A Femtomolar-acting Neuroprotective Peptide

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Abstract

A novel 14–amino acid peptide, with stress-protein–like sequences, exhibiting neuroprotection at unprecedented concentrations, is revealed. This peptide prevented neuronal cell death associated with the envelope protein (GP 120) from HIV, with excitotoxicity (*N*-methyl d-aspartate), with the beta amyloid peptide (putative cytotoxin in Alzheimer's disease), and with tetrodotoxin (electrical blockade). The peptide was designed to contain a sequence derived from a new neuroprotective protein secreted by astroglial cells in the presence of vasoactive intestinal peptide. The neurotrophic protein was isolated by sequential chromatographic methods combining ion exchange, size separation, and hydrophobic interaction. The protein (mol mass, 14 kD and pI, 8.3±0.25) was named activity-dependent neurotrophic factor, as it protected neurons from death associated with electrical blockade. Peptide sequencing led to the synthesis of the novel 14–amino acid peptide that was homologous, but not identical, to an intracellular stress protein, heat shock protein 60. Neutralizing antiserum to heat shock protein 60 produced neuronal cell death that could be prevented by cotreatment with the novel protein, suggesting the existence of extracellular stress-like proteins with neuroprotective properties. These studies identify a potent neuroprotective glial protein and an active peptide that provide a basis for developing treatments of currently intractable neurodegenerative diseases. (*J. Clin. Invest.* 1996. 97:2299–2307.) Key words: activity-dependent neurotrophic factor • vasoactive intestinal peptide • neuronal survival • astrocyte

Introduction

Neuronal division, survival, and differentiation are dependent on protein and peptide growth factors during development. The increasing number and diversity of these molecules has become apparent. Included in this group of regulatory molecules are recognized trophic factors such as nerve growth factor (1), ciliary neurotrophic factor (2), fibroblast growth factor (3), IGFs 1 and 2 (4), brain derived neurotrophic factor (5), glial derived neurotrophic factor (6), and neurotrophin-3 and neurotrophin-4/5 (7). In addition, cytokines also have neurotrophic properties (8, 9). Although many of the classic growth factors were first recognized to play important trophic roles in neuron/target cell interactions, it is now clear that glial cells in the central nervous system (CNS) express most of these growth factors/cytokines, and that these support cells have significant roles during development and nerve repair.

Our interest has been to understand the role of neuropeptides in regulating the release/expression of glia-derived trophic substances and to identify new glial molecules that contribute to the survival of developing CNS neurons. Our focus in this area has been the investigation of trophic support for activity-dependent neurons in the CNS. The activity-dependent neurons are a class of neurons that die during electrical blockade due to a reduction of soluble trophic materials in their environment (10, 11). Electrical blockade has been demonstrated to inhibit the synthesis and release of trophic materials into the extracellular milieu of CNS cultures (12, 13). Included in this trophic mixture is vasoactive intestinal peptide (VIP)[1] (13, 14).

The 28–amino acid peptide VIP, originally isolated by Said and Mutt (15), has been associated with cellular protection in sensory neurons, axotomized sympathetic neurons, and acutely injured lung and airways. Indeed, the upregulation of VIP expression observed in these injured or inflamed systems probably represents an adaptive response that limits damage and promotes recovery (for short recent review, see reference 16).

VIP has been shown to interact with high affinity receptors present on glial cells (17), resulting in the release of survival-promoting substances (18, 19), among which are a glial-derived cytokine (IL-1–α references 8, 20), and protease nexin I, a serine protease inhibitor (21). However, the neuronal survival-promoting effects of the VIP-conditioned medium were observed at very low concentrations that could not be attributed to IL-1 or protease nexin I released from astroglia. We therefore searched for other survival-promoting proteins released from glial cells stimulated by VIP.

In the present study, the biochemical purification of a novel protein of extraordinary potency is presented. This protein was named activity-dependent neurotrophic factor (ADNF) for two reasons: (*a*) a blockade of spontaneous electrical activity was necessary to detect the neuroprotective action of this substance in dissociated spinal cord cultures; and (*b*) VIP, a secretagogue for ADNF, was released during electrical activity, making the presence of ADNF in the extracellular milieu indirectly dependent on spontaneous activity. Furthermore, the structure of an active peptide fragment of ADNF is re-

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1. *Abbreviations used in this paper:* ADNF, activity-dependent neurotrophic factor; ADNF-14, 14–amino acid peptide derived from ADNF; FPLC, fast performance liquid chromatography; hsp 60, heat-shock protein 60; NMDA, *N*-methyl D-aspartate; VIP, vasoactive intestinal peptide.

vealed. This active peptide, 14–amino acids derived from ADNF (ADNF-14) was shown to mimic the potency of the parent protein while exhibiting a broader range of effective concentrations as compared to the parent protein.

