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Flunarizine Protects Neurons from Death After Axotomy or NGF Deprivation

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Systemically administered flunarizine enhanced neuronal survival in lumbar sensory ganglia in newborn rats after axotomy. Flunarizine-treated rats lost 71 percent fewer neurons than the untreated control rats at the end of 1 week. In cell culture, flunarizine at 30 to 40 μM also prevented neuronal death in nerve growth factor–dependent embryonic sensory and sympathetic neurons after the abrupt withdrawal of neurotrophic support. The drug may cause this effect by acting at an intracellular site, one distinct from its blockade of voltage-dependent calcium channels.

SENSORY AND SYMPATHETIC NEURONS depend on nerve growth factor (NGF) for maintenance or survival (or both) in vivo and in vitro. Characterization of the relation between these neural crest–derived cells and NGF provides much of the basis for the neurotrophic theory (*1*). Neuronal death is a highly controlled and intricate process that occurs naturally during development in multiple neural groups in the nervous system. Neurotrophic factors are important determinants in this process (*2*). NGF is critical during development for the survival of dorsal root ganglion (DRG) and superior cervical ganglion (SCG) neurons (*3*). Postnatally, sympathetic SCG neurons remain dependent on NGF for survival, whereas sensory DRG neurons remain dependent on NGF only for maintenance of their morphologic features and biochemical properties, but not for survival (*4*). Interruption of the physiologic trophic support between end-organ and neuron can result in neuronal death. After axotomy, death in DRG neurons occurs to varying degrees contingent on several factors, such as the age of the animal, location and type of lesion, and the success of subsequent regeneration (*5*). Exogenous NGF can prevent the neuronal death that occurs in the DRG after axotomy in newborn (*6*) or adult rats (*7*). The mechanism whereby NGF promotes survival in these neurons is not known. The ability of the Ca^{2+} channel blocking agent flunarizine [(*E*)-1-bis-(4-fluorophenyl)methyl-4-(3-phenyl-2-propenyl)piperazine dihydrochloride] to protect neurons from death after withdrawal of trophic support was examined both in vitro and in vivo. Two other Ca^{2+} channel–blocking agents, nimodipine and lidoflazine, were tested in vitro.

Embryonic day 15 (E15) DRG neurons were dissociated and maintained for 10 days in the presence of NGF. The neurons were then acutely deprived of NGF by removal of the trophic factor from the medium and treatment of the cultures with 2% guinea pig antiserum to mouse NGF (*8*). The E21 SCG sympathetic neurons were dissociated and maintained in culture for 7 days, at which time the neurons were deprived of NGF in the same manner as described for the sensory neurons. A dose-response relation for the protective effects of flunarizine after withdrawal of NGF support was determined by adding various concentrations (1 to 80 μM) of the drug to the cell cultures. On each culture plate, both positive (NGF-maintained) and negative (NGF-deprived) control groups of neurons were grown without flunarizine. Neuronal death was determined by histologic examination with phase-contrast microscopy and scanning electron microscopy. Neurons were examined at regular intervals by phase-contrast microscopy for evidence of neuronal death, that is, neuronal atrophy, loss of perikaryal morphology, and presence of pyknotic cells. To further assess neuronal protection, we measured protein synthesis with an [^{35}S]methionine incorporation assay (*8*). In other groups of dissociated neurons after NGF deprivation, the cultures were fixed with glutaraldehyde, and prepared for examination by scanning electron microscopy.

The E15 DRG neurons, maintained in culture in the presence of NGF for 10 days, consistently died 48 to 72 hours after NGF deprivation. However, neurons similarly NGF deprived but treated with flunarizine at concentrations ≥30 μM were protected from death, both morphologically and biochemically. The protection of neurons and neurites is most clearly shown by scanning electron microscopy (Fig. 1). Dose-response studies showed incomplete neuronal protection at lower concentrations of flunarizine

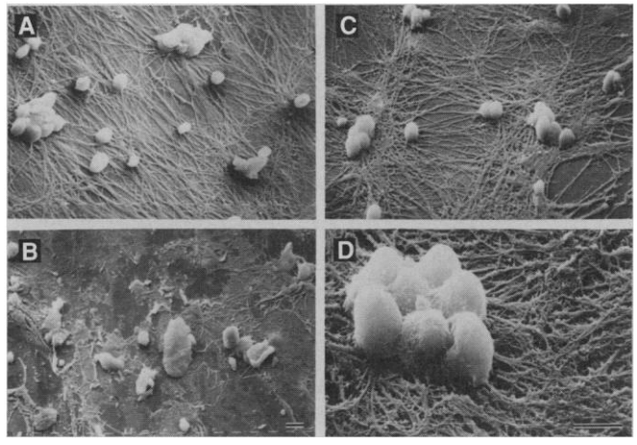

Fig. 1. Representative scanning electron micrographs from cell cultures of DRG neurons. (**A**) DRG neurons maintained in an NGF-containing medium. (**B**) DRG neurons deprived of NGF for 8 hours show loss of neurons and extensive disruption of neuritic network. (**C**) DRG neurons deprived of NGF for 48 hours in the presence of 30 μM flunarizine demonstrate protection of the neurons and their neurites. (**D**) Higher magnification of DRG neurons protected after NGF deprivation with 30 μM flunarizine. Scale bar in (B) and (D), 20 μm.

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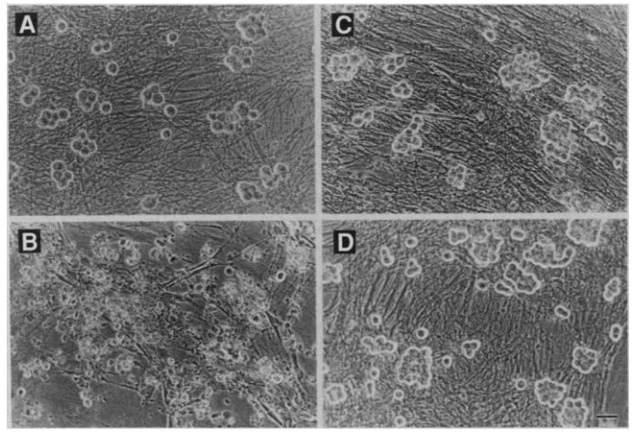

Fig. 2. Phase-contrast micrographs from cell cultures of DRG neurons. (**A**) DRG neurons maintained in an NGF-containing medium. (**B**) DRG neurons deprived of NGF for 72 hours show extensive neuronal death and disruption of the neuritic network. (**C**) DRG sensory neurons deprived of NGF for 72 hours in the presence of 40 μM flunarizine. Neurons have developed a granular appearance. (**D**) The same group of neurons shown in (C) after removal of flunarizine and return to NGF-containing medium for an additional 96 hours. The changes seen in neurons in (C) have resolved and neurons are indistinguishable in appearance from the control neurons in (A). Scale bar in (D), 40 μm.

Table 1. Effect of flunarizine on the number of neurons in the DRG of newborn rats after axotomy. Neurons with nucleoli were counted and expressed as a percent decrease of the injured compared to uninjured DRG. Significant difference by t test, $P < 0.02$ and $P < 0.001$ in 1- and 7-day groups, respectively.

Treatment	Percent neuronal loss ± SEM for days after injury	
	1	7
Control	32 ± 2 (n = 8)	35 ± 3 (n = 8)
Flunarizine	14 ± 6 (n = 11)	10 ± 3 (n = 8)

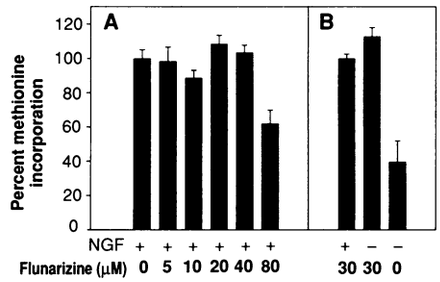

Fig. 3. Effect of flunarizine on protein synthesis. Incorporation of [^{35}S]methionine is expressed as percentage of control cells in NGF-containing media. (**A**) No significant alteration of protein synthesis until 80 μM flunarizine is reached, correlating with morphological evidence of toxicity and cell death. (**B**) Flunarizine (30 μM) preserves neuronal protein synthesis after NGF deprivation. Mean ± SEM, n = 3.

from 10 to 20 μM, and no protective effect was seen below 10 μM. Parallel studies of E21 sympathetic SCG neurons maintained in NGF-containing media for 7 days showed similar protective effects of flunarizine. The extensive neuronal death seen 48 to 72 hours after NGF deprivation in the sympathetic neurons was again prevented by the addition of 30 μM flunarizine to the media. A prolonged protective effect of flunarizine was demonstrated by maintaining the cells for 4 days in NGF-containing media after 72 hours of NGF deprivation with flunarizine protection. These neurons also were protected from death as shown by phase-contrast microscopy (Fig. 2) and protein synthesis assay. These "rescued" neurons showed no quantitative decrease in protein synthesis compared with the control neurons maintained in an NGF-containing media and 30 μM flunarizine (Fig. 3B). Preliminary studies with a second diphenylpiperazine class Ca^{2+} channel blocker (lidoflazine) also at relatively high concentrations (10 to 60 μM) showed similar protective effects in sensory neurons after NGF deprivation, whereas nimodipine (a dihydropyridine Ca^{2+} channel blocker) at 0.5 to 60 μM concentration, failed to demonstrate protective effects in cell culture.

