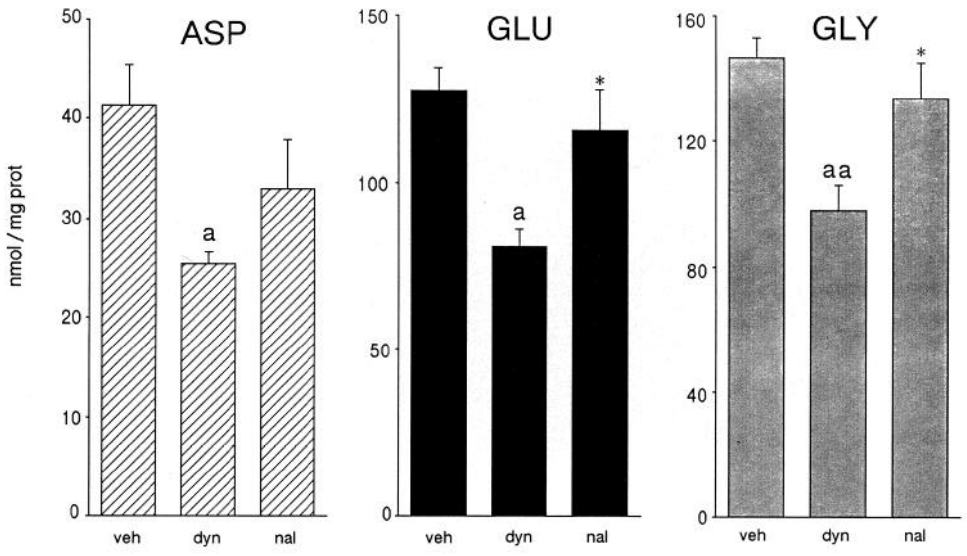

Figure 5. Total rat spinal cord tissue levels of aspartate (*ASP*), glutamate (*GLU*), and glycine (*GLY*) at the infusion site after intrathecal administration of vehicle [saline + saline (*veh*)], saline + Dyn A (*dyn*), or nalmefene + Dyn A (*nal*) at 24 hr postinfusion. Values are expressed as means ± SEM ($n = 7$ per group). *a,* different from veh ($p < 0.05$); *, different from dyn ($p < 0.05$). *aa,* different from veh ($p < 0.005$).

Table 1. Effects of nalmefene pretreatment on outcome after intrathecal administration of Dyn A[a]

	60 min		24 hr	
	Vehicle	Nalmefene	Vehicle	Nalmefene
Median neuroscore	1	5*	2	6*
Percent walkers	0	86*	40	100*
Angle-board score	48 ± 4	13 ± 5*	21 ± 3	7 ± 2*
Survival rate	—	—	41.7%	100%*

[a] Data represent behavioral scores and survival rates from animals killed at 24 hr for analysis of lipids or amino acids in Study 1.

* $p < 0.05$ compared to vehicle.

al., 1981), hypoglycemia (Agardh et al., 1981), and trauma (Faden et al., 1987). FFAs may cause cellular damage either directly or through the actions of their metabolites (Chan et al., 1983; Hsu et al., 1985). High concentrations of FFAs may directly serve as detergents that disrupt cell membranes (Lucy, 1970), facilitate osmotic hemolysis (Raz and Livine, 1973), inhibit synaptosomal uptake of amino acids, reduce synaptosomal Na^+,K^+-ATPase activity (Rhoads et al., 1982; Faden et al., 1987), and impair mitochondrial function (Lazarewicz et al., 1972; Wojtczak, 1976). In addition, FFAs may contribute to free-radical-induced tissue injury, believed to be important in the pathophysiology of TSCI (Hall and Wolf, 1986). The decrease in cholesterol may also reflect Dyn A-induced membrane breakdown. Cholesterol is known to have a condensing effect on the acyl-chain region of fluid bilayers, contributing to structural stability; a decrease in cholesterol affects the permeability of lipid bilayers to water and ions such as K^+, Na^+, and Ca^{2+} (Blok et al., 1977; Houslay and Stanley, 1982). Spinal cord trauma causes a decline in tissue cholesterol (Demopoulos et al., 1982; Segler-Stahl et al., 1985), as well as changes in tissue cations (Lemke et al., 1987; Kwo et al., 1989).