The significance of the current study lies in the potential clinical relevancy of the ADNF peptides. Although isolated using a strategy of protection against a known neurotoxin (tetrodotoxin), the protective properties of ADNF are now shown to extend against other neurotoxic substances as well. The ADNF secretagogue, VIP, has been shown to prevent neurotoxicity associated with the HIV envelope protein (GP120, reference 22) as well as against neurotoxicity associated with the beta amyloid peptide which is deposited in the brains of Alzheimer's disease patients (23). The mechanisms of these VIP effects are probably due to its ability to promote the secretion of neuroprotective substances from glial cells (18–21). Here, we test the hypothesis that VIP's neuroprotective actions are mediated in part through ADNF.

Patients infected with HIV exhibit neural degeneration and loss despite the presence of relatively few infected cells (24, 25), suggesting the production of a toxic substance associated with HIV. Such a toxic agent was proposed to be the viral envelope protein GP120, released from HIV-infected cells (26). Neuronal cell killing has been observed after treatment of CNS cultures with low concentrations of GP120 (22, 27, 28) and neurodystrophy was observed after GP120 treatment in vivo (28). The deleterious action of this viral protein is thought to involve a synergy with the glutamate receptor *N*-methyl d-aspartate (NMDA), associated with excitotoxicity (29). Because of the demonstrated protective actions of VIP against GP120 neurotoxicity, the current study explored the possibility that ADNF-14 would be effective against GP120 or NMDA-associated neurotoxicity.

Furthermore, we have recently demonstrated that VIP and a new lipophilic VIP analogue (30, 31) provided potent and effective protection from neuronal cell death produced by beta amyloid peptide in vitro (23). The mechanism of neuroprotection elicited by VIP is not known. In the present study, the potential role of ADNF in this neuroprotection is explored with the active peptide of ADNF. The beta amyloid peptide is excessively deposited in the brains of Alzheimer's disease patients, contributing to the neurodegenerative process which leads to senility (32–34). Despite apparent progress in the understanding of this disease, successful treatment of neurodegeneration associated with Alzheimer's senile dementia (afflicting 3–5 million people in the United States, references 35, 36) remains elusive (37, 38). We now demonstrate that ADNF-14 exhibited an unprecedented potency in protecting neurons from beta amyloid toxicity.

Methods

Cell culture. The source of the neurotrophic activity was rat cerebral cortical astrocytes, a superior source for astroglia because of rapid growth characteristics and established cellular composition (17, 39). For measurements of neuroprotective actions, two cell cultures were used. Dissociated mouse spinal cord cultures (obtained from 12-d old embryos) were plated (0.5 million cells/35-mm dish) in a medium consisting of 10% FCS and 10% heat-inactivated horse serum in MEM. After 24 h, the medium was changed to 5% horse serum in MEM, supplemented with defined medium components (40). Neuronal cell counts were conducted after fixation with glutaraldehyde. Neuronal identity was established with sister cultures immunocytochemically stained with antiserum against neuronal specific enolase (41). A second system, used for quantitation of neuronal survival, was dissociated cerebral cortical cultures, derived from newborn rats (23, 28).

Purification of ADNF. 2-wk old astroglial (17, 39) cultures (confluent 75-cm^2 flasks) were washed three times with PBS and conditioned medium was collected (10 ml PBS/flask) during a 3-h incubation with 0.1 nM VIP (an amount previously shown to be optimal for releasing neurotrophic activity from astroglial cells, references 18, 19). The medium was centrifuged (3,000 *g* for 10 min) and dialyzed (3.5-kD cutoff) against 50 mM sodium phosphate buffer, pH 7.0, 4°C. Neuroprotection was assayed initially in tetrodotoxin-blocked spinal cord cultures. The rationale for choosing tetrodotoxin-blocked culture cells for assays of survival-promoting activities secreted from glial cells in the presence of VIP was that treatment with 1 μM tetrodotoxin blocked spontaneous synaptic activity, thereby inhibiting the synthesis (12) and release (13) of endogenous VIP, rendering the system dependent on exogenous VIP.

