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The Role of Excitatory Amino Acid Receptors and Intracellular Messengers in Persistent Nociception After Tissue Injury in Rats

Terence J. Coderre

Pain Mechanisms Laboratory, Clinical Research Institute of Montreal, Département de Médecine, Université de Montréal, Department of Psychology, McGill University, Montréal, Quebec, Canada

Contents

Abstract

Increased pain sensitivity (hyperalgesia) and persistent nociception following peripheral tissue injury depends both on an increase in the sensitivity of primary afferent nociceptors at the site of injury (peripheral sensitization), and on an increase in the excitability of neurons in the central nervous system (central sensitization). We will review evidence that central sensitization, and the persistent nociception it leads to, are dependent on an action of glutamate and aspartate at excitatory amino acid (EAA) receptors. Additional evidence will be

presented implicating a role of various intracellular second messengers that are coupled to EAA receptors (nitric oxide, arachidonic acid, and protein kinase C) to central sensitization and persistent nociception following tissue injury. Finally, we will examine the evidence for a contribution of molecular events, including noxious stimulus-induced expression of immediate-early genes such as c-*fos* to persistent nociception.

Index Entries: Pain; hyperalgesia; glutamate; aspartate; *N*-methyl-D-aspartate (NMDA); intracellular calcium; nitric oxide; arachidonic acid; protein kinase C; c-*fos*.

Introduction

Recent evidence suggests that following tissue injury there are specific changes in central neuron function that contribute to persistent pain or nociception (*see* Woolf, 1991; Dubner and Ruda, 1992; Coderre et al., 1993 for reviews). Furthermore, there is evidence that this injury or noxious stimulus-induced plasticity is influenced by activity at excitatory amino acid (EAA) receptors and by the intracellular messengers coupled to these receptors. In the present review, we will examine the mechanisms that may underlie the central neuroplasticity generated by noxious stimulation or injury. Specifically, we will assess the contribution of EAA transmitters (glutamate, aspartate), receptors (NMDA, AMPA/kainate, metabotropic), and intracellular messengers (nitric oxide, arachidonic acid, protein kinase C) to noxious stimulus-induced changes in central neural function. We will also evaluate the role of these transmitters and second messengers in animal models of secondary hyperalgesia and persistent pain.

Contribution of Excitatory Amino Acids to Nociception

Recent evidence implicates a contribution of EAAs to nociceptive processes. EAAs have widespread activity in the CNS, including the spinal cord (Watkins and Evans, 1981; Davies and Watkins, 1983) and thalamus (Eaton and Salt, 1990). The role of EAAs in nociception is suggested since noxious stimulation causes the release of glutamate and asparatate in spinal cord dorsal horn (Skilling et al., 1988; Sorkin et al., 1992). Furthermore, iontophoretic application of EAAs produces an excitation of dorsal horn neurons (Curtis and Watkins, 1960; Willcockson et al., 1984a; Schneider and Perl, 1988), whereas intrathecal treatment produces behavioral hyperalgesia and nociceptive behaviors (Aanonsen and Wilcox, 1986, 1987).

Sensitization in Spinal Cord

A contribution of EAAs to sensitization of spinal cord dorsal horn neurons following noxious stimulation is suggested by several findings. Repetitive C-fiber stimulation produces a "wind-up" of dorsal horn neuron activity that is mimicked by the application of L-glutamate or *N*-methyl-D-aspartate (NMDA) (Gerber and Randic, 1989; King et al., 1985), and blocked by application of either competitive (Dickenson and Sullivan 1987; Thompson et al., 1990a) or noncompetitive (Davies and Lodge, 1987; Thompson et al., 1990b) NMDA antagonists. Iontophoretic application of EAAs produces receptive field changes in dorsal horn neurons (Zieglgänsberger and Herz, 1971), as well as enhanced dorsal horn neuron responses to nonnoxious and noxious mechanical stimulation (Aanonsen et al., 1990; Dougherty and Willis, 1991a). Dorsal horn neurons that are sensitized following peripheral tissue injury/inflammation show increased responsiveness to the iontophoretic application of EAAs (Dougherty and Willis, 1992), and exhibit a reduction in responsiveness or sensitization following intravenous administration of ketamine or iontophoretic application of ketamine or APV (Schaible et al., 1991), or the administration of CNQX or AP-7 to dorsal horn neurons by microdialysis (Dougherty et al., 1992). Intrathecal

administration of the EAAs L-glutamate or L-aspartate produces an increase in the excitability of flexor efferents (Woolf and Wiesenfeld-Hallin, 1986), whereas competitive or noncompetitive NMDA antagonists reduce the facilitation of flexion reflexes induced by electrical (C-fiber) stimulation or cutaneous application of the chemical irritant mustard oil (Woolf and Thompson, 1991)

Secondary Hyperalgesia

In recent studies (Coderre and Melzack, 1991), we have examined the contribution of excitatory amino acids to the development of secondary hyperalgesia. In these studies, foot-withdrawal latencies from 48°C water were assessed in the same rats, prior to and following intrathecal administration of EAA receptor agonists and antagonists both before a thermal injury, and in the hindpaw contralateral to an injury. The injury was produced by immersing the left hindpaw in 55°C water for 15 s. As shown in Fig. 1, injury of a hindpaw produced significant hyperalgesia in the contralateral hindpaw of rats treated with intrathecal saline as a control. This hyperalgesia was unaffected by deafferentation of the injured hindpaw 15 min after injury, indicating that central, and not peripheral, changes underlie the contralateral hyperalgesia. A similar degree of hyperalgesia could be produced in uninjured rats by intrathecal treatment with NMDA or quisqualate, but not AMPA or kainic acid. Furthermore, contralateral hyperalgesia was prevented in rats pretreated with the NMDA receptor antagonist APV, but not by the non-NMDA EAA receptor antagonist CNQX. These results suggest that EAAs contribute to secondary hyperalgesia by acting at NMDA receptors in spinal cord dorsal horn.

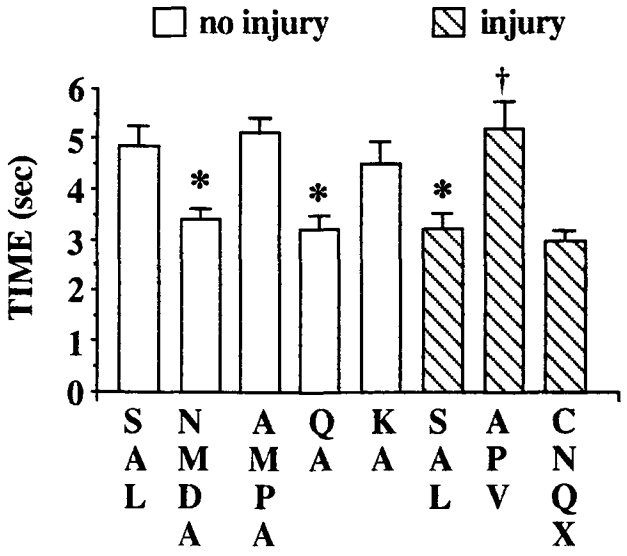


Fig. 1. Paw-withdrawal latencies (± SEM) from water at 48°C in uninjured rats and in the hindpaw contralateral to a thermal injury (paw immersed in water at 55°C for 15 s) in injured rats, after intrathecal pretreatment with excitatory amino acid receptor agonists and antagonists. Compared to uninjured saline-treated rats, there is a significant reduction in paw-withdrawal latencies in the hindpaw contralateral to an injury in injured saline-treated rats. Pretreatment with intrathecal NMDA or quisqualate (QA) in uninjured rats produced reductions in paw-withdrawal latencies similar to that seen in the contralateral hindpaw of injured rats. Intrathecal pretreatment with the NMDA antagonist APV, but not the non-NMDA EAA antagonist CNQX, produced a reversal of the hyperalgesia in the contralateral hindpaw of injured rats. (* significant difference from uninjured saline-treated group, $p < 0.01$; † significant difference from injured saline-treated group, $p < 0.01$). Data from Coderre and Melzack, 1991.

Persistent Nociception after Tissue Injury

Recent studies suggest that EAAs also contribute to persistent pain that develops following tissue injury induced by formalin injection. Subcutaneous injection of formalin into a rat's hindpaw evokes an increased release of glutamate and aspartate in spinal cord dorsal horn (Skilling et al., 1988; Sorkin et al., 1992), whereas the sustained responses of spinal nociceptive cells to noxious peripheral stimulation produced by sc formalin injection are reduced by intrathecal administration of selective NMDA antagonists (Haley et al., 1990). In our laboratory (Coderre, and Melzack, 1992a), we have examined the contribution of EAAs to the development of persistent pain in the formalin test. In these studies, rats were injected with 50 μL of 2.5% formalin

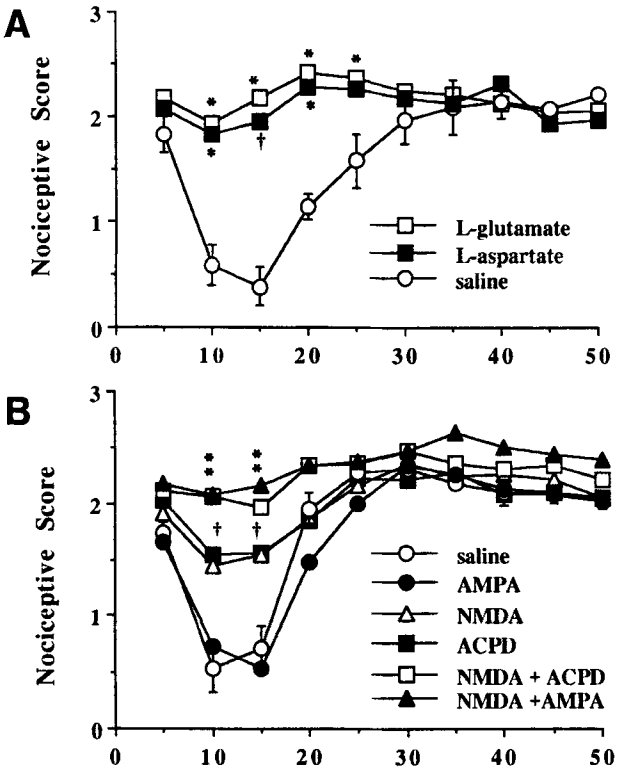
into the hindpaw 10 min after intrathecal injection of EAA agonists (L-glutamate, L-aspartate, NMDA, AMPA, and trans-ACPD) and antagonists (CNQX, AP-3, APV, and MK-801). As shown in Fig. 2A and B, pretreatment with L-glutamate, L-aspartate, NMDA, and trans-ACPD, but not AMPA, significantly enhanced nociceptive responses to formalin. In addition, we found that a combined treatment with half the dose of NMDA and either AMPA or trans-ACPD produced a significant enhancement in formalin responses over that produced by the full dose of either agent alone. This suggests there is an interactive effect of EAA action at both NMDA and non-NMDA receptor sites, and may reflect the fact that activation of non-NMDA receptors may be required to reduce a voltage-gated block of the NMDA receptor by extracellular Mg^{2+} (Mayer et al., 1984; Norwalk et al., 1984).

Consistent with the above findings, pretreatment with competitive (APV) and noncompetitive (MK-801) NMDA antagonists, but not the non-NMDA EAA antagonists CNQX or AP-3, significantly reduced formalin nociceptive responses during the formalin test (Fig. 2C). Importantly, NMDA antagonists more effectively relieve nociceptive responses in the late phase of the formalin test than the early phase, and are less effective if administered after the early phase is completed, a finding that has been supported by other investigators (Yamamoto and Yaksh, 1992; Vaccarino et al., 1992). It is our hypothesis that EAA transmitters (aspartate and glutamate) are released in response to noxious inputs associated with the early phase of the formalin test, and that predominantly through activity at the NMDA receptor these transmitters lead to the development of hypersensitivity in spinal cord dorsal horn, which contributes significantly to nociceptive responses in the late phase of the formalin test.

NMDA antagonists have also been particularly effective at reducing persistent pain produced by other stimuli. MK-801 reduces the hyperalgesia that develops in rats with peripheral neuropathy (Davar et al., 1991; Mao et al., 1992) or adjuvant-induced inflammation (Ren et al., 1991) and reduces autotomy behavior in rats with peripheral nerve sections (Seltzer et al., 1991). MK-801 also reduces the adjuvant inflammation-induced expansion of the receptive fields of nociceptive neurons in spinal cord dorsal horn (Dubner and Ruda, 1992). In humans, ischemic and postoperative pain is suppressed by subanesthetic doses of ketamine (Maurset et al., 1989), whereas in the rat, the increased activity in dorsal horn in response to ischemia associated with femoral artery occlusion is inhibited by intrathecal application of APV (Sher and Mitchell, 1990).

Interactions of EAAs and Neuropeptides

An interaction of EAAs and neuropeptides in central nociceptive processing is also suggested by several findings. Neuropeptides and EAAs are found to be colocalized in the central terminals of primary afferent neurons (DeBiasi and Rustioni, 1988). Substance P produces a prolonged enhancement of the responses of dorsal horn neurons to ionotophoretically applied glutamate (Willcockson et al., 1984b) or NMDA (Dougherty and Willis, 1991b). Combined treatment with substance P and NMDA produces a profound enhancement of the responses of dorsal horn neurons to nonnoxious and noxious mechanical stimulation (Dougherty and Willis, 1991b), as well as the behavioral responses to noxious chemical stimulation (Mjellen-Joly et al., 1992). These effects likely depend on both presynaptic and postsynaptic actions of neuropeptides on EAA neurotransmission since substance P, neurokinin A, or calcitonin gene-related peptide have been found to enhance the release of glutamate and aspartate from spinal cord dorsal horn (Kangrga and Randic, 1990; Smullin et al., 1990), whereas substance P produces a potentiation of glutamate- and NMDA-induced currents in rat spinal dorsal horn neurons in vitro (Randic et al., 1990). Recent evidence also indicates that the amount of substance P required to increase this EAA release is reduced in rats with peripheral neuropathy following partial sciatic nerve ligation (Skilling et al., 1992).



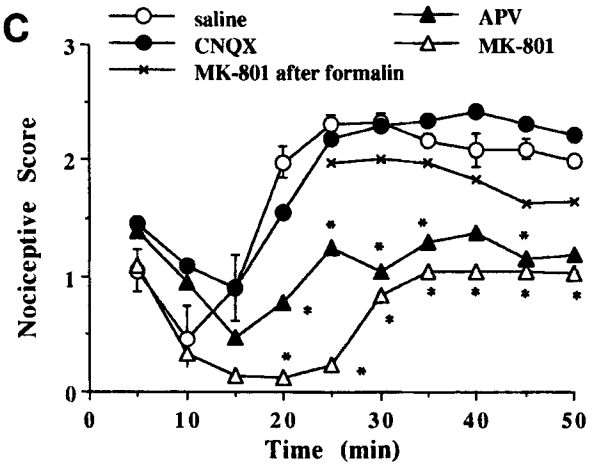


Fig. 2. Nociceptive responses (± SEM) to formalin injury in rats that were pretreated with intrathecal (A) endogenous EAAs, (B) receptor selective EAA agonists, and (C) receptor selective EAA antagonists. Nociceptive responses were significantly elevated by glutamate, aspartate (A), NMDA, trans-ACPD, NMDA + trans-ACPD, and NMDA + AMPA. The effects of NMDA + trans-ACPD and NMDA + AMPA were also significantly greater than that of NMDA, AMPA, or trans-ACPD alone. (B) Nociceptive responses were significantly reduced by the NMDA antagonists APV and MK-801, but not by the non-NMDA antagonist CNQX. (C) The antinociceptive effects of MK-801 were lost if MK-801 was given 10 min after formalin injection. (Significant differences from the saline control group, † $p < 0.05$, * $p < 0.01$). Modified from Coderre and Melzack, 1992a, with permission.

Cellular Mechanisms of Persistent Nociception

The above data suggest that EAAs may contribute to persistent pain following tissue injury. However, the manner by which these substances produce these central changes is not clear. It is possible that EAAs trigger alterations in membrane excitability through interactions with second messenger systems and protein kinases that phosphorylate membrane-bound proteins (Nestler and Greengard, 1983). Evidence suggests that there is a contribution of intracellular calcium (Ca^{2+}), second messenger systems, and protein kinases to the development of noxious stimulus-induced neuroplasticity.

Intracellular Calcium

Recent evidence suggests that noxious stimulation results in an increase of intracellular Ca^{2+}, which influences the excitability of the cell. Neurotransmitters released in response to noxious stimulation are known to affect the intracellular levels of Ca^{2+}. Glutamate and aspartate stimulate the influx of Ca^{2+} through NMDA receptor-operated channels (MacDermott et al., 1986), as well as increasing Ca^{2+} influx through voltage-gated Ca^{2+} channels associated with AMPA/kainate receptors (Murphy and Miller, 1989), and a mobilization of Ca^{2+} from internal stores after activation of metabotropic receptors (Berridge and Galione, 1988).

Studies assessing the behavioral effects of agents affecting Ca^{2+} availability suggest that Ca^{2+} influx is more critical in persistent pain models where central sensitization and plasticity are present. These studies indicate that brief, phasic nociceptive tests, such as the tail-flick and hot-plate tests, are unaffected by Ca^{2+} (Harris et al., 1975; Chapman and Way, 1982), Ca^{2+} chela-

tors (Harris et al., 1975; Ben-Sreti et al., 1983), or Ca^{2+} channel antagonists (Benedek and Szikszay, 1984; Contreras et al., 1988), whereas tonic nociceptive tests, such as the formalin and acetic acid-induced writhing tests, are sensitive to Ca^{2+}, Ca^{2+} ionophores, and Ca^{2+} channel agonists (Chapman and Way, 1982; Coderre and Melzack, 1992b) that increase nociceptive responses, or Ca^{2+} chelators and Ca^{2+} channel antagonists (Coderre and Melzack, 1992b; Del Pozo et al., 1987; Miranda et al., 1992) that reduce nociceptive responses.

Recently, we have assessed the effects on formalin-induced nociceptive behaviors of intrathecal treatment with agents affecting intracellular levels of Ca^{2+} (Coderre and Melzack, 1992b). These studies compared the effects of a Ca^{2+} ionophore (A23187), a Ca^{2+} chelator (Quin 2), a Ca^{2+} channel activator (Bay K8644), and Ca^{2+} channel antagonists (nifedipine and verapramil) on nociceptive response to formalin injury. As shown in Fig 3A, increasing intracellular Ca^{2+} by treatment with either the Ca^{2+} ionophore A2187 or the Ca^{2+} channel activator Bay K8644 produced a dramatic increase in formalin nociceptive responses. Conversely, blocking increases in intracellular Ca^{2+} with either a Ca^{2+} chelator Quin 2 or the Ca^{2+} channel antagonists nifedipine and verapramil produced a reduction in formalin nociceptive responses.

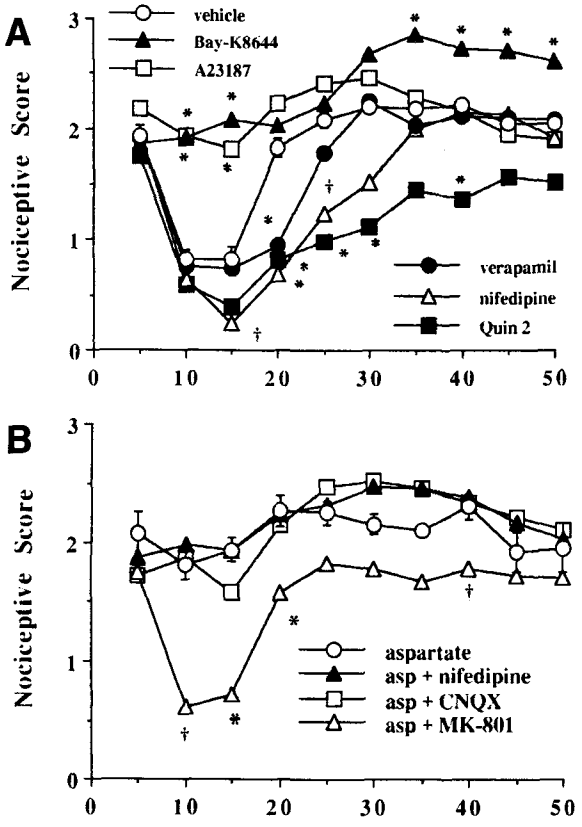
Although the behavioral effects of agents affecting Ca^{2+} availability may depend, in part, on the effect of Ca^{2+} influx on presynaptic transmitter release, it is possible that their effects may also depend on Ca^{2+} influx in the postsynaptic cell. Although nociceptive responses to formalin are significantly suppressed by voltage-gated Ca^{2+} channel antagonists, they are more effectively suppressed by Ca^{2+} chelators that reduce all available extracellular Ca^{2+} (not just that which enters through voltage-gated Ca^{2+} channels) (Fig. 3A), and noncompetitive NMDA antagonists that block Ca^{2+} influx through NMDA receptor-operated Ca^{2+} channels (Fig. 2C). Furthermore, we (Coderre and Melzack, 1992b) have shown that the enhancement of nociceptive responses to formalin injury following intrathecal treatment with L-aspartate or L-glutamate is prevented by agents that block NMDA receptor-operated Ca^{2+} channels, but not by those that block voltage-gated Ca^{2+} channels (Fig. 3B and C). These data suggest an important role of Ca^{2+} influx into postsynaptic cells, particularly through NMDA receptor-operated channels (MacDermott et al., 1986), to the development of persistent nociception after formalin injury.

Intracellular Second Messengers

In addition to increases in intracellular calcium, EAA receptor activation produces an activation of various intracellular second messengers. The influx of Ca^{2+} through NMDA receptor channels activates nitric oxide (NO) synthase, which generates NO from free L-arginine (Garthwaite et al., 1988), which in turn activates soluble guanylate cyclase and increases cGMP (Southam et al., 1991). The Ca^{2+} influx also activates neuronal phospholipase A_2, which triggers the production of arachidonic acid (Dumuis et al., 1988, 1990), which is metabolized into various eicosanoids. In addition, activity at metabotropic receptors triggers an increase in polyphosphoinositide metabolism, resulting in the production of inositol trisphosphate (Sladeczek et al., 1985), which leads to the release of Ca^{2+} from intracellular stores (Murphy and Miller, 1988), as well as diacylglycerol, which stimulates the translocation and activation of protein kinase C (pKC) (Manzoni et al., 1990), as well as producing additional arachidonic acid when metabolized (Gammon et al., 1989).

Nitric Oxide

In the central nervous system, NO is released in response to increases in intracellular Ca^{2+} following activation of NMDA receptors. After stimulation by aspartate or glutamate, NMDA receptors open ion channels to admit extracellular Ca^{2+}, intracellularly the Ca^{2+} binds to calmodulin that activates NO synthase. NO synthase converts L-arginine to NO, and the released NO stimulates the production of cGMP from soluble guanylate cyclase (Synder, 1992). In addition to its function in the regulation of ion channels (Doerner and Alger, 1988), the NO stimulated



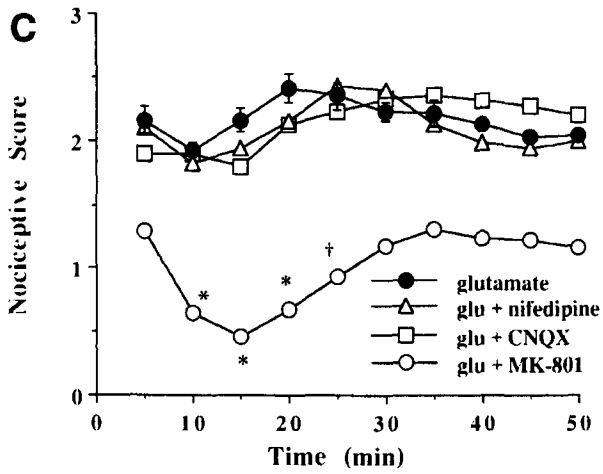


Fig. 3. (**A**) Nociceptive responses (± SEM) to formalin injury in rats pretreated intrathecally with a calcium channel activator (Bay K-8644), a calcium ionophore (A23187), a calcium chelator (Quin 2), and calcium channel antagonists (verapramil, nifedipine). Nociceptive responses were increased compared to controls in rats pretreated with Bay K-8644 and A23187, and were decreased by verapramil, nifedipine, and Quin 2. (Significant differences from the vehicle control group,† $p < 0.05$, * $p < 0.01$). (**B** and **C**) Nociceptive responses (± SEM) induced by formalin in rats pretreated with intrathecal L-aspartate (B) or L-glutamate (C) alone or in combination with nifedipine, CNQX, or MK-801. Only MK-801 was capable of reversing the increase in nociceptive responses produced by L-aspartate or L-glutamate. (Significant differences from the L-aspartate or L-glutamate group, † $p < 0.05$, * $p < 0.01$). Modified from Coderre and Melzack, 1992b, with permission.

cGMP has been proposed to contribute to EAA-induced neurotoxicity (Dawson et al., 1991), and has been proposed as a mediator in two models of synaptic plasticity, long-term depression (Shibuki and Okuda, 1991), and long-term potentiation (Haley et al., 1992b).

Evidence that NO and cGMP contribute to nociceptive processing is indicated by finding that NO synthase inhibitors and inhibitors of soluble guanylate cyclase produce analgesic effects in various nociceptive tests. Moore et al. (1991) demonstrated that systemic and icv treatment with L-N^G-nitro arinine methyl ester (L-NAME) produced analgesia in the formalin test, whereas systemic L-NAME produced analgesia in the acetic acid-induced abdominal constriction model. Of interest, L-NAME pretreatment produced more significant analgesic effects in the late phase of the formalin test than in the early phase, suggesting that NO may be involved in the development of persistent pain associated with central sensitization. This possibility is reinforced by the finding of Meller et al. (1992a), who demonstrated that L-NAME and a soluble guanylate cyclase inhibitor (Methylene Blue) block the facilitation of tail-flick reflex induced by intrathecal NMDA. In addition, Haley et al. (1992) demonstrated that L-NAME reduced the persistent increase in dorsal horn neuron activity following sc adminstration of formalin. Furthermore, it has recently been shown that both L-NAME and Methylene Blue reduce the thermal hyperalgesia that develops in rats with chronic nerve constriction injury (Meller et al., 1992b). In our own studies (Yashpal and Coderre, 1993), we have demonstrated that intrathecal treatment with sodium nitroprusside, which degrades into NO, produces an enhancement of nociceptive responses to formalin, whereas L-NAME pro-

duces antinociceptive effects, as well as a reduction in the hyperalgesic effects of glutamate, in the formalin test.

Arachidonic Acid

EAA activity also results in the stimulation of arachidonic acid. The increase in intracellular Ca^{2+} by influx of extracellular Ca^{2+} through NMDA receptor operated Ca^{2+} channels (Dumuis et al., 1988), as well as its mobilization from internal stores after activation of metabotropic EAA receptors, produces a phospholipase A_2-catalyzed stimulation of arachidonic acid (Dumuis et al., 1990). Further increases in arachidonic acid occur following the breakdown of diacyclygerol (Gammon et al., 1989), which is produced following stimulation of phospholipase C with activity at metabotropic EAA receptors. A role of intracellular arachidonic acid in the development of neuronal plasticity is consistent with the finding that arachidonic acid regulates a number of ionic conductances either directly (Kim and Clapham, 1989), or following its metabolism into various eicosanoids (Kurachi et al., 1989). Evidence for a role of arachidonic acid in synaptic plasticity is suggested since arachidonic acid has been found to induce a long-term activity-dependent enhancement of synaptic transmission in the hippocampus (Williams et al., 1990a).

Evidence suggest that arachidonic acid metabolites also contribute to nociceptive processing. Although antinociceptive effects associated with inhibition of cyclooxygenase metabolism of arachidonic acid with nonsteroidal anti-inflammatory drugs (NSAIDs) are typically attributed to their peripheral anti-infammatory action, recent evidence also indicates that spinal intrathecal administration of NSAIDs produces analgesia in animals subjected to intraperitoneal (acetic acid) or sc (formalin) injection of irritant chemicals (Yaksh, 1982; Malmberg and Yaksh, 1992a). Like NO synthase inhibitors, the intrathecal NSAIDs are more effective at inhibiting the late phase of the formalin response than they are at inhibiting the early phase. Once again this suggests that prostanoids may be involved in the development of persistent pain associated with central sensitization. This notion is supported by recent findings that intrathecal NSAIDs block the facilitation of the tail-flick response induced by intrathecal treatment with NMDA or substance P (Malmberg and Yaksh, 1992b). In our own studies (Yashpal and Coderre, 1993), we have demonstrated that intrathecal treatment with arachidonic acid produces an enhancement of nociceptive responses to formalin, whereas inhibition of arachidonic acid with dexamethasone produces antinociceptive effects, as well as a reduction in the hyperalgesic effects of glutamate, in the formalin test.

Protein Kinase C

Activity at metabotropic EAA receptors by glutamate and aspartate (Murphy and Miller, 1988; Sugiyama et al., 1987) also stimulates the hydrolysis of ionositol phospholipids by activating a polyphosphoinositide-specific PLC. PLC is an enzyme that catalyzes the hydrolysis of the phospholipid polyphosphatidylinositol into the intracellular messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). Following its production, IP_3 stimulates the release of Ca^{2+} from internal stores; on the other hand, DAG stimulates the translocation and activation of protein kinase C (pKC). When activated by DAG, pKC phosphorylates specific substrate proteins that contribute to various cellular processes, including neurotransmitter release and transduction (Nishizuka, 1986). Stimulation of pKC with phorbol esters or synthetic DAG, or the intracellular microinjection of pKC, have been found to enhance Ca^{2+} currents (DeRiemer et al., 1985), which may increase neuronal excitability as well as presynaptic transmitter release, and to reduce both Ca^{2+}-dependent K^+ currents (Alkon et al., 1986) and Cl^- currents (Madison et al., 1986), which may result in the prolongation of depolarization and afterdischarges associated with an inhibiton of spike accomodation. These changes in ionic conductances may underlie long-term changes in synaptic plasticity, and may account for finding that pKC contributes to long-term potentiation in the hippocampus (Malenka et al., 1986; Hu et al., 1987).

Recently, Gerber et al. (1989) have shown that activators of pKC enhance the basal and evoked release of glutamate and aspartate in the spinal cord slice, as well as the depolarizing responses of dorsal horn neurons to exogenous glutamate and NMDA. Furthermore, Chen and Huang (1992) have demonstrated that pKC increases NMDA activated currents in isolated trigeminal cells by increasing the probability of channel openings and by reducing the voltage-dependent Mg^{2+} block of NMDA-receptor channels. The resultant increased activity at the NMDA receptor would permit more Ca^{2+} ions to enter the cell, raising intracellular Ca^{2+}, and so further increasing pKC activity and its effects on NMDA receptor channels. This positive feedback loop may be important for the induction or maintenance of sensitization in central neurons.

It has been shown recently that both activation of metabotropic EAA receptors with quisqualate or trans-ACPD produces an enhancement of NMDA currents in both hippocampal neurons (Anikstejn et al., 1991; Ben-Ari et al., 1992) and oocytes injected with rat brain RNA (Kelso et al., 1992). Furthermore, since in both these studies the effects of metabotropic EAA receptor activation were blocked by pKC inhibitors, it is likely that activity at the metabotropic receptor also enhances NMDA currents by stimulating intracellular pKC.

In recent studies (Yashpal and Coderre, 1993; Coderre, 1992) we have assessed the role of phosphoinositide hydrolysis to the development of persistent nociception following formalin injury. In these studies, we assessed the effects of agents that inhibit the phospholipase C (neomycin) or pKC (pKC [19–36] H-7) activity, as well as agents that stimulate pKC (phorbol esters, SC-10). As shown in Fig. 4, it was found that inhibiting phospholipase C activity with neomycin produces a substantial reduction in nociceptive responses during the formalin test. In addition, formalin nociceptive responses were enhanced by the phorbol ester PMA and the pKC stimulant SC-10, suggesting that pKC is involved in the development of persistent pain after formalin injury. Furthermore, nociceptive responses to formalin injury were suppressed following

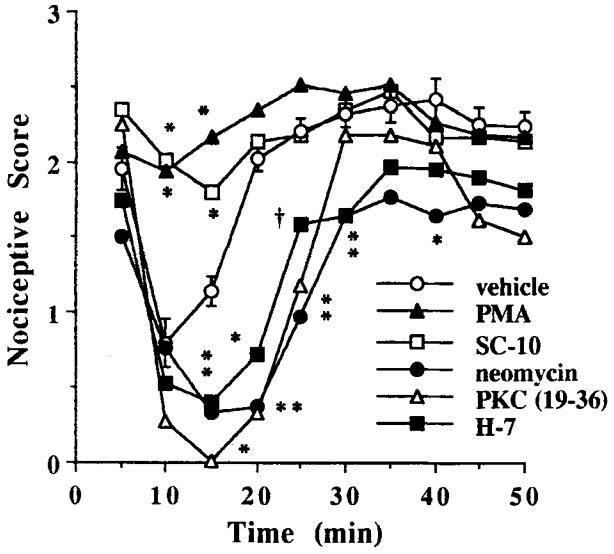


Fig. 4. Nociceptive responses (± SEM) to formalin injury in rats pretreated with a phorbol ester (phorbol 12-myristate 13-acetate, PMA), a protein kinase C (pKC) activator (SC- 10), an inhibitor of phospholipase C (PLC) (neomycin), and inhibitors of pKC (pKC [19-36] and H-7). Nociceptive responses were significantly increased with agents that increase pKC activity and decreased with agents that reduce PLC or pKC activity. (Significant differences from the vehicle control group, † $p < 0.05$, * $p < 0.01$). Modified from Coderre, 1992, with permission, additional data from Yashpal and Coderre, 1993.

intrathecal treatment with pKC (19–36) and H-7, which inhibit pKC activity. These results suggest that pKC contributes to persistent nociceptive response elicited by sc injection of formalin. Furthermore, a contribution of pKC to persistent pain is consistent with the findings of Hayes et al. (1992), who demonstrated that monosialoganglioside, which inhibits the translocation of pKC (Vaccarino et al., 1987), reduces behavioral hyperalgesia in rats with peripheral neuropathy.

Molecular Mechanisms of Persistent Nociception

In addition to altering membrane permeability, increases in intracellular Ca^{2+} and the activation of pKC result in the increased expression of

immediate-early genes such as c-*fos* (Morgan and Curran, 1991). The protein products of these immediate-early genes (e.g., Fos) act as third messengers that are believed to be involved in the transcriptional control of genes that encode a variety of neuropeptides, including enkephalins and tachykinins.

Expression of c-fos and Other Immediate-Early Genes

Noxious stimulation leads to the expression of immediate-early genes and their protein products. Hunt et al. (1987) first demonstrated that the c-*fos* protein product Fos is expressed in postsynaptic dorsal horn neurons following noxious thermal or chemical stimulation of the skin. The expression of Fos has also been demonstrated in rat spinal dorsal horn in response to noxious pinch of the hindpaws (Bullit, 1989), the injection of formalin (Presley et al., 1990; Kehl et al., 1991) or carageenan (Draisci and Iadorola, 1989) into a hindpaw or sodium urate crystals into joints (Menétrey et al., 1989), the injection of acetic acid into viscera (Menétrey et al., 1989), the induction of polyarthritis with Freund's adjuvant (Menétrey et al., 1989), and the development of a neuroma following nerve injury (Chi et al., 1990). Noxious stimulation also leads to the spinal cord expression of other immediate-early gene products, including Fos B, Jun, Jun B, Jun D, NGF1-A, NGF1-B, and SRF (Herdegen et al., 1990a,b; Wisden et al., 1990; Herdegen et al., 1991a,b,c). Furthermore, following noxious stimulation there is an expression of Fos in CNS structures involved in pain transmission, including the periaqueductal gray, thalamus, habenula, and somatosensory cortex (Bullit, 1989; Herdegen et al., 1991b; Iadorola et al., 1988). Importantly, there is a strong correlation between pain behavior and the number of cells expressing Fos (Presley et al., 1990). Moreover, morphine pretreatment produces a dose-dependent suppression of Fos expression which corresponds with its analgesic effects (Presley et al., 1990; Tolle et al., 1991).

Triggers of c-fos Expression in Neuronal Cells

Since an elevation of intracellular Ca^{2+} is crucial to the transcriptional activation of the c-*fos* gene (Morgan and Curran, 1986, *see also* Morgan and Curran, 1991 for general review of immediate-early genes), it is possible that c-*fos* is induced in spinal cord dorsal horn following Ca^{2+} entry through NMDA receptor-operated Ca^{2+} channels, or through voltage-gated Ca^{2+} channels following the activation of AMPA/kainate receptors. Indeed, NMDA receptor activation with glutamate or NMDA leads to increases in c-*fos* mRNA or Fos protein in rat cerebellar granule cells (Szekely et al., 1989), dentate gyrus (Lerea et al., 1992), and cortical neurons (Hisanaga et al., 1992) in culture. Furthermore, the effect of glutamate is blocked by competitive or noncompetitive NMDA receptor antagonists, or by Mg^{2+}, which blocks NMDA-receptor-operated Ca^{2+} channels (Szekely et al., 1989; Lerea et al., 1992). Although c-*fos* activity can also be elevated by increasing Ca^{2+} availability with the Ca^{2+} ionophore ionomycin (Szekely et al., 1989; Lerea et al., 1992) or reduced by the chelation of extracellular Ca^{2+} with EGTA (Lerea et al., 1992), the importance of NMDA-receptor operated Ca^{2+} channels is indicated by the finding that the glutamate or NMDA-induced increase in c-*fos* expression is unaffected by nifedipine or nitrendipine, selective blockers of voltage-gated Ca^{2+} channels (Szekely et al., 1989; Lerea et al., 1992).

Although it has been demonstrated that noxious stimulation-induced expression of Fos in the spinal dorsal horn is substantially reduced by pretreatment with the NMDA receptor antagonist MK-801 (Kehl et al., 1991; Birder and de Groat, 1992), others have found that NMDA antagonists do not affect the distribution of Fos labeled neurons in spinal cord (Wisden et al., 1990; Tolle et al., 1991). This discrepancy may depend on differences in the nature of the noxious stimulus applied to the periphery (chemical vs thermal stimulation), or specific differences in experimental methods, such as the type of anesthetic agent used.

Recently, Lerea et al. (1992) have demonstrated that c-*fos* mRNA is also induced in dentate gyrus neurons following the activation of kainate/AMPA receptors with kainic acid. Although both NMDA- and kainic acid-induced c-*fos* expression were eliminated by chelation of extracellular Ca^{2+} with EGTA, only kainic acid-induced c-*fos* expression was reduced with the voltage-gated Ca^{2+} channel blocker nifedipine. These findings suggest that c-*fos* induction depends on increases in intracellular Ca^{2+} that results from an influx of Ca^{2+} through either NMDA receptor-operated Ca^{2+} channels after NMDA receptor activation, or through voltage-gated Ca^{2+} channels after activation of non-NMDA EAA receptors.

There is also an increase in c-*fos* mRNA in cerebellar granule cells in response to non-NMDA EAA receptor agonists (Szekely et al., 1987), such as quisqualate. Quisqualate acts at metabotropic glutamate receptor sites to stimulate the second messengers IP_3 and DAG following the activation of PLC (Murphy and Miller, 1988; Sugiyama et al., 1987). Furthermore, c-*fos* expression is also induced in cultures of neuronal cells (Naranjo et al., 1991) by stimulation of pKC with phorbol esters or DAG. Interestingly, the ability of quisqualate to induce the expression of c-*fos* in cerebral granule cells is lost when Mg^{2+} is added to block NMDA receptor-operated Ca^{2+} channels (Szekely et al., 1989). This suggests that the expression of c-*fos* depends on an interaction between Ca^{2+} influx through NMDA receptor-operated Ca^{2+} channels and the stimulation of second messengers, such as DAG, that are linked to metabotropic receptors and activate pKC (Nishizuka, 1986). Perhaps this is not surprising, since the DAG-induced translocation and activation of pKC depends on an accompanying Ca^{2+} influx (*see* Rasmussen, 1986). Thus, it is possible that the expression of early immediate genes, such as c-*fos*, is induced by Ca^{2+} influx and the translocation and stimulation of pKC, following the activation of both NMDA and metabotropic receptors, which by activating PLC stimulate the production of DAG. These molecular events may trigger prolonged cellular changes (i.e., protein phosphorylation and new gene expression) which could account for a long-lasting sensitization of central cells and behavioral signs of persistent pain or hyperalgesia.

Relationship Between c-fos and Persistent Nociception

Although there is evidence that noxious stimulation leads to the expression of Fos, this does not necessarily mean that c-*fos* is involved in central sensitization and persistent nociception. However, there is growing evidence of a relationship between noxious stimulus-induced Fos expression, central sensitization, and behavioral hyperalgesia. First, the noxious stimuli that produce Fos expression (heat injury, formalin injection, and inflammatory agents) also produce behavioral hyperalgesia, which is associated with central neuroplasticity (Coderre and Melzack, 1987; Kayser and Guilbaud, 1987; Coderre et al., 1990). Second, the time course of Fos expression coincides with the development of behavioral hyperalgesia. This cooccurrence is evident in cases where noxious inputs are driven by a specific peripheral lesion. For example, peripheral inflammation induced by carrageenan produces both an increase in c-*fos* mRNA and behavioral hyperalgesia that peak over a similar time course (Draisci and Iadorola, 1989).

However, the cooccurrence of Fos expression and behavioral hyperalgesia is also evident in cases where a peripheral stimulus initiates but does not apparently maintain the hyperalgesia. Thus, heat injury of a rat's hindpaw produces an immediate hyperalgesia in the injured hindpaw and hyperalgesia in the uninjured contralateral hindpaw, which develops between 4–24 h after injury (Coderre and Melzack, 1987, 1991). Similarly, heat injury of a rat's hindpaw, or C-fiber stimulation of the sciatic nerve, not only produces an immediate expression of Fos in the spinal cord dorsal horn ipsilateral to the injury, but also produces a "second wave" of Fos (Williams et al., 1990), Jun (Herdegen et al., 1991a), Jun D (Herdegen et al., 1991c), and NGF1A (Herdegen et al., 1990b) activity in both ipsilateral and contralateral dorsal horns 4–24 h after the injury. An

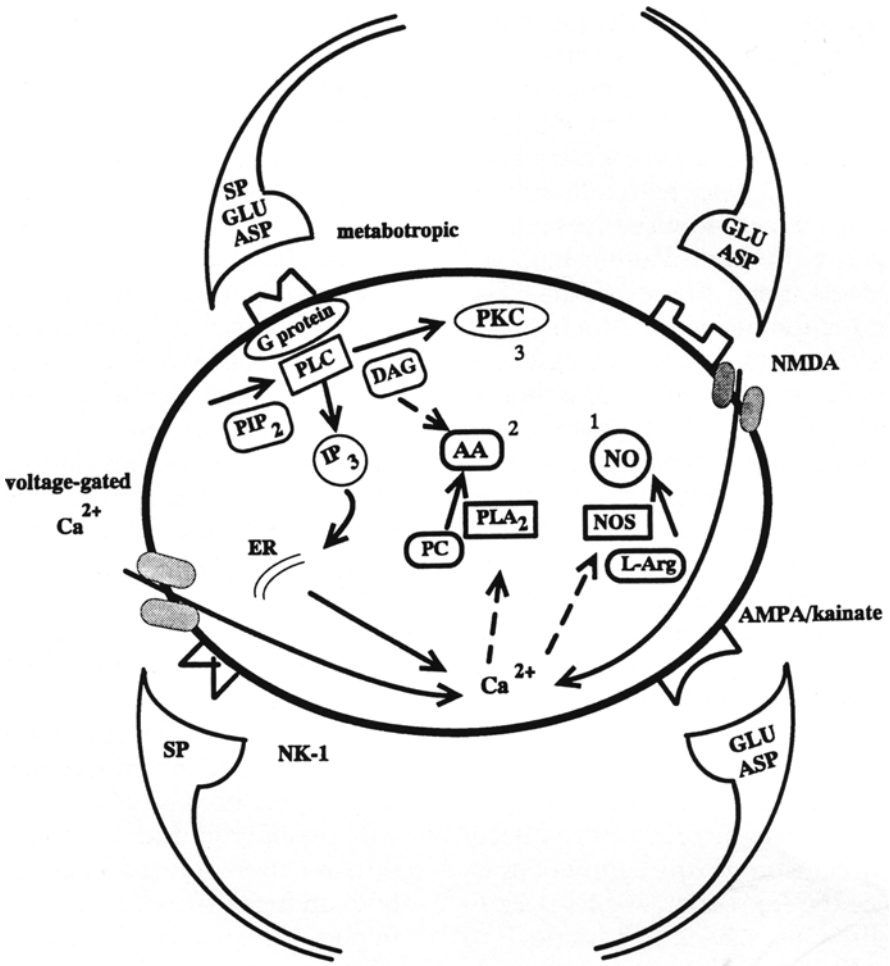


Fig. 5. Schematic diagram indicating possible mechanisms by which noxious stimulation or injury leads to central sensitization of spinal cord dorsal horn neurons and contributes to persistent nociception. High levels of afferent input cause the release of aspartate, glutamate, and substance P (SP) within the dorsal horn. Repetitive fast-transmitter activity of aspartate and glutamate at AMPA/kainate receptors produces a membrane depolarization that would counter a voltage-dependent blockade of the NMDA receptor by Mg^{2+}. Activation of neurokinin-1 (NK-1) receptors by SP produces a slow, prolonged depolarization and enhances influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels. A further action of aspartate and glutamate at NMDA and metabotropic receptors, respectively, would produce an influx of Ca^{2+} (through NMDA receptor-operated Ca^{2+} channels), and the activation of phospholipase C (PLC). PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) producing ionositol trisphosphate (IP_3) and diacyclglycerol (DAG). The production of IP_3 causes the release of Ca^{2+} from intracellular stores within the endoplasmic reticulum (ER). The increases in intracellular Ca^{2+} produced by the influx of Ca^{2+} through voltage-gated channels, NMDA receptor-operated channels, and by the release of Ca^{2+} from internal stores, stimulates the nitric oxide (NO) synthase-induced conversion of L-arginine to NO and the phospholipase A_2 (PLA_2)-induced release of arachidonic acid (AA). The production of DAG further stimulates AA release and induces the translocation and activation of protein kinase C (pKC), which is activated during high rates of Ca^{2+} influx. Activation of NO, AA, and pKC induces sustained alterations in the cellular membrane affecting membrane permeability for prolonged periods. pKC also interacts with Ca^{2+} to stimulate increases in the expression of the immediate early genes c-*fos* and c-*jun*. The protein products of these immediate early genes participate in the regulation of mRNA encoding various peptides in spinal cord, and can influence long-term changes in cellular function.

association of the behavioral hyperalgesia and Fos expression with neural plasticity after heat injury is suggested since both the contralateral hyperalgesia (Coderre and Melzack, 1987) and the Fos expression in the contralateral dorsal horn (Williams et al., 1990b) still develop when the injured hindlimb is locally anesthetized shortly after the injury. Furthermore, Herdegen et al. (1990a) have shown that although "low level" noxious cutaneous stimulation of one hindpaw induced Fos in only a few neurons, the same stimulus repeated 1 h later in the contralateral hindpaw induced dense Fos labeling in many neurons. The "second wave" expression of immediate-early genes in the contralateral dorsal horn, and the enhanced expression of Fos if there has been prior noxious stimulation of the contralateral hindpaw, are consistent with the idea that central sensitization results in the spread of hyperalgesia to a limb contralateral to a heat injury. Interestingly, although heat injury of a rat's hindpaw leads to the development of both contralateral hyperalgesia and a second wave of immediate-early gene products in the contralateral dorsal horn, inflammatory lesions with formalin (Coderre et al., 1990) or carrageenan (Hargreaves et al., 1988) produce minimal contralateral behavioral responses and little or no immediate-early gene activity in the contralateral dorsal horn (Herdegen et al., 1991c; Williams et al., 1990; Noguchi et al., 1991, 1992).

Conclusions

In addition to peripheral changes, tissue injury or noxious stimulation leads to changes in central neuronal function that contribute to persistent pain and hyperalgesia. The evidence presented suggests that EAA receptors and intracellular messengers to which they are coupled play a significant role in the development of noxious stimulus-induced plasticity and the persistent nociception it causes. As illustrated in Fig. 5, we propose that intense noxious stimulation leads to a release of EAA transmitters (Skilling et al., 1988) within spinal dorsal hom. Following the activation of ionotropic (NMDA) and metabotropic receptors, these transmitters produce an increase in intracellular Ca^{2+}, NO, phospholipase A_2, and phospholipase C, leading to the activation or production of soluble guanylate cyclase, arachidonic acid, and polyphosphoinosites (IP_3, DAG) (Garthwaite et al., 1988; Dumuis et al., 1988; Sladeczek et al., 1985), which in turn trigger the release or production of cGMP, ecosanoids, and protein kinase C, respectively (Southam et al., 1991; Manzoni et al., 1990; Gammon et al., 1989). The activation of each of these second messengers is proposed to produce intracellular changes, including the phosphorylation of substrate proteins and membranebound receptor channels (Mayer and Miller, 1991). Importantly, it has been suggested that Ca^{2+} influx, as occurs following NMDA receptor activation, is required to transform protein kinase C to its activated state (Rasmussen, 1986). The activated protein kinase C would not only contribute to membrane phosphorylation, but along with Ca^{2+}, would trigger the production of immediate-early genes such as c-*fos* (Naranjo et al., 1991). This chain of events would lead to prolonged alterations in the neurons' responses to further stimulation, and would thus account for the ability of a brief injury or intense noxious stimulus to exert a long-term influence on subsequent pain sensitivity.

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