Administration of Dyn A caused sustained (at 24 hr) decreases in total tissue glutamate, aspartate, and glycine, suggesting early release of these amino acids into the extracellular space followed by transport into the circulation or metabolic transformation

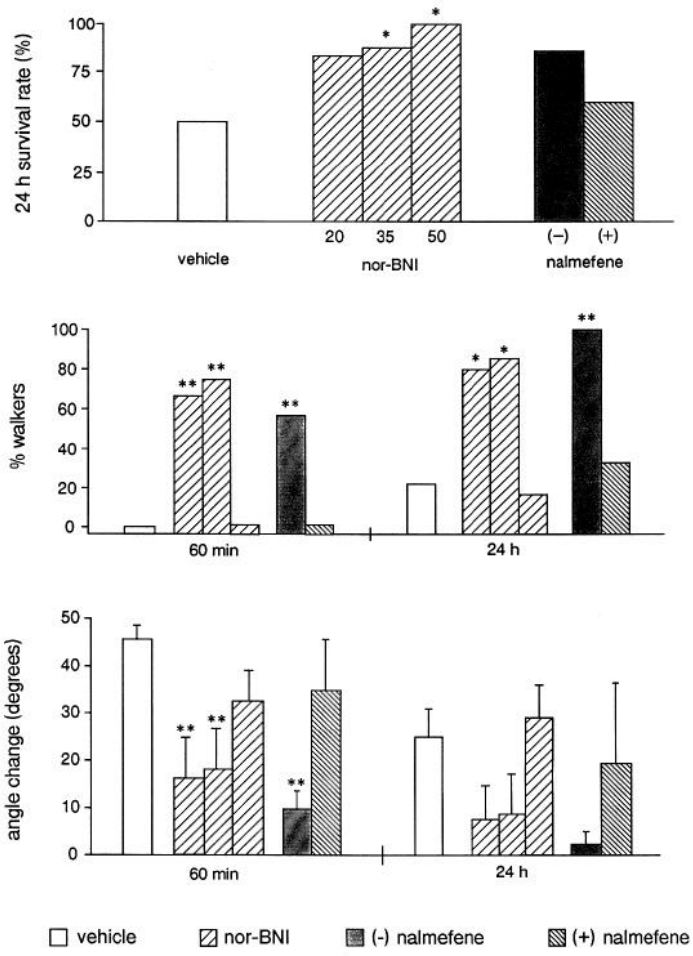

Figure 7. In animals from Figure 6, nor-BNI and nalmefene significantly improved corresponding effects of Dyn A, including 24-hr survival rate (*upper panel*), functional ability (*middle panel*), and angle-board score (*lower panel*, mean ± SEM). *Asterisks*, different from vehicle-pretreated controls (*, $p < 0.05$; **, $p < 0.005$).

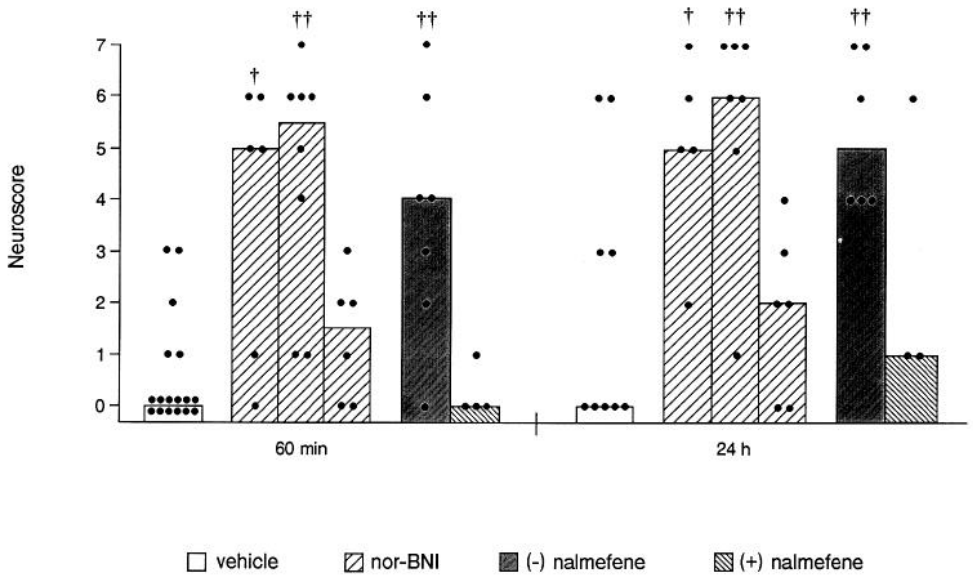

Figure 6. Comparison of effect of intrathecal pretreatment with nor-BNI (doses from *left* to *right*: 20, 35, 50 nmol), nalmefene (16 nmol), or vehicle on rat hindlimb neuroscore (at 60 min and 24 hr) after infusion of 24 nmol Dyn A. Each treatment significantly improved neuroscores compared to controls (†, $p < 0.05$; ††, $p < 0.005$), with protective effects of nalmefene specific for the (−) stereoisomer. *Histograms* represent median scores; *dots* indicate individual animal neuroscores.

(Erecinska et al., 1984). Spinal cord trauma causes sustained decreases in total tissue levels of glutamate and aspartate (Demediuk et al., 1989), as well as early transitory increases in extracellular concentrations of these amino acids (Panter et al., 1990). Preliminary studies from our laboratory utilizing *in vivo* microdialysis indicate that Dyn A administration to the rat spinal cord causes early extracellular increases of glutamate, aspartate, and glycine (A. Faden and P. Halt, unpublished observations). Glutamate and aspartate have a neurotoxic action that has long been recognized (Lucas and Newhouse, 1957; Olney et al., 1971). Among the known EAA receptors, the NMDA receptor appears to mediate this "excitotoxic" action (Olney et al., 1971; Choi et al., 1988). Treatment with NMDA antagonists limits tissue damage after spinal cord trauma (Faden et al., 1990). Extracellular increases in glycine may also contribute to excitotoxicity: studies *in vitro* (Johnson and Ascher, 1987) and *in vivo* (Danysz et al., 1989) have shown that glycine potentiates glutamate-induced and NMDA-induced responses by an allosteric binding site located on the NMDA receptor complex. The hypothesis that extracellular increases in EAA and glycine contribute to Dyn A-induced irreversible paralysis and histological damage is consistent with studies showing that NMDA receptor antagonists limit Dyn A-induced neurological dysfunction, including those acting at the glycine binding site (Bakshi and Faden, 1990b), glutamate binding site (Caudle and Isaac, 1988; Bakshi and Faden, 1990a), and ion channel (Long et al., 1989a; Bakshi and Faden, 1990a).

The mechanism by which dynorphin induces NMDA-receptor-mediated actions remains speculative. Dyn A causes marked reduction in spinal cord blood flow upon intrathecal administration (Long et al., 1987; Thornhill et al., 1989). Thus, Dyn A-induced ischemia may trigger the release of EAAs, leading to biomembrane breakdown and the liberation of FFAs, activation of NMDA receptors, and cell death with subsequent functional deficits. It is also possible that Dyn A causes release of EAAs through opiate-receptor mechanisms independent of ischemia, possibly via presynaptic actions. However, the relationship between dynorphin and NMDA-receptor-mediated activity is complicated, with *in vitro* evidence that dynorphin may serve through "nonopioid" actions as an NMDA *antagonist* (Choi et al., 1989; Massardier and Hunt, 1989).

Involvement of opiate receptors in the neurologic effects of Dyn A was first suggested by Han and Xie (1982), who demonstrated that naloxone partially blocks the tail-flick effects of Dyn A-(1-13); the low efficacy of naloxone to reverse these effects and the absence of cross-tolerance between morphine analgesia and Dyn A-induced reflex loss led them to propose that Dyn A was acting not at μ-receptors, but at κ-receptors. Subsequently, a number of groups have demonstrated that opioid antagonists attenuate Dyn A-induced neurologic dysfunction, including the nonselective antagonist naloxone (Przewlocki et al., 1983; Spampinato and Candeletti, 1985), the κ-active antagonist MR1452 (Spampinato and Candeletti, 1985), and the κ-selective antagonist nor-BNI (Faden, 1990). In contrast, other observations suggest that the effects of Dyn A are not entirely opioid. Dyn A fragments (2-17) and (3-13), apparently devoid of opiate-receptor binding activity (Chavkin and Goldstein, 1981; Walker et al., 1982), also cause a clinically similar paralysis, though with less potency (Faden and Jacobs, 1984; Stevens and Yaksh, 1986; Long et al., 1988; Bakshi and Faden, 1990a). Furthermore, some groups have failed to block Dyn A-induced paralysis with naloxone (Faden and Jacobs, 1984; Stevens and Yaksh, 1986; Long et al., 1988). Long et al. (1989b), using an *anesthetized* rat model involving intrathecal injection by lumbar puncture, failed to block Dyn A-induced paralysis with nor-BNI; however, only a single, relatively low dose of nor-BNI was evaluated. Because the κ-*agonist* U50488H, even at high doses, does not cause alterations in motor function, some have argued that Dyn A-induced paralysis is entirely nonopioid (Stevens and Yaksh, 1986; Long et al., 1988).

Nalmefene is an opiate-receptor antagonist with increased activity at κ-opiate receptors (Michel et al., 1985), which is strongly neuroprotective after spinal cord trauma (Faden et al., 1988). Nalmefene pretreatment attenuated the lipid and amino acid changes after Dyn A infusion and stereospecifically limited the paralytic effects. In addition, the highly specific κ-opiate receptor antagonist nor-BNI (Takemori et al., 1988), which limits paralysis after spinal cord trauma (Faden, 1990), reduced Dyn A-induced motor dysfunction. Taken together, these findings strongly suggest that the consequences of Dyn A administration are, at least in part, mediated by opiate receptors, and more specifically, the κ-opiate receptor. Consistent with this hypothesis, Dyn A binds well to opiate receptors, with preference for κ subtypes (Chavkin et al., 1982); such receptors are widely distributed in the spinal cord (Traynor et al., 1982; Czlonkowski et al., 1983; Mack et al., 1984; Gouarderes et al., 1985). There is also mounting evidence to support the existence of separate populations of κ-receptors (isoreceptors) within the CNS (Attali et al., 1982; Gouarderes and Cros, 1984; Iyengar et al., 1985; Nock et al., 1988; Zukin et al., 1988); therefore, Dyn A and U50488H may well be acting through different isoreceptors. At least 2 subpopulations of κ-receptors have been identified in the rat spinal cord: high-affinity (κ_1) and low-affinity (κ_2) sites (Gouarderes and Cros, 1984). Similarly, Zukin et al. (1988) have identified κ_1 and κ_2 sites in the rat brain; though Dyn A and U50488H show similar potencies at κ_1, Dyn A is nearly 300 times more active then U50488H at the low-affinity κ_2 site.

Our findings may have particular application to spinal cord or brain trauma, which cause the accumulation of immunoreactive Dyn A (Faden et al., 1985; McIntosh et al., 1987) and a decrease in blood flow (Young et al., 1981; McIntosh et al., 1987), as well as the release of FFAs (Faden et al., 1987) and EAAs (Faden et al., 1989; Panter et al., 1990). Neurological dysfunction after such injuries can be limited by treatment with Dyn A antiserum (Faden, 1990), NMDA antagonists (Faden and Simon, 1988), or opiate antagonists (Faden et al., 1988). The similar neurochemical, physiological, and pharmacological profiles between dynorphin-induced and traumatic spinal cord injuries strengthen the hypothesis that endogenous dynorphin is a secondary injury factor, contributing to the pathogenesis of CNS trauma. These studies also suggest that dynorphin-induced tissue damage may result, in part, from the release of EAAs and phospholipid hydrolysis, providing a potential linkage among opioid, excitotoxin, and membrane lipid mechanisms of secondary injury after neurotrauma.