The first purification step in the isolation of ADNF was DEAE–Sephacel chromatography (Pharmacia Diagnostics AB, Uppsala, Sweden) of VIP-stimulated astroglia-conditioned medium. Dialyzed (50 mM sodium phosphate buffer, pH 7.0) conditioned medium (300 ml, 6–8 mg protein) was loaded onto a DEAE-Sephacel column (0.75 cm in diameter and 3 cm in length) preequilibrated with 50 mM sodium pyrophosphate buffer, pH 7.0. The column was washed sequentially with 40 ml of 50 mM sodium pyrophosphate buffer (pH 7.0) and then the same buffer supplemented with increasing concentrations of NaCl, 0.1 M, 0.26 M, 0.5 M, 1.0 M, 2 M, and 3 M. Column fractions, after dialysis against water (1:10,000), were added together with 1 μM tetrodotoxin to the spinal cord test cultures. Neuroprotective activity was determined by gauging the effects on the number of surviving spinal cord neurons. Significant increases in neuronal cell counts were observed in the 2-M NaCl eluate. The second purification step was size separation of the active DEAE fraction (2-M NaCl eluate) on fast performance liquid chromatography (FPLC; Pharmacia Diagnostics AB). The 2-M NaCl fraction (corresponding to 300 ml original conditioned medium preparation) was dialyzed against water, lyophilized, and resuspended in 0.5 ml of 50 mM sodium phosphate (pH 7.3) containing 0.15 M NaCl. 0.25-ml aliquots were loaded on a Superose™ (Pharmacia Diagnostics AB) 12-column (prepacked HR 10/30) FPLC. Fractions (0.5 ml, 0.4 ml/min) were collected from the column, diluted (1:10,000), and tested in the neuronal survival assay. Significant increases in neuronal cell counts were observed in column fractions 22 and 31. A third purification step of the low molecular weight neuroprotective activity included hydrophobic interaction (Alkyl Superose™ HR5/5. Pharmacia Diagnostics AB) FPLC. The column was washed with 0.1 M phosphate buffer (pH 7.0) and equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 2.0 M $(NH_4)_2SO_4$. The sample (0.5 ml of eluted fraction 31 from the size fractionation FPLC) was dialyzed extensively against deionized water, lyophilized, and resuspended in 0.1 M sodium phosphate buffer, pH 7.0, containing 1.43 M $(NH_4)_2SO_4$. Elution (1-ml fractions, 0.5 ml/min) was performed with a linear gradient of salt removal (2.0–0 M) initiated 10 min after injection and lasting 50 min. Protein samples were dialyzed extensively against deionized water and analyzed for protein concentrations (protein assay; Bio-Rad Laboratories, Richmond, CA). After hydrophobic interaction chromatography, the amount of protein in the active fraction was determined by total amino acid analysis on an instrument (model 7300, Beckman Instrs., Fullerton, CA) after hydrolysis (24 h/110°C) in 6 N HCl containing 0.2% phenol. Samples eluted from the hydrophobic interaction column by salt removal were tested for biological activity and absorbance at 280 nm after dialysis against water.

Isoelectric focusing, HPLC and gel electrophoresis. For isoelectric focusing, the MinipHor™ (Protein Technologies, Tucson, AZ) apparatus was used. Eluted fractions were assayed for survival-promoting activity after dilution 1:1,000 with PBS. Before SDS electrophoresis, ADNF was tested for purity by reverse-phase HPLC

chromatography using a LiChroCART 125-4, Lichrospher RP8, 5 μm (Merck, Darmstadt, Germany). ADNF was solubilized in 2 ml of 50% acetonitrile containing 0.1% trifluoroacetic acid and eluted with a gradient of 0–100% acetonitrile containing 0.1% trifluoroacetic acid. The ADNF appeared as a single peak eluting at 50% acetonitrile. The ADNF then was subjected to electrophoresis on a 12% polyacrylamide SDS gel containing 0.1% SDS, according to Laemmli (42). Gels were stained with silver stain (Bio-Rad Laboratories). Extraction of the 14-kD protein band from the polyacrylamide gel was performed as described (43). Molecular weight determination was obtained by the parallel analysis of molecular weight markers.

Peptide sequencing and synthesis. For peptide sequencing, HPLC-eluted ADNF (3–5 μg) was subjected to V8 protease digestion (Boehringer Mannheim, Indianapolis, IN). The reaction was carried out in 50 mM ammonium hydrogen carbonate, pH 7.8, with an enzyme to substrate ratio of 1:50 at 37°C for 16 h. Resulting peptides were resolved by HPLC (see above) and sequenced on Model 470 and 477 (Applied Biosystems Inc., Foster City, CA). For sequencing, peptides were dried onto Biobrene-coated cartridge filters (Applied Biosystems Inc.) and the tube that contained the peptide was rinsed with 30 μl of trifluoroacetic acid, which was also dried on top of the filter. For peptide synthesis, the solid phase strategy employing optimum side chain protection was used (23, 30, 31). Products were purified on Sephadex G-25 (Sigma Chemical Co., St. Louis, MO) and reverse-phase HPLC. Peptides showed the desired molar ratios of constitutive amino acids.

Results

Purification of ADNF. Secreted proteins (> 3,500 D, which excluded VIP) were collected from astroglial cultures treated with 0.1 nM VIP for 3 h (Fig. 1). The secreted proteins exhibited distinct electrophoretic patterns which were clearly distinguished from total cellular proteins (19). Furthermore, incubation with VIP was necessary to induce release of survival-promoting substances (18, 19). Survival-promoting activity was tested initially on tetrodotoxin-treated neurons from dissociated spinal cord cultures (13, 14).

The factor responsible for survival activity in the conditioned medium was trypsin sensitive and heat inactivated. Fig. 1 compares the potency of unfractionated conditioned medium (*triangles*, EC_{50}, 125 pg/ml) to that of purified ADNF (*circles*, EC_{50}, 0.075 pg/ml or 5 fM, see Methods and below), indicating a 1,650-fold purification (see Table I for isolation scheme). The initial protein purification step by DEAE-Sephacel ion exchange chromatography yielded maximal biological activity in the fraction eluting with 2 M NaCl (Fig. 2 *A*). A 10-fold purification of the survival-promoting substance from the unfractionated conditioned medium was observed after anion exchange (Table I). As shown in Fig. 2 *B*, the 2 M NaCl DEAE-Sephacel eluate was further purified by gel filtration (Superose 12) using FPLC. Two peaks of survival-promoting activity were discovered, one with an apparent molecular mass of 150 kD and the other with an estimated mass of 14–16 kD. The lower molecular weight fraction from the gel filtration column was purified further using hydrophobic interaction chromatography. Fig. 2 *C* shows that the factor responsible for neuronal survival activity was confined to a single peak that eluted in the middle of the desalting gradient (0.94–1.04 M salt).

Biochemical characterization of ADNF. The isolated protein was subjected to isoelectric focusing using the Mini-pHor™ apparatus. Eluted fractions were subjected to pH as well as biological activity assays, revealing a single peak of survival-promoting activity eluting at pH 8.1 (Fig. 3 *A*). Isoelectric focusing by electrophoresis on polyacrylamide gels confirmed a single protein band with a pI of 8.3±0.25. Isolated ADNF had an apparent molecular mass of 14 kD as determined by SDS polyacrylamide gel electrophoresis (Fig. 3 *B*). The protein band eluted from the SDS gel retained a similar specific activity in the survival assay to that observed after hydrophobic interaction chromatography.

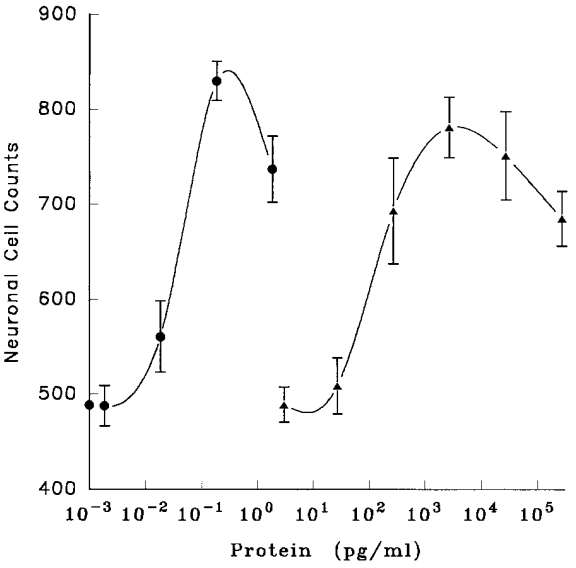


Figure 1. Dose response of the survival-promoting activity of ADNF as determined by effects on spinal cord neurons: comparison between conditioned medium and purified ADNF. Test cultures (spinal cord neurons) were treated for 5 d with varying amounts of conditioned medium in the presence of 1 μM tetrodotoxin. Neuronal cell counts were made in 30 fields from predetermined coordinate locations without knowledge of the treatment group. Each value is the mean of six determinations from three experiments (error bars are the SEM). The survival-promoting activity in unfractionated conditioned medium from astroglial cultures is depicted by triangles and the survival-promoting activity of purified ADNF (see Methods and Results) by circles. Significant increases from control cell counts were observed at concentrations ≥ 0.1 pg/ml for purified ADNF ($P < 0.001$) and at concentrations ≥ 0.3 ng/ml for unfractionated conditioned medium ($P < 0.05$).

Table I. Summary of Purification of Activity-Dependent Neurotrophic Factor

Step	Protein	Units	Specific activity	Recovery	Fold purification
	μg	*million*	*U*/ng*	%	
Conditioned medium	5,400	43.0	8	100	
DEAE	467	41.5	89	96.5	11.12
Sizing	5	5.5	1,100	12.8	137.5
Hydrophobic interaction	0.28	3.7	13,200	8.6	1,650

The conditioned medium (300 ml) was from rat astroglia cultures stimulated with 0.1 nM VIP. *A unit was determined by the amount of protein which elicited a half maