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A serine protease inhibitor, protease nexin I, rescues motoneurons from naturally occurring and axotomy-induced cell death

(spinal motoneurons/chicken/mouse)

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ABSTRACT **Protease nexin I (PNI) is a member of the family of serine protease inhibitors (serpins) that have been shown to promote neurite outgrowth *in vitro* from different neuronal cell types. These include neuroblastoma cells, hippocampal neurons, and sympathetic neurons. Free PNI protein is markedly decreased in various anatomical brain regions, including hippocampus, of patients with Alzheimer disease. Here, we report that PNI rescued spinal motoneurons during the period of naturally occurring (programmed) cell death in the chicken in a dose-dependent fashion. Furthermore, PNI prevented axotomy-induced spinal motoneuron death in the neonatal mouse. The survival effect of PNI on motoneurons during the period of programmed cell death was not associated with increased intramuscular nerve branching. PNI also significantly increased the nuclear size of motoneurons during the period of programmed cell death and prevented axotomy-induced atrophy of surviving motoneurons. These results are consistent with the possible role of PNI as a neurotrophic agent. They also support the idea that serine proteases or, more precisely, the balance of proteases and serpins may be involved in regulating the fate of neuronal cells during development.**

During normal development, ≈50% of postmitotic lumbosacral motoneurons undergo naturally occurring (programmed) cell death during a period when these cells are forming synaptic connections with their target muscles (1, 2). Naturally occurring motoneuron death has been described in many vertebrate species, including chicken (2, 3), mouse (4, 5), rat (6), and human (7) embryos or fetuses. For example, programmed motoneuron death occurs between embryonic day (E)6 and E10 in the chicken (2). This system has been used as a biological model for testing different neurotrophic agents on motoneuron survival *in vivo* (8, 9).

Although programmed cell death is completed before birth in mammals, the maintenance of motoneurons continues to be dependent on support from the target for some time after birth. Thus, if transection of motor axons is performed in neonatal mammals and reinnervation is prevented, a large number of motoneurons degenerate and die (10–13). Axotomy-induced death of motoneurons has also been extensively used as a model for testing the survival effects of various agents, including neurotrophic and growth factors on motoneurons (14–17).

Protease nexin I (PNI) (18), also known as glia-derived nexin (19), is a 43- to 47-kDa protein that was first found secreted by cultured fibroblasts (20) but is also produced by glial (glioma and primary) and skeletal muscle (21, 22) cells. PNI has been shown to promote neurite outgrowth *in vitro* from different neuronal cell types. These include neuroblastoma cells as well as primary hippocampal and sympathetic neurons (19, 21). The neurite-promoting activity of PNI *in vitro* is mediated by inhibition of thrombin, a potent serine protease (21). PNI (mRNA and protein) is transiently up-regulated in rat sciatic nerve after axotomy, and PNI-producing cells are localized distal to the lesion site (23). This up-regulation of PNI occurs 2–3 days after a similar up-regulation of prothrombin and thrombin in the distal stump (I. V. Smirnova, J. A. Ma, and B.W.F., unpublished data). Other bona fide neurotrophic agents such as nerve growth factor, brain-derived neurotrophic factor, and insulin-like growth factor I exhibit similar responses to peripheral nerve injury. Free PNI protein is significantly decreased, while endogenous PNI–thrombin complexes are increased, in various anatomical brain regions, including hippocampus, of patients with Alzheimer disease (24). When considered together with the recent demonstration that PNI can promote the *in vitro* survival of mixed mouse spinal cord neurons and that PNI is released from glial cells by neuropeptides such as vasoactive intestinal polypeptide (25), these observations suggest that PNI may play a physiological role in neuronal survival, differentiation, and/or axonal regeneration *in vivo*.

In the present report, we examined whether PNI could promote the survival of spinal motoneurons in two different models—i.e., during the period of programmed cell death in the embryonic chicken and after axotomy in the neonatal mouse. The data show that PNI prevented motoneuron death during development in both of these models. Moreover, PNI increased motoneuron cell sizes during programmed cell death and prevented atrophy of motoneurons after axotomy.

EXPERIMENTAL PROCEDURES

Naturally Occurring Motoneuron Cell Death. White Leghorn chicken eggs were obtained as a gift from Hubbard Farms (Statesville, NC) and were incubated in the laboratory at 37°C and 60% humidity. On E5, a small window was made in the shell, which was then sealed with a piece of tape, after which eggs were returned to the incubator. Embryos were treated once daily from E6 to E9—i.e., the period of programmed motoneuron death in the chicken (e.g., see ref. 2), either with saline (0.9% NaCl, pH 7.2) or with different concentrations (0.1–5 μg) of purified mouse myotube PNI (26) or of recombinant human PNI (gift of R. Scott, Incyte Pharmaceuticals, Palo Alto, CA) by dropping 0.2-ml solutions onto the vascularized chorioallantoic membrane as described (8). Assuming

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Abbreviations: PNI, protease nexin I; serpin, serine protease inhibitor; L4, fourth lumbar segment; E and P, embryonic and postnatal day, respectively.

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a uniform distribution of PNI in the embryonic fluids (total volume, ≈12 ml), the amounts of PNI used here (i.e., 0.1–5 μg) range from 0.2 to 10 nM in embryonic aqueous fluids. Embryos were killed on E10, and their spinal cords were dissected, fixed in Carnoy's fluid overnight, and processed for serial 10-μm-thick paraffin sections and histology. Motoneuron cell counts were performed as described below.

Axotomy-Induced Motoneuron Death. Five-day-old BALB/cByJ mice (The Jackson Laboratory) were anesthetized by hypothermia and the right sciatic nerve was sectioned in the mid-thigh region and a 1- to 2-mm-piece of the nerve was removed to prevent reinnervation. After the nerve was sectioned, a 2-mm^3 piece of gelfoam (Upjohn), soaked in a 5-μl solution of PNI (1 ng or 1 μg/μl), was implanted at the axotomy site. Four days later, 5 ng or 5 μg of PNI (in 5 μl) was injected (using a microsyringe) into the site of axotomy. Animals were killed on postnatal day (P)12, which is 7 days postsurgery, and their spinal cords were processed for histology. We have shown previously that this experimental paradigm—i.e., axotomy on P5—results in a 45% decrease in fourth lumbar segment (L4) motoneuron numbers by P12 and can be used to assess the trophic effects of different agents on axotomized motoneurons (17). Lumbar spinal cords with attached vertebrae and dorsal root ganglia were dissected out and fixed by immersion in Bouin's fixative for ≈2 weeks (this procedure also results in decalcification). The unoperated contralateral side of the spinal cord was used as a control. Tissues were embedded in paraffin, serially sectioned at 12 μm, and stained with hematoxylin and eosin. Motoneuron counts were performed as described below.

Motoneuron Cell Counts and Morphometric Analysis. Motoneurons in the lateral motor column were counted blindly on every 10th (chicken) or 5th (mouse) section throughout the entire chicken lumbar spinal cord and in segment L4 of mouse tissue as described (17, 27). Briefly, only cells with a well-stained cytoplasm and a large nucleus containing one or two distinct nucleoli were counted. Using these rather stringent criteria, we have found previously that <1% of the cells appear on two successive sections and, therefore, only an insignificant number of cells would be counted twice (17, 27). Two sections were selected from the middle of L4 spinal cord of each animal. From these, the outlines of motoneurons (nuclei and soma), which met the same criteria established for cell counts, were traced with a camera lucida attached to the microscope and digitized with a Summagraphics bit pad interfaced to a computer, and the areas were calculated with SIGMASCAN software (17). Statistical analysis of cell numbers was made by Student's *t* test.

Analysis of Avian Muscle Innervation. Previously, it has been suggested that increased intramuscular nerve branching and synaptogenesis may regulate motoneuron survival by providing greater access to target-derived trophic agents (28). To determine whether PNI treatment alters skeletal muscle innervation, we have examined the pattern of intramuscular nerve branches in iliotibialis, iliofibularis, and sartorius muscles on E10. Muscles were stained with C-2 (a mouse IgG monoclonal antibody to a chicken-specific neuronal cytoskeletal element) using the whole-mount technique as described (28). After treatment (E6–E9) of chicken embryos and sacrifice on E10, limb muscles, still attached to the pelvis, were dissected free of skin and connective tissue, prefixed in cold (−20°C) acetone for 2–3 min, washed (3 × 5 min) in phosphate-buffered saline (PBS), and incubated in C-2 antibody (a gift from L. Landmesser, Case Western Reserve, Cleveland) diluted 1:4 in 1% Triton X-100 for 1 hr at room temperature (or overnight at 4°C). Tissues were washed in PBS, fixed in 3.7% formaldehyde for 30 min, washed in PBS, incubated in rhodamine isothiocyanate-labeled secondary (i.e., goat anti-mouse) antibody for 1 hr at room temperature or overnight at 4°C, and washed in PBS. The iliotibialis, iliofibularis, and sartorius muscles were then individually dissected out from the limb and mounted in 50% (wt/vol) glycerol containing (1 mg/20 ml) phenylenediamine. Tissues were examined with a Nikon light microscope equipped with an appropriate filter. The pattern of intramuscular nerve sprouting was determined by directly counting under the microscope the number of side branches per mm of the 2 or 3 major intramuscular nerve trunks (see Fig. 2).

RESULTS

Effects of PNI on Programmed Motoneuron Cell Death. Daily treatment of chicken embryos *in ovo* from E6 to E9 with PNI resulted in a substantial reduction of naturally occurring death of spinal motoneurons when examined on E10 (Fig. 1). The survival effect of PNI was dose dependent (Fig. 1) and comparable to that obtained in this model with previously examined neurotrophic agents such as ciliary neurotrophic factor (29) and brain-derived neurotrophic factor (30). Treatment of chicken embryos with 5 μg of PNI (the highest dose tested) increased motoneuron numbers by 45–50% (Fig. 1) and significantly increased motoneuron nuclear size (Table 1)

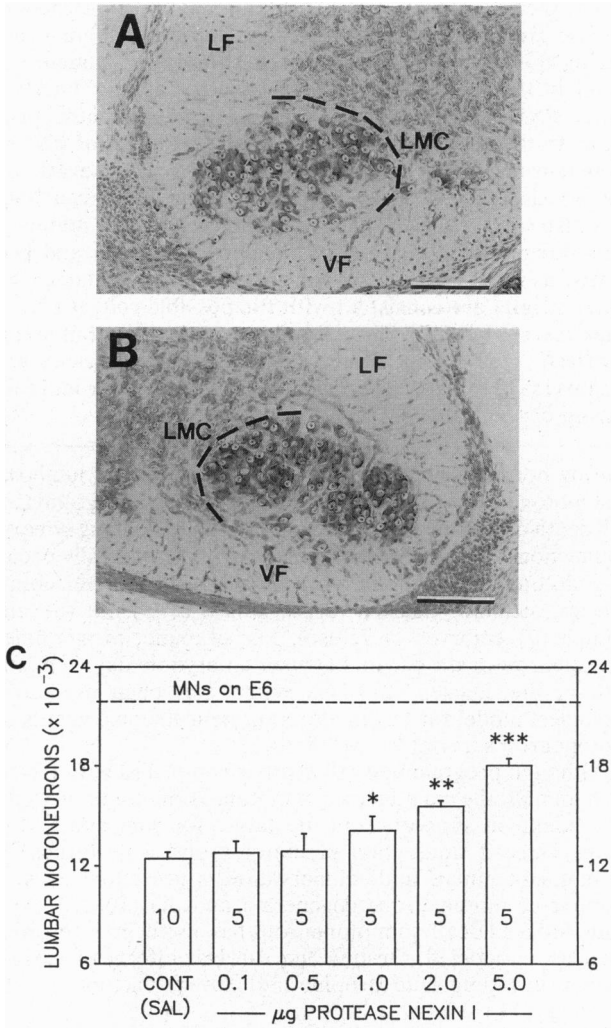

FIG. 1. Photomicrographs of the lateral motor column (LMC) of segment L4 from control (*A*) and PNI-treated (5 μg) (*B*) chicken embryos on E10. LF and VF, later and ventral funiculus. (Bars = 100 μm.) (*C*) Motoneuron numbers (means ± SEM) in the LMC of E10 chicken lumbar spinal cords after treatment with either saline (control) or PNI. Dashed line, number of motoneurons (MNs) on E6. Numbers in bars are sample sizes. *, $P < 0.05$; **, $P < 0.0001$ vs. control. ***, $P < 0.002$ vs. 2 μg of PNI (Student's *t* test).

Table 1. Nuclear and soma areas (mean ± SEM) of motoneurons in the LMC of lumbar spinal cords from chicken embryos on E10 and axotomized mice on P12 after treatment with either saline or PNI

	Nuclear area (*n*)	Soma area (*n*)
Normal MN death (chicken)		
Control	77.56 ± 0.68 (344)	—
PNI (5 μg)	82.23 ± 0.69 (320)*	—
Axotomy-induced MN death (mouse)		
Contralateral	142.84 ± 2.15 (64)	504.99 ± 9.25 (64)
Ipsilateral + saline	120.52 ± 3.97 (42)**	433.45 ± 13.88 (42)**
Ipsilateral + PNI (5 ng)	143.27 ± 2.35 (64)***	472.82 ± 9.78 (64)****

Nuclear and soma areas are expressed as μm^2 (n = sample size). Outlines of motoneuron (MN) soma and nuclei were traced with a drawing tube attached to the microscope, and the areas were determined by the SIGMASCAN program (Jandel, Corte Madera, CA). Because motoneurons were densely clustered in embryonic chicken LMC (see Fig. 1 *A* and *B*), we were unable to reliably determine chicken motoneuron soma sizes.

*, $P < 0.0001$ vs. control; **, $P < 0.001$ vs contralateral; ***, $P < 0.001$ vs. ipsilateral + saline; ****, $P < 0.05$ vs. ipsilateral + saline (Student's t test).

when compared to E10 controls.

One mechanism regulating motoneuron survival may be intramuscular nerve branching to increase the sites of trophic factor uptake (28). Coupled with the reported neurite-promoting activity of PNI *in vitro* on various neuronal cell types (21, 31, 32), we examined the patterns of intramuscular nerve branching by the whole-mount technique described previously (28). Daily treatment of chicken embryos, from E6 to E9, with 5 μg of PNI did not alter the number of side branches occurring on intramuscular nerve trunks in the iliotibialis, iliofibularis, or sartorius muscle (Fig. 2).

Axotomy-Induced Motoneuron Death and Effect of PNI. In neonatal mammals, nerve section results in a massive motoneuron cell loss in the corresponding spinal cord segments (11). Recently, we have shown that unilateral sciatic nerve section in the mouse on P5 results in a substantial (≈45%) loss of motoneurons in the ipsilateral L4 by P12 when compared to the contralateral side (17). We have also shown that treatment of animals after axotomy with different neurotrophic agents can prevent significant numbers of these cells from injury-induced death (17). Therefore, in the present study, we used this experimental paradigm to assess further the rescue effects of PNI on axotomized mouse motoneurons. Remarkably, as we found in avian embryonic programmed motoneuron cell death, PNI significantly rescued mouse motoneurons from axotomy-induced death. As little as 5 ng of PNI prevented the death of >95% (virtually all) of axotomized motoneurons (Fig. 3) and also prevented the atrophy of surviving axotomized motoneurons (Table 1).

DISCUSSION

This report shows that a member of the serine protease inhibitor (serpin) superfamily (33)—i.e., PNI—can promote the *in vivo* survival of spinal motoneurons in two different cell death models: during the period of programmed cell death in the chicken and after neonatal axotomy in the mouse. One obvious possible explanation for these results is that PNI may indirectly rescue motoneurons by inhibiting one or more serine proteases involved in the cascade of intracellular events leading to cell death. Consistent with this idea, it has been recently shown that thrombin, a prominent serine protease, induces degeneration and death of neurons and astrocytes *in vitro* (34). Furthermore, the deleterious effect of thrombin can be prevented by specific thrombin inhibitors (34). Similar to other serpins, PNI forms stable complexes with target serine proteases (20, 33). These include urokinase, tissue plasminogen activator, trypsin, plasmin, thrombin, and kallikrein, all of which are serine proteases that cleave after an arginine residue (18, 33). Once formed, PNI–protease complexes are recognized by a specific receptor and are rapidly internalized into the cells and degraded (20, 33).

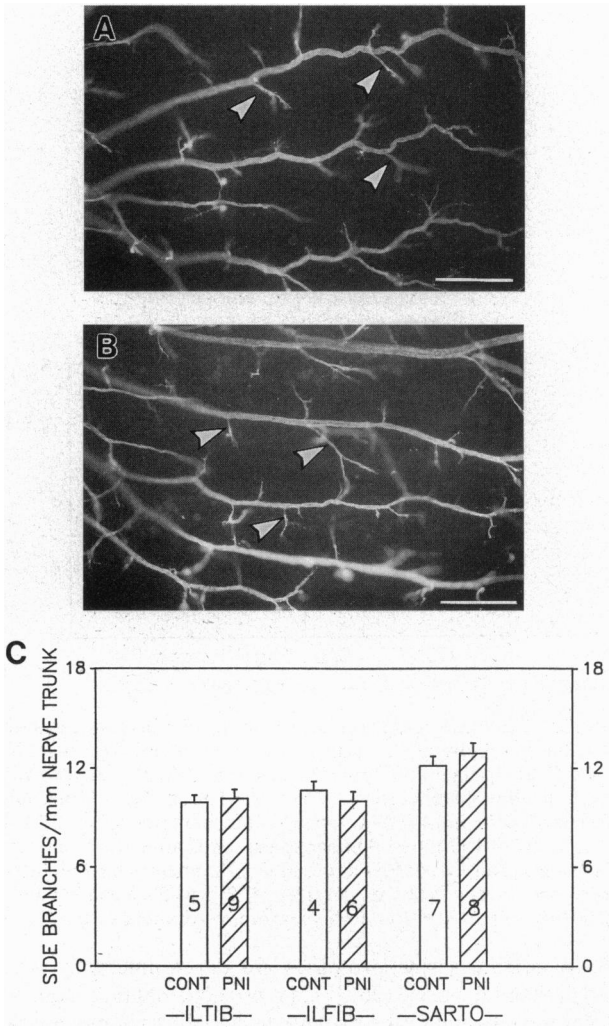

FIG. 2. Photomicrographs of whole-mount sartorius muscle from embryos treated with either saline (*A*) or PNI (5 μg) (*B*) on E10. Arrowheads indicate nerve side branches. (Bars = 200 μm.) (*C*) Numbers of side branches (means ± SEM) of intramuscular nerves in iliotibialis (ILTIB), iliofibularis (ILFIB), and sartorius (SARTO) muscles on E10 after treatment with either saline (CONT) or 5 μg of PNI (PNI). Muscles were dissected free of connective tissue and stained with C-2 (neurofilament specific) antibodies (see ref. 28). Numbers in bars represent sample sizes.

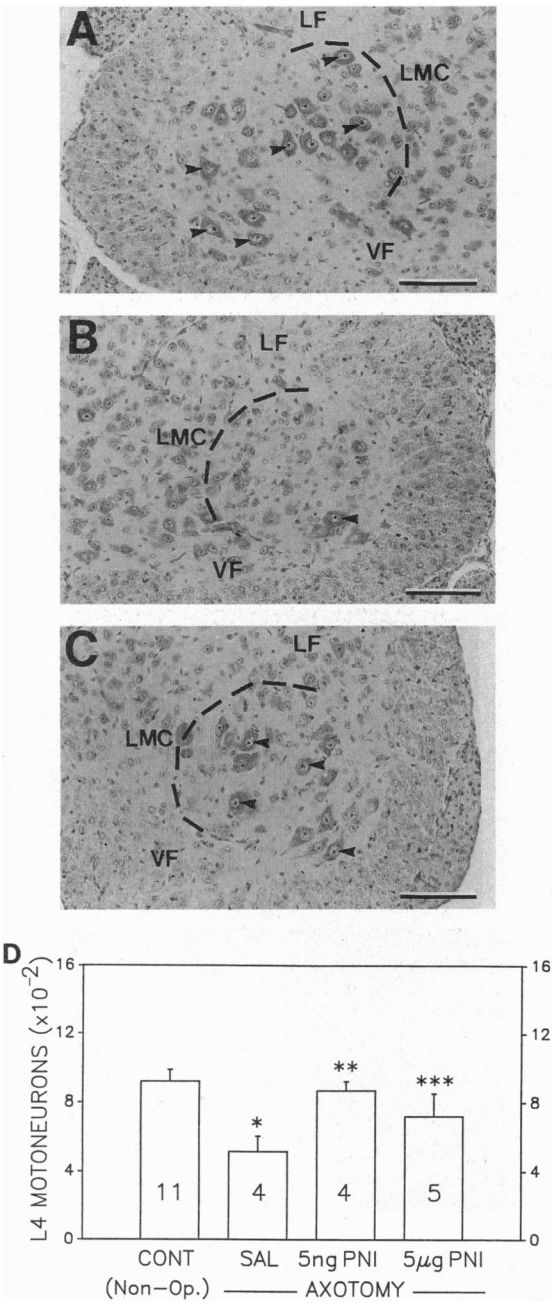

FIG. 3. Photomicrographs of lateral motor column (LMC, segment L4) from contralateral (*A*), saline-treated ipsilateral (*B*), and PNI-treated (5 ng) ipsilateral (*C*) spinal cords from P12 mice. Arrowheads indicate healthy motoneurons. LF and VF, lateral and ventral funiculus. (Bars = 100 μm.) (*D*) Motoneuron numbers (means ± SEM) in the L4 LMC of P12 mouse lumbar spinal cords after treatment with either saline (control) or PNI as described. Numbers in bars represent sample sizes. *, $P < 0.001$ vs. control; **, $P > 0.05$ vs. control and $P < 0.001$ vs. saline; ***, $P < 0.05$ vs. axotomy (Student's *t* test).

Since certain serine proteases are developmentally regulated in skeletal muscle (26, 35), it is possible that motoneurons die naturally owing to the proteolytic activity of some muscle-derived serine proteases. Accordingly, treatment with exogenous PNI might inhibit this proteolytic activity leading to the survival of more motoneurons. The fact that α_1-antichymotrypsin, another serpin that inhibits cathepsin G and similar serine proteases, promotes the survival of cultured rat cortical and hippocampal neurons (36, 37) is consistent with this conclusion. Previously, however, treatment with other protease inhibitors failed to rescue rat sympathetic neurons *in vitro* (38) or embryonic chicken motoneurons *in vivo* (39). In fact, in early experiments, glia-derived nexin or PNI failed to promote neuronal survival in culture (40). One possible explanation for this discrepancy may be the concentration of glia-derived nexin involved (40), since recent *in vitro* studies demonstrate biphasic curves with PNI (25). It is not yet known whether spinal motoneurons express receptors that are able to recognize and internalize protease–PNI complexes or whether serine proteases can directly result in the death of motoneurons *in vitro* and *in vivo*. However, spinal cord astrocytes do possess a similar receptor for α_1-antichymotrypsin–cathepsin G complexes (41) and serine proteases have been implicated in apoptosis in the immune system (42).

Alternatively, PNI may directly regulate the survival and differentiation of motor neurons *in vivo* and, therefore, may be considered a putative neurotrophic agent. One potential mechanism for this may be the posttranslational production of a trophic PNI fragment from its C terminus after it inhibits thrombin or another target protease. Production of complexes with their cognate proteases, with the release of a variably sized fragment, occurs with most serpins (33). One such fragment, angiotensin, has hormonal actions mediated via a specific receptor. Consistent with this idea, we previously showed survival and neurite outgrowth in enriched cultures of chicken spinal cord motoneurons treated with chicken sciatic nerve extracts enriched in PNI (43) and recently confirmed this with purified PNI (E. D. Lloyd, B.W.F., and L.J.H., unpublished data). A number of other purified glia-derived polypeptides, including the neurotrophins, have been suggested to play a physiological role in the survival of neuronal cells during development and after injury (14, 15, 29, 30). Since PNI is also synthesized and secreted by skeletal muscle cells in culture (22) and is expressed in adult mouse skeletal muscle, where it is colocalized with acetylcholine receptor clusters at the neuromuscular junctions (22), these may be physiologically relevant sites for trophic agent uptake by motoneurons. However, it is not yet known whether target muscles express physiologically relevant levels of PNI during the period of programmed cell death or in the neonatal period when mammalian motoneurons are susceptible to axotomy. PNI mRNA is markedly expressed after human myoblast fusion to myotubes (M. Verdiere-Sahuqué, M. Akaaboune, S. Lachkar, L. Garcia, G. Barlovatz-Meimon, B.W.F., and D. Hantai, unpublished data), suggesting that PNI might also be expressed in embryonic chicken myofibers. Despite these uncertainties, the evidence for PNI expression and localization in skeletal muscle (22), together with the reported neurite-promoting (21, 31, 32) and neuronal survival-promoting activities (refs. 25 and 43; this work) of PNI, suggest that this serpin may function as a neurotrophic agent *in vivo* at specific times during development of the neuromuscular system.

In conclusion, we have shown that PNI, a prominent member of the serpin family with arginine specificity, has survival and growth-promoting effects on spinal cord motoneurons during the period of programmed cell death in the chicken and after axotomy in the mouse. The present data, together with the previously reported effects of PNI on neuronal differentiation (21, 31, 32) and the reduction of active PNI protein in brains of patients with Alzheimer disease (24), indicate that PNI, and possibly other members of the serpin family known, or yet to be described (44), may function as important regulators in neuronal development, including survival, differentiation, and axonal regeneration (45).

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