Flunarizine toxicity was determined by maintaining dissociated sensory neurons in various concentrations of flunarizine, up to 80 μM. Protein synthesis, determined in these cultures 48 hours after the addition of flunarizine at concentrations of up to 40 μM, remained the same as control neurons not treated with flunarizine (Fig. 3A). In neurons treated with flunarizine at ≥5 μM, phase-contrast microscopy revealed morphological changes, including intracellular vesicles and a granular appearance when compared with control neurons. These changes were noted in neurons both in the presence and absence of NGF and were reversible after flunarizine removal (Fig. 2, C and D). Concentrations of 60 μM flunarizine resulted in significant loss of neurons, and 80 μM concentrations caused extensive neuronal death and consequent marked decrease of protein synthesis (Fig. 3A).

The in vivo experiments tested for protective effects of flunarizine on DRG neurons in neonatal rats after sciatic nerve axotomy. Beginning on the first postnatal day (PND-1), newborn rats were injected subcutaneously with flunarizine (25 mg per kilogram of body weight) every 12 hours. Unilateral sciatic nerve axotomy (either transection or crush injury) was performed under hypothermic anesthesia on PND-2, with continuation of the twice daily injections of flunarizine. On PND-3, 24 hours after sciatic nerve injury, the L4 and L5 DRG were dissected and removed bilaterally. The ganglia were fixed, paraffin-embedded, serially sectioned, and stained with toluidine blue. Total neuronal counts were compared between paired ganglia of lesioned and uninjured sides (*6*). In other animals we increased the twice daily flunarizine dose to 50 mg per kilogram of body weight and dissected the bilateral L4 and L5 DRGs on PND-8. The rats tolerated the flunarizine well with normal weight gain. The DRG were prepared and the surviving neurons were counted as described above.

Newborn rats subjected to sciatic nerve crush on PND-0 lose 45% of their L4 DRG neurons within 24 hours of injury (*6*). Our untreated rats, undergoing sciatic section on PND-1, experienced a 32% neuronal loss in the L4 and L5 DRG during the subsequent 24 hours. In contrast, sciatic-sectioned rats treated with systemic flunarizine had only a 14% loss of neurons in the L4 and L5 DRG after 24 hours. Rats treated with a higher dose of flunarizine for 1 week after axotomy showed a similar protective effect with only a 10% loss of neurons in the treated group compared with a 35% loss in the untreated, control group (Table 1). Thus, flunarizine protected injured sensory neurons from death in vivo during the first week after axotomy. The mechanism by which flunarizine protects neurons from death after axotomy is not known.

Cycloheximide treatment prevents neuronal death in chicken DRG 24 hours after sciatic nerve axotomy (*9*). The protective effect may be related to inhibition of protein synthesis, similar to the finding in cell culture that shows protein synthesis is necessary for the death of SCG neurons after NGF withdrawal (*10*). Drugs, such as cycloheximide or actinomycin D, that block protein or RNA synthesis can protect dissociated sympathetic neurons after withdrawal of NGF from the medium (*10*). This finding has led to the speculation that after trophic factor deprivation, neurons undergo an "active" process requiring specific protein synthesis that results in neuronal death (*10*). Flunarizine protection, however, does not appear to be related to a decrease in protein

synthesis as noted from the results of protein assays.

Flunarizine is a lipophilic, weakly basic diphenylalkylamine Ca^{2+} channel antagonist that has been used to treat vertigo, epilepsy, migraine headaches, and peripheral vascular insufficiency (*11*). Experimentally, flunarizine has a cerebral protective effect in an ischemic-hypoxic model in rats (*12*), possibly because of its ability to block intracellular entry of Ca^{2+} (*13, 14*). The inhibitory effect of flunarizine on contractile smooth muscle protein has an intracellular site of action (*15*). Flunarizine also inhibits the important intracellular regulatory protein calmodulin at concentrations greater than 10 μM (*11*). Secondary inhibition by flunarizine of calmodulin-phosphodiesterase may alter levels of adenylate cyclase with potential beneficial effects. Our data indicate that neuronal death after neurotrophic deprivation can be prevented by flunarizine in vivo and in vitro. In cell culture, the dose required for protection (20 to 40 μM) was much higher than required for block of the voltage-dependent Ca^{2+} channels (3 to 10 μM) (*11*). Studies in sympathetic neurons in culture with various Ca^{2+} entry-blocking agents at concentrations capable of blocking the voltage-dependent channels offered no protection from neuronal death (*16*). Similar high concentrations of nimodipine in DRG cell culture failed to protect neurons. On the basis of the concentration of flunarizine required for protection and the lack of protection afforded by the Ca^{2+} channel blocker nimodipine, we believe an alternative intracellular mechanism of action may be responsible. The protective ability of flunarizine in the in vivo and in vitro models provides opportunities to study the mechanisms involved in neuronal death after injury and trophic factor deprivation. Such pharmacological agents have promise in future clinical approaches.

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Inhibition of Factor VIIa–Tissue Factor Coagulation Activity by a Hybrid Protein

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Lipoprotein-associated coagulation inhibitor (LACI) appears to inhibit tissue factor (TF)–induced blood coagulation by forming a quaternary inhibitory complex containing factor Xa, LACI, factor VIIa, and TF. A genetically engineered hybrid protein consisting of the light chain of factor Xa and the first Kunitz-type inhibitor domain of LACI is shown to directly inhibit the activity of the factor VIIa–TF catalytic complex. Unlike inhibition of factor VIIa–TF activity by native LACI, inhibition by the hybrid protein is not dependent on factor Xa. In an assay of TF-induced coagulation, 50% TF inhibition occurs with hybrid protein at 35 nanograms per milliliter, whereas LACI at 2.5 micrograms per milliliter is required for an equivalent effect. γ-Carboxylation of glutamic acid residues in the factor Xa light chain portion of the hybrid protein is required for inhibitory activity, indicating that the first Kunitz-type domain of LACI alone is not sufficient for inhibition of factor VIIa–TF.

BLOOD COAGULATION CAN BE INITIated when factor VII or VIIa, a plasma protease, binds to its cell membrane–associated cofactor, tissue factor (TF), and proteolytically activates its substrates, factors IX and X (*1*), triggering a cascade of events that leads to the formation of a fibrin clot. TF-initiated coagulation has been implicated in the pathogenesis of thrombohemorrhagic disorders associated with tissue damage, sepsis, malignancy, and obstetrical complications (*2*). Thus, an agent that inhibits factor VIIa–TF activity may be useful as a specific antithrombotic agent.

Plasma contains a lipoprotein-associated coagulation inhibitor (LACI) that inhibits activated factor X (Xa) directly and, in a factor Xa–dependent fashion, inhibits factor VIIa–TF activity, presumably by forming a quaternary inhibitory complex consisting of factor Xa, LACI, factor VIIa, and TF (*3, 4*). LACI contains three Kunitz-type serine protease inhibitory domains (Kunitz domains) (*5, 6*), of which both the first and second are necessary for inhibition of the factor VIIa–TF complex. We proposed that in the putative quaternary inhibitory complex, the first Kunitz domain of LACI is bound to the active site of the factor VIIa, whereas the second Kunitz domain is bound to the active site of factor Xa (*7*). Coagulation factor X consists of two peptide chains covalently linked by a disulfide bridge. Proteolytic release of a peptide from the heavy chain of factor X by the factor VIIa–TF catalytic complex produces the active enzyme factor Xa. Factor X can also be activated by factor IXa with its cofactor VIIIa. The heavy chain of factor Xa contains the catalytic site. The light chain of factor Xa (and X) contains the γ-carboxyglutamic acid (gla) domain, which is responsible for Ca^{2+} binding, followed by two growth factor–like domains, which may in part mediate the interaction with specific coagulation cofactors (Fig. 1A). Chymotrypsin treatment cleaves the gla domain from the NH_2-terminus of factor Xa. LACI binds to and inhibits factor Xa without the gla domain, but, in the presence of factor Xa without the gla domain, LACI does not inhibit factor VIIa–TF activity (*3*).

Since neither factor Xa nor LACI alone significantly inhibit factor VIIa–TF activity, the binding of factor Xa to the second Kunitz domain in LACI may serve to juxtapose the gla domain of the factor Xa light

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