References

Agardh CD, Chapman AG, Nilsson B, Siesjo BK (1981) Endogenous substrates utilized by rat brain in severe insulin-induced hypoglycemia. J Neurochem 36:490–500.

Allen KG, Fellows ME, Tornheim PA, Wagner KR (1984) A new procedure to analyze free fatty acids. Application to 20-mg brain tissue samples. J Chromatogr 309:33–42.

Allison LA, Mayer GS, Shoup RE (1984) *o*-Phthalaldehyde derivatives

of amines for high speed liquid chromatography/electrochemistry. Anal Chem 56:1089–1096.
Arias MJ (1985) Effect of naloxone on functional recovery after experimental spinal cord injury in the rat. Surg Neurol 23:440–442.
Attali B, Gouarderes C, Mazurguil H, Audigier Y, Cros J (1982) Evidence for multiple "kappa" binding sites by use of opioid peptides in the guinea-pig lumbosacral spinal cord. Neuropeptides 4:53–64.
Bakshi R, Faden AI (1990a) Competitive and non-competitive NMDA antagonists limit dynorphin A-induced rat hindlimb paralysis. Brain Res 507:1–5.
Bakshi R, Faden AI (1990b) Blockade of the glycine modulatory site of NMDA receptors modified dynorphin-induced behavioral effects. Neurosci Lett 110:113–117.
Blok MC, Van Deenen LLM, De Gier J (1977) The effect of cholesterol incorporation on the temperature dependence of water permeation through liposomal membranes prepared from phosphatidylcholines. Biochim Biophys Acta 464:509–518.
Bowman RE, Wolf RC (1962) A rapid ultramicro method for total serum cholesterol. Clin Chem 8:302–309.
Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal Biochem 72:248–254.
Caudle RM, Isaac L (1987) Intrathecal dynorphin (1-13) results in an irreversible loss of the tail flick reflex in rats. Brain Res 435:1–6.
Caudle RM, Isaac L (1988) A novel interaction between dynorphin (1-13) and an *N*-methyl-D-aspartate site. Brain Res 443:329–332.
Chan PH, Fishman RA, Carronna J, Schmidley JW, Piroleau G, Lee J (1983) Induction of brain edema following intracerebral injection of arachidonic acid. Ann Neurol 13:625–632.
Chavkin C, Goldstein A (1981) Specific receptor for the opioid peptide dynorphin: structure-activity relationships. Proc Natl Acad Sci USA 78:6543–6547.
Chavkin C, James IF, Goldstein A (1982) Dynorphin is a specific endogenous ligand of the κ-opioid receptor. Science 215:413–415.
Choi DW, Koh J, Peters S (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. J Neurosci 8:185.
Choi DW, Rose K, Weiss JH, Faden AI (1989) Dynorphin attenuates NMDA receptor-mediated cortical neuronal injury *in vitro*. Soc Neurosci Abstr 15:1113.
Czlonkowski A, Costa T, Przewlocki R, Pasi A, Herz A (1983) Opiate receptor binding sites in human spinal cord. Brain Res 267:392–396.
Danysz W, Wroblewski JT, Brooker G, Costa E (1989) Modulation of glutamate receptors by phencyclidine and glycine in the rat cerebellum: cGMP increases *in vivo*. Brain Res 479:270–276.
Demediuk P, Saunders RD, Anderson DK, Means ED, Horrocks LA (1985) Membrane lipid changes in laminectomized and traumatized cat spinal cord. Proc Natl Acad Sci USA 82:7071–7075.
Demediuk P, Daly MP, Faden AI (1989) Effect of impact trauma on neurotransmitter and non-neurotransmitter amino acids in rat spinal cord. J Neurochem 52:1529–1536.
Demopoulos HB, Flamm ES, Seligman ML, Pietronigro DD, Tomasula J, DeCrescito V (1982) Further studies on free-radical pathology in the major nervous system disorders: effect of very high doses of methylprednisolone on the functional outcome, morphology, and chemistry of experimental spinal cord impact injury. Can J Physiol Pharmacol 60:1415–1424.
Erecinska M, Nelson D, Wilson DF, Silver IA (1984) Neurotransmitter amino acids in the CNS. I. Regional changes in amino acid levels in rat brain during ischemia and reperfusion. Brain Res 304:9–22.
Faden AI (1990) Opioid and non-opioid mechanisms may contribute to dynorphin's pathophysiologic actions in spinal cord injury. Ann Neurol 27:67–74.
Faden AI, Jacobs TP (1984) Dynorphin-related peptides cause motor dysfunction in the rat through a non-opiate action. Br J Pharmacol 81:271–276.
Faden AI, Simon RP (1988) A potential role for excitotoxins in the pathophysiology of spinal cord injury. Ann Neurol 23:623–626.
Faden AI, Jacobs TP, Holaday JW (1981) Opiate antagonist improves neurologic recovery after spinal injury. Science 211:493–494.
Faden AI, Molineaux CJ, Rosenberger JG, Jacobs TP, Cox BM (1985) Increased dynorphin immunoreactivity in spinal cord after traumatic injury. Reg Pept 11:35–41.
Faden AI, Chan PH, Longar S (1987) Alterations in lipid metabolism, Na^+,K^+-ATPase activity, and tissue water content of spinal cord following experimental traumatic injury. J Neurochem 48:1809–1816.
Faden AI, Sacksen I, Noble LJ (1988) Opiate receptor antagonist nalmefene improves neurological recovery after traumatic spinal cord injury in rats through a central mechanism. J Pharmacol Exp Ther 245:742–748.
Faden AI, Demediuk P, Panter SS, Vink R (1989) Role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science 244:798–800.
Faden AI, Ellison JA, Noble LJ (1990) Effects of competitive and non-competitive NMDA receptor antagonists in spinal cord injury. Eur J Pharmacol 175:165–174.
Flamm ES, Young W, Demopoulos HB, DeCrescito V, Tomasula JJ (1982) Experimental spinal cord injury: treatment with naloxone. Neurosurgery 10:227–231.
Gardiner M, Nilsson B, Rehncrona S, Siesjo BK (1981) Free fatty acids in moderate and severe hypoxia. J Neurochem 36:1500–1505.
Gouarderes C, Cros J (1984) Opioid binding sites in different levels of rat spinal cord. Neuropeptides 5:113–116.
Gouarderes C, Cros J, Quiron R (1985) Autoradiographic localization of mu, delta and kappa opioid receptor binding sites in rat and guinea pig spinal cord. Neuropeptides 6:331–342.
Hall ED, Wolf DL (1986) A pharmacological analysis of the pathophysiological mechanisms of post-traumatic spinal cord ischemia. J Neurosurg 64:951–961.
Han J-S, Xie C-W (1982) Dynorphin: potent analgesic effect in spinal cord of the rat. Life Sci 31:1781–1784.
Hara A, Radin NS (1978) Lipid extraction of tissues with a low toxicity solvent. Anal Biochem 90:420–426.
Hayes RL, Galinet BJ, Kulkarne P, Becker D (1983) Effects of naloxone on systemic and cerebral responses to experimental concussive brain injury in cats. J Neurosurg 58:720–728.
Hayes RL, Jenkins LW, Lyeth BG (1988) Pretreatment with phencyclidine, an *N*-methyl-D-aspartate receptor antagonist, attenuates long-term behavioral deficits in the rat produced by traumatic brain injury. J Neurotrauma 5:259–274.
Herman BH, Goldstein A (1985) Antinociception and paralysis induced by intrathecal dynorphin A. J Pharmacol Exp Ther 232:27–32.
Houslay MD, Stanley KK (1982) Dynamics of biological membranes. Influence on synthesis, structure and function, pp 71–81. New York: Wiley.
Hsu CY, Halushka PV, Hogan EL, Banik NL, Lee WA, Perot LP (1985) Alteration of thromboxane and prostacyclin levels in experimental spinal cord injury. Neurology 35:1003–1009.
Inoue Y (1986) Evoked spinal potentials in the Wistar rat: effect of cord compression and drugs. J Japan Orthop Assoc 60:777–785.
Iyengar S, Kim H, Wood PL (1985) *In vivo* evidence for kappa isoreceptors in rat: neurochemical and neuroendocrine indices. J Neurochem 44:S89.
Johnson JW, Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325:529–531.
Jones J, Keenan RW, Horowitz P (1982) Use of 6-*p*-toluidine-2-naphthalenesulfonic acid to quantitate lipids after thin layer chromatography. J Chromatogr 237:522–524.
Kwo S, Young W, DeCrescito V (1989) Spinal cord sodium, potassium, calcium and water concentration changes in rats after graded contusion injury. J Neurotrauma 6:13–24.
Lazarewicz JW, Strosznajder J, Gromek A (1972) Effects of ischemia and exogenous fatty acids on the energy metabolism in brain mitochondria. Bull Acad Pol Sci [Biol] 20:599–606.
Lemke M, Demediuk P, McIntosh TK, Vink R, Faden AI (1987) Alterations in tissue Mg^{++}, Na^+ and spinal cord edema following impact trauma in rats. Biochem Biophys Res Commun 147:1170–1175.
Lemke M, Yum SW, Faden AI (1990) Lipid alterations correlate with tissue magnesium decrease following impact trauma in rabbit spinal cord. Mol Chem Neuropath, in press.
Long JB, Kinney RC, Malcolm DS, Graeber GM, Holaday JW (1987) Intrathecal dynorphin A (1-13) and dynorphin A (3-13) reduce rat spinal cord blood flow by nonopioid mechanisms. Brain Res 436: 374–379.
Long JB, Petras JM, Mobley WC, Holaday JW (1988) Neurological dysfunction after injection of dynorphin A-(1-13) in the rat. II. Non-opioid mechanisms mediate loss of motor, sensory and autonomic function. J Pharmacol Exp Ther 246:1167–1174.
Long JB, Rigamonti DD, Martinez-Arizala A, Holaday JW (1989a) Non-competitive *N*-methyl-D-aspartic acid receptor inhibitors pre-

vent persistent dynorphin A-induced hindlimb paralysis in rats. J Neurotrauma 6:59–60.

Long JB, Rigamonti DD, de Costa B, Rice KC, Martinez-Arizala A (1989b) Dynorphin A-induced rat hindlimb paralysis and spinal cord injury are not altered by the κ-opioid antagonist nor-binaltorphimine. Brain Res 497:155–162.

Lucas DR, Newhouse JP (1957) The toxic effect of sodium L-glutamate on the inner layers of the retina. Arch Ophthalmol 58:193–201.

Lucy JA (1970) The fusion of biological membranes. Nature 227:815–817.

Mack KJ, Killian A, Weyhanmeyer JA (1984) Comparison of mu, delta, and kappa opiate binding sites in rat brain and spinal cord. Life Sci 34:281–285.

Massardier D, Hunt PF (1989) A direct non-opiate interaction of dynorphin-(1-13) with the *N*-methyl-D-aspartate (NMDA) receptor. Eur J Pharmacol 170:125–126.

McIntosh TK, Hayes RL, DeWitt DS, Agura V, Faden AI (1987) Endogenous opioids may mediate secondary damage after experimental brain injury. Am J Physiol 253:E565–E574.

Michel ME, Bolger G, Weissman B-A (1985) Binding of a new opiate antagonist, nalmefene, to rat brain membranes. Meth Find Exp Clin Pharmacol 7:175–177.

Nock B, Rajpara A, O'Connor LH, Cicero TJ (1988) Autoradiography of [^{3}H]U-69593 binding sites in rat brain: evidence for κ opioid receptor subtypes. Eur J Pharmacol 154:27–34.

Olney JW, Ho OL, Rhee V (1971) Cytotoxic effects of acidic and sulfur containing amino acids on the infant mouse central nervous system. Exp Brain Res 14:61–76.

Panter SS, Yum SW, Faden AI (1990) Alterations in extracellular amino acids after traumatic spinal cord injury. Ann Neurol 27:96–99.

Przewlocki R, Shearman GT, Herz A (1983) Mixed opioid/non-opioid effects of dynorphin and dynorphin-related peptides after their intrathecal injection in rats. Neuropeptides 3:233–240.

Raz A, Livine A (1973) Differential effects of lipids on the osmotic fragility of erythrocytes. Biochim Biophys Acta 311:222–229.

Rehncrona S, Westerberg E, Akesson B, Siesjo BK (1982) Brain cortical fatty acids and phospholipids during and following complete and severe incomplete ischemia. J Neurochem 38:84–93.

Rhoads DE, Kaplan MA, Peterson NA, Raghupathy E (1982) Effects of free fatty acids on synaptosomal amino acid uptake systems. J Neurochem 38:1255–1260.

Rice KC, Newman AH (1986) Total synthesis of northebaine, normorphine, noroxymorphone enantiomers and derivatives via *N*-nor-intermediates. Application for U.S. Patent (filed 10/31/86).

Rivlin AS, Tator CH (1977) Objective clinical assessment of motor function after experimental spinal cord injury in the rat. J Neurosurg 47:577–581.

Rouser G, Siakotos V, Fleischer S (1969) Quantitative analysis of phospholipids by thin layer chromatography and phosphorus analysis of spats. Lipids 1:85–86.

Saunders RD, Horrocks LA (1984) Simultaneous extraction and preparation for HPLC of prostaglandins and phospholipids. Anal Biochem 143:71–75.

Segler-Stahl K, Demediuk P, Castillo R, Watts C, Moscatelli EA (1985) Phospholipids of normal and experimentally injured spinal cord of the miniature pig. Neurochem Res 10:563–569.

Spampinato S, Candeletti SU (1985) Characterization of dynorphin A-induced antinociception at the spinal level. Eur J Pharmacol 110: 21–30.

Stevens CW, Yaksh TL (1986) Dynorphin A and related peptides administered intrathecally in the rat: a search for putative kappa opiate receptor activity. J Pharmacol Exp Ther 238:833–838.

Takemori AE, Ho BY, Naesbeth JS, Portoghese PS (1988) Nor-binaltorphimine, a highly selective κ-opioid antagonist in analgesic and receptor binding assays. J Pharmacol Exp Ther 246:255–258.

Thornhill JA, Gregor L, Mathison R, Pittman Q (1989) Intrathecal dynorphin A administration causes pressor responses in rats associated with an increased resistance to spinal cord blood flow. Brain Res 490:174–177.

Traynor JR, Kelley PD, Rance MJ (1982) Multiple opiate binding sites in rat spinal cord. Life Sci 31:1377–1380.

Walker JM, Moises HC, Coy DH, Baldrighi G, Akil H (1982) Non-opiate effects of dynorphin and des-Tyr-dynorphin. Science 218:1136–1138.

Wojtczak L (1976) Effect of long-chain fatty acids and acyl-CoA on mitochondrial permeability, transport and energy-coupling processes. J Bioenerg Biomembr 8:293–311.

Yaksh TL, Rudy TA (1976) Chronic catheterization of the spinal subarachnoid space. Physiol Behav 17:1031–1036.

Yoshida S, Abe K, Busto R, Watson BO, Kogure K, Ginsberg MD (1982) Influence of transient ischemia on lipid-soluble antioxidants, free fatty acids and energy metabolites in rat brain. Brain Res 245: 307–316.

Yoshimura K, Huidobro-Toro JP, Lee NM, Loh HH, Way EL (1982) Kappa-opioid properties of dynorphin and its peptide fragments on the guinea pig ileum. J Pharmacol Exp Ther 222:71–79.

Young W, Flamm ES, Demopoulos HB, Tomasula JJ, DeCrescito V (1981) Naloxone ameliorates posttraumatic ischemia in experimental spinal contusion. J Neurosurg 55:209–219.

Zukin RS, Eghbali M, Olive D, Unterwald EM, Tempel A (1988) Characterization and visualization of rat and guinea pig brain κ-opiate receptors: evidence for κ_1 and κ_2 opioid receptors. Proc Natl Acad Sci USA 85:4061–4065.