(*2, 3*). As pointed out above, however, there is less discrepancy in the results than in the conclusions, for in these experiments lymphocyte activities were studied in vitro, whereas we have assessed tissue tolerance in vivo. Results similar to ours were obtained in several other systems (*10*), including manipulations of amphibian embryos (*11*), also tested by tissue grafts. The fact that nude mice engrafted with an allogeneic thymic epithelium may produce T cells that respond to donor lymphocytes, yet simultaneously accept skin grafts (referred to as "split tolerance"), also suggests that a complete clonal deletion is not necessary for inducing physiological tolerance to tissue grafts. Split tolerance, which extends to divergent reactivities in proliferative versus cytolytic tests (*12*), has been frequently observed, and the very use of the term indicates the unjustified comparison of heterogeneous categories of phenomena, as well as our current limitations in the understanding of these processes. "Peripheral tolerance" without thymic clonal deletion has been shown in transgenic mouse models (*13*) and embryonic allogeneic grafts (*14*). All of this evidence indicates that, in addition to the well-established process of clonal deletion in the thymus (*15*), other mechanisms, such as clonal "anergy" or "suppression," exist that do not involve the thymus. These prevent autoimmune aggression by self-reactive clones that escaped thymic negative selection.

Our experimental model is a complement to other systems that address the problem of self-nonself descrimination. Thus, it allows for a quasi-normal ontogeny of immunocompetent cells, exposing them to allogeneic antigens that are exclusively expressed by thymic epithelium. Transgenic mouse models targeting expression of MHC antigens to selected tissues have shown some degree of "leakiness" (*16*) and are limited to the genes isolated thus far. In contrast, the appropriate choice of thymic epithelium donor and nude recipient strain combinations provides a means for addressing any antigenic differences. Furthermore, by varying the age of euthymic recipients of E10 thymic epithelium, it is possible to test the importance of developmental state of the host immune system on the ability of thymic epithelial grafts to induce tolerance. Finally, interactions between lymphocytes that have developed in different MHC thymic environments can also be investigated by multiple allogeneic thymic epithelial grafts into nude mice.

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The Calcium Channel Blocker Nifedipine Attenuates Slow Excitatory Amino Acid Neurotoxicity

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High concentrations of potent *N*-methyl-D-aspartate (NMDA) agonists can trigger degeneration of cultured mouse cortical neurons after an exposure of only a few minutes; in contrast, selective non-NMDA agonists or low levels of NMDA agonists require exposures of several hours to induce comparable damage. The dihydropyridine calcium channel antagonist nifedipine was used to test whether this slow neurotoxicity is mediated by a calcium influx through voltage-gated channels. Nifedipine had little effect on the widespread neuronal degeneration induced by brief exposure to high concentrations of NMDA but substantially attenuated the neurotoxicity produced by 24-hour exposure to submaximal concentrations of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate, kainate, or quinolinate. Calcium ion influx through dihydropyridine-sensitive, voltage-dependent calcium channels may be an important step in the neuronal injury induced by the prolonged activation of NMDA or non-NMDA glutamate receptors.

EXPOSURE TO EXCESS GLUTAMATE OR related compounds can destroy neurons in the central nervous system (*1*) and may be responsible for neuronal loss in some neurological diseases (*2*). High concentrations of glutamate or selective NMDA agonists can trigger neuronal degeneration after an exposure of only a few minutes (*3–5*), possibly due to excess Ca^{2+}

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influx through NMDA receptor–activated channels (*6, 7*). Such rapidly triggered neurotoxicity may contribute to the acute brain injury associated with hypoxia-ischemia (*8*), prolonged seizures (*9*), or trauma (*10*).

In contrast, even high concentrations of agonists selectively active at non-NMDA glutamate receptors, such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) or kainate (*11*), require exposures of several hours to destroy substantial numbers of cultured cortical neurons (*3, 12, 13*). Similar prolonged exposure is needed for the neurotoxicity of the weak NMDA agonist quinolinate (*14*). NMDA receptor–mediated toxicity has been postulated to cause striatal neuronal loss in Huntington's disease (*15*). Non-NMDA receptors mediate the toxic injury induced by ingestion of the lathyrus toxin β-*N*-oxalylaminoalanine (*16*) and the shellfish toxin domoate (*17*) and might also have a role in amyotrophic lateral sclerosis or Alzheimer's disease (*18*).

Like rapid NMDA receptor–mediated neurotoxicity, slow neurotoxicity may be mediated by excess Ca^{2+} influx. Kainate neurotoxicity in hippocampal cultures (*19*) and cerebellar slices (*20*) is dependent on the presence of extracellular Ca^{2+}. However, unlike channels gated by NMDA receptors, channels gated by non-NMDA receptors exhibit little permeability to Ca^{2+} (*7*). A more likely pathway for toxic Ca^{2+} entry into neurons exposed to non-NMDA agonists is voltage-gated Ca^{2+} channels, activated by Na^{+} entry and resultant membrane depolarization. We tested the hypothesis that the voltage-gated Ca^{2+} channel blocker nifedipine would not attenuate the neocortical neuronal damage induced by intense exposure to NMDA agonists but would attenuate slow excitotoxicity, at least that induced by non-NMDA agonists (*21*).

Mixed cortical cell cultures, containing both neuronal and glial elements, were prepared from fetal mice at 14 to 17 days gestation (*3*). After dissociation, cells were plated in media consisting of Eagle's minimal essential medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, 2 m*M* glutamine, and glucose (total, 21 m*M*). Cultures were exposed to excitatory amino acids after 15 to 21 days in vitro (*22*). One day (20 to 24 hours) after exposure initiation, neuronal injury was assessed by morphological examination and the measurement of lactate dehydrogenase (LDH) released by damaged cells to the bathing medium (*23*).

Exposure to either 10 μ*M* AMPA or 30 μ*M* kainate for 20 to 24 hours reliably destroyed somewhat more than half of the neuronal population (Fig. 1). When 100 μ*M* nifedipine in dimethyl sulfoxide (DMSO) (final DMSO concentration, 1%) was included in the media during the exposure, there was a marked attenuation of this neuronal damage (Fig. 1) (>15 experiments); control experiments showed that DMSO itself did not alter neurotoxicity. This protection was concentration-dependent between 3 and 100 μ*M* nifedipine (Fig. 2) (>5 experiments).

In contrast, nifedipine provided little protection against the neurotoxicity resulting from a 5-min exposure to 500 μ*M* concentrations of the NMDA agonists glutamate, NMDA, or homocysteate (HCA) (*5, 24*) (Fig. 3A). However, nifedipine did partially attenuate the neuronal loss and LDH efflux resulting from prolonged submaximal exposures to the endogenous weak NMDA agonist quinolinate (*14*). Protection against quinolinate toxicity was somewhat less and more variable than against AMPA or kainate toxicity: in four experiments, the injury (assessed by LDH efflux) seen with addition of 30 μ*M* nifedipine ranged between 33 and 74% of that produced by a 24-hour exposure to 1 m*M* quinolinate alone (Fig. 3B). Similar weak protection was observed in five experiments involving 24-hour exposure to 15 μ*M* NMDA; summed over the five experiments, 30 μ*M* nifedipine reduced mean LDH efflux in 20 cultures to 75 ± 8% (SEM) of the mean value in 27 control cultures (different at $P < 0.01$ by two-tailed *t* test).

We used whole-cell recordings to test whether the protective effects of nifedipine might be due to direct interference with glutamate receptor–activated channel currents (*25*). Our results indicated that this was not the case: the whole-cell currents produced by 10 μ*M* AMPA (three cells), 30

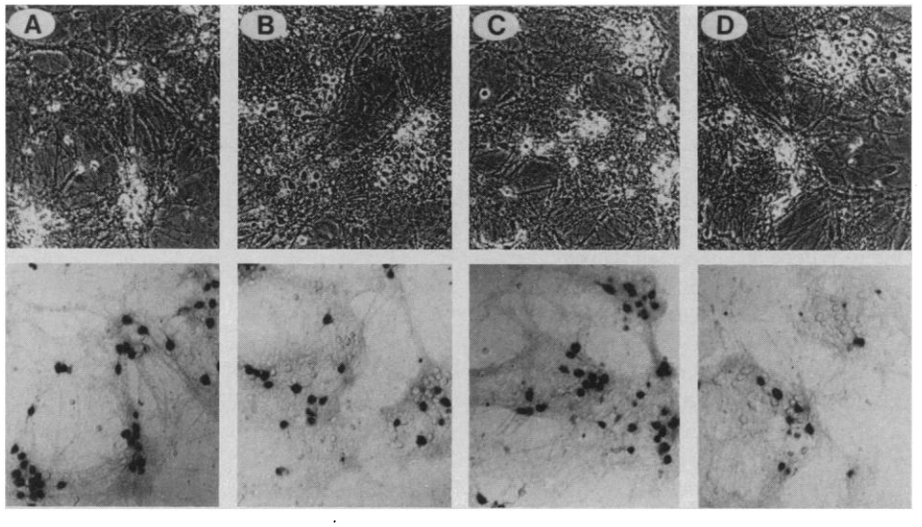

Fig. 1. Nifedipine attenuates AMPA- and kainate-induced neuronal damage. (**A**) Photomicrographs taken 24 hours after exposure to 10 μ*M* AMPA plus 1% DMSO. Top, phase contrast; bottom, bright field after 5-min incubation in trypan blue dye, which stains debris and nonviable cells. (**B**) Same as (A) but with addition of 100 μ*M* nifedipine to the exposure solution. (**C**) Same as (A) but after exposure to 30 μ*M* kainate rather than AMPA. (**D**) Same as (C) but with addition of 100 μ*M* nifedipine to the exposure solution.

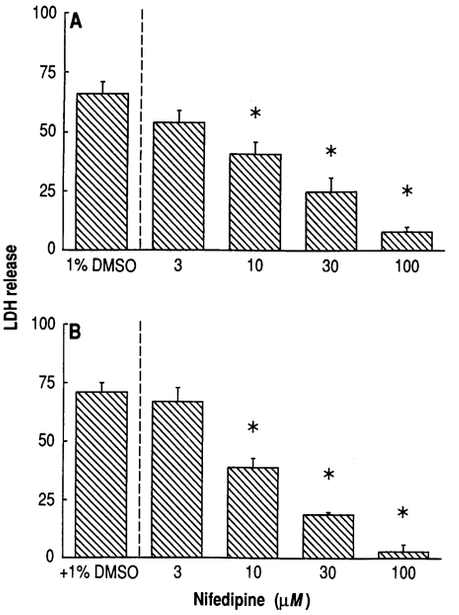

Fig. 2. Nifedipine attenuates AMPA neurotoxicity. (**A**) Sister cultures were exposed for 24 hours to 10 μ*M* AMPA in the presence of either 1% DMSO or the indicated concentration of nifedipine. LDH release (mean ± SEM, $n = 4$ cultures at each condition) is scaled to that produced by exposure of sister cultures to 300 μ*M* NMDA for 24 hours (= 100), an exposure sufficient to reliably destroy most (about 93 ± 3%) of the neurons in the cultures (*35*). An asterisk indicates difference from vehicle control at $P < 0.05$ by two-tailed *t* test with Bonferroni correction for four comparisons. (**B**) Nifedipine attenuates kainate neurotoxicity. Same as (A) but after exposure to 30 μ*M* kainate for 24 hours.

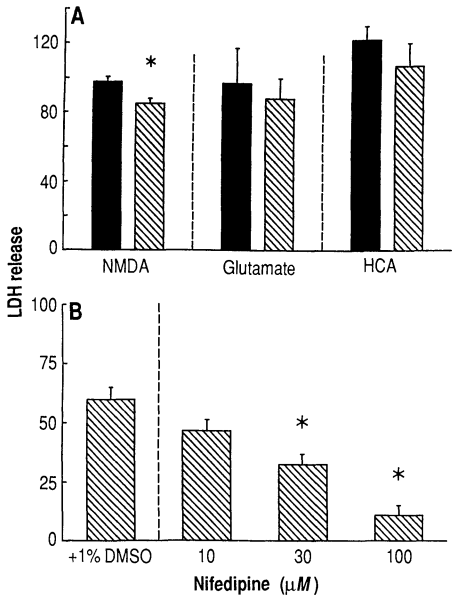

Fig. 3. Effect of nifedipine on NMDA receptor–mediated toxicity. (**A**) Sister cultures were exposed for 5 min to 500 μM NMDA in the presence of vehicle alone (1% DMSO) (solid bars) or vehicle plus 100 μM nifedipine (hatched bars). Sister cultures were similarly exposed to 500 μM glutamate or to 500 μM HCA in the presence or absence of nifedipine. LDH release (24 hours after exposure) (mean ± SEM, $n = 4$ cultures at each condition) was scaled to the near maximal signal produced by exposure of other sister cultures to 500 μM NMDA for 5 min (= 100). An asterisk indicates difference from vehicle control at $P < 0.05$ by two-tailed t test. (**B**) Similar to (A) but cultures were exposed for 24 hours to 1 mM quinolinate. Bars depict pooled data from four experiments ($n = 16$ cultures at each condition). LDH release (mean ± SEM) is scaled to the near maximal signal found in sister cultures exposed to 300 μM NMDA for 24 hours (= 100). An asterisk indicates difference from vehicle control at $P < 0.05$ by two-tailed t test with Bonferroni correction for three comparisons.

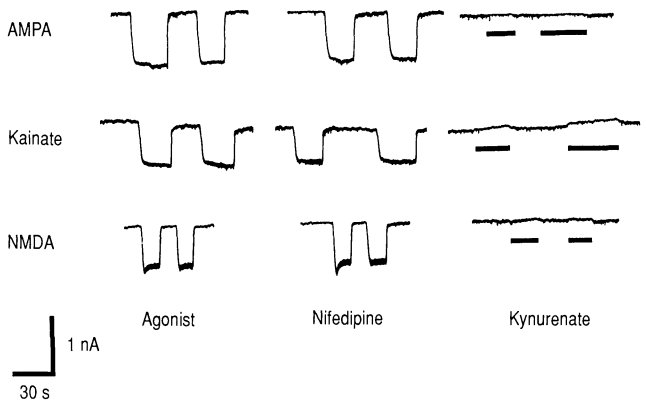

Fig. 4. Effect of nifedipine on agonist-induced whole-cell currents. Whole-cell voltage clamp (−60 mV), recordings from cortical neurons exposed to 10 μM AMPA, 30 μM kainate, or 15 μM NMDA. In each case, a single neuron was exposed to agonist alone or to agonist plus 100 μM nifedipine or 2 mM kynurenate. Nifedipine also did not block kainate- or NMDA-induced currents when tested at a positive membrane holding potential.

μM kainate (seven cells), or 15 μM NMDA (eight cells) could be blocked by the broad spectrum glutamate antagonist kynurenate but were little affected by even 100 μM nifedipine (Fig. 4).

The observed ability of nifedipine to reduce the neuronal injury associated with prolonged exposure to glutamate agonists likely reflects, at least in part, reduction of Ca^{2+} influx through slowly inactivating voltage-gated Ca^{2+} channels (*26*). In other experiments, the related dihydropyridine nimodipine could also reduce slow glutamate receptor–mediated neuronal damage (*27*). The 10 to 30 μM concentrations of dihydropyridines required to produce significant protection is higher than predicted by binding studies but may be consistent with the 10 μM concentrations that block L-channel–mediated Ca^{2+} currents (*28*). High-threshold Ca^{2+} currents in central neurons, particularly from neocortex, may be relatively insensitive to dihydropyridine block (*29*); and substantial blockade of these currents may be needed to improve neuronal survival. It is also possible that high concentrations of nifedipine could reduce Ca^{2+} influx into cortical neurons through non–L-type Ca^{2+} channels or the Na^{+}-Ca^{2+} exchanger (*30*); other mechanisms cannot be excluded.

Further support for the idea that non-NMDA agonists induce Ca^{2+} influx largely through voltage-gated Ca^{2+} channels is the finding that the dihydropyridine nitrendipine markedly reduced buildup of intracellular free Ca^{2+} in striatal neurons exposed to kainate (*31*). The additional ability of nifedipine to reduce the neuronal damage associated with prolonged low-level exposure to NMDA agonists suggests that Ca^{2+} entry through voltage-gated channels may also predominate under those conditions, perhaps because of NMDA receptor downregulation or desensitization. Nifedipine had expectedly little protective effect against the injury produced by intense exposure to NMDA agonists in our system, although greater effect may occur on young cultured neurons with little sensitivity to NMDA agonists (*3, 32*).

Nifedipine and nimodipine are clinically available and are apparently well tolerated with long-term administration (*33*). Although our in vitro observations lack direct connection to pathogenesis in vivo, they suggest that dihydropyridines could have therapeutic value in disease states characterized by slow glutamate neurotoxicity. Nifedipine serum levels in humans may approach 1 μM after a routine single oral dose (*34*). This concentration would be too low to produce substantial neuroprotection in our experiments, but dihydropyridines are lipophilic and might attain higher local levels in the central nervous system. Furthermore, the effects of the 24-hour exposures studied here may be more intense than the excitotoxicity associated with a neurodegenerative disease; under even longer term excitotoxic conditions, concentrations below 10 μM might be neuroprotective. Our observations add support to the idea that some types of excitotoxicity can be attenuated by pharmacological approaches directed at targets other than glutamate receptors.

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23. J. Koh and D. W. Choi, *J. Neurosci. Methods* **20**, 83 (1987). The small amount of LDH present in the media of cultures carried through the exposure protocol but without addition of excitatory amino acids was subtracted from values obtained in treated cultures. Other control experiments showed that the 24-hour exposure to 10 μ*M* AMPA, 30 μ*M* kainate, or 1 m*M* quinolinate produced little or no LDH efflux from cultures of cortical glia alone.
24. In other experiments, 5-min exposure to either 500 μ*M* glutamate or 500 μ*M* NMDA was carried out in the medium used for 24-hour exposures (MEM plus glucose). The medium was warmed to 37°C and gassed with CO_2 to *p*H 7.2; after brief room-air manipulation, cultures were placed in the 37°C incubator for most of the 5-min exposure period. This change in procedure did not affect outcome: 100 μ*M* nifedipine still produced little improvement in neuronal survival.
25. Whole-cell recordings were achieved with patch electrodes as described by O. P. Hamill *et al.*, *Pfluegers Arch.* **391**, 85 (1981). Before an experiment, the culture medium was replaced by a salt solution containing 140 m*M* NaCl, 2.8 m*M* KCl, 1 m*M* $CaCl_2$, 10 m*M* Hepes, and 5 m*M* glucose. The patch pipette was filled with a solution containing 140 m*M* CsCl, 0.5 m*M* $CaCl_2$, 5 m*M* EGTA (*p*Ca ≈ 7.3), 10 m*M* Hepes, and 5 m*M* Mg^{2+}–adenosine triphosphate. The perfusion solution was the same as the extracellular solution, plus 5 μ*M* glycine, 1 μ*M* tetrodotoxin, and any indicated drug; delivery was from a large-bore pipette fed by a rotary selector valve.
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HIP-70: A Protein Induced by Estrogen in the Brain and LH-RH in the Pituitary

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Estrogen and luteinizing hormone–releasing hormone (LH-RH) interact to influence both behavior and gonadotropin release. However, little is known about the biochemical mechanisms that mediate the effects of these hormones or their interactions. The most prominent protein induced by estrogen in the ventromedial hypothalamus has the same amino-terminal sequence as the most prominent protein induced by LH-RH in the pituitary in vitro and in vivo; these proteins comigrate on two-dimensional gels. Furthermore, the hormonal induction may be caused by modification of a constitutive protein with the same molecular weight (70,000) but a slightly more acidic isoelectric point, whose level is inversely related to the level of the induced form after estrogen treatment. Thus estrogen and LH-RH may interact by additively or synergistically inducing this protein, which is called HIP-70.

ESTROGEN ACTS ON HYPOTHALAMIC neurons to regulate sexual behavior and gonadotropin secretion (*1*). LH-RH acts on neurons in the hypothalamus and central gray area to facilitate female sexual behavior, but only in the presence of estrogen (*1, 2*). Similarly, LH-RH acts on the pituitary to facilitate gonadotropin secretion both in vivo and in vitro (*3, 4*); this facilitatory effect of LH-RH is enhanced by estrogen in vivo and in vitro (*3, 5*). However, the biochemical mechanisms by which estrogen and LH-RH facilitate behavior and gonadotropin secretion are unknown, as are the mechanisms by which these hormones synergize.

The most prominent estrogen-induced protein synthesized in the rat ventromedial hypothalamus (VMH) is a 70-kD protein (EI70A) that is transported to the midbrain central gray (MCG) (*6*). Estrogen appears to influence lordosis by inducing a polypeptide in the VMH that is transported to the MCG (*1, 7*). Although several proteins are induced by estrogen in the ventromedial nucleus of the hypothalamus and may play a role in lordosis, EI70A is the only induced protein known to be transported to the MCG. The most prominent LH-RH–induced protein synthesized in the rat anterior pituitary gland is a protein (LHRH70A) originally reported to have a molecular size of 69 kD (*4*). This protein represents about 0.1% of the total protein from pituitaries treated with LH-RH in vitro, but it is not detected in pituitaries not treated with LH-RH (*4, 8*). The physical similarity of the proteins induced by the pituitary and hypothalamus and the similar induction kinetics [1 to 4 hours after hormone treatment (*6, 8*)] suggested that the same protein might mediate some effects of both hormones. This could provide a basis for interaction; for example, each hormone could independently cause a 10-fold induction of the protein, leading to a potential multiplicative 100-fold induction and a magnification of the effects of both hormones.

The induction of EI70A by estrogen tends to be correlated with a reduction of a protein (EI70B) which, on two-dimensional gels, migrates to a slightly more acidic isoelectric point (*p*I) than EI70A (*6, 9*). This finding suggests that estrogen induces EI70A by modifying its acidic isoform. There has been no clear evidence that LH-RH induces LHRH70 by such a mechanism, since the pituitary protein equivalent to EI70B (LHRH70B) has not been reported to be influenced by LH-RH. To examine if EI70A is the same protein as LHRH70A, and to examine if EI70A and LHRH70A are modifications of the slightly more acidic proteins EI70B and LHRH70B, we used recently developed techniques (*10*) to determine the amino-terminal sequences of EI70A and LHRH70A, and their corresponding acidic neighbors, directly after separation on two-dimensional gels.

By means of two-dimensional gel electrophoresis we separated proteins from the VMH of ovariectomized rats that had been implanted with estrogen-filled silicone elastomer capsules 14 hours before they were killed by decapitation (after carbon dioxide anesthesia), and proteins from pituitary glands from intact rats anesthetized with sodium pentobarbitone and injected intravenously with LH-RH (50 ng per 100 g of body weight) at 1230 hours on proestrus. The proteins were then transferred to a polyvinyldifluoride membrane and stained with 0.1% Coomassie blue in 50% MeOH (Fig. 1). Spots corresponding to EI70A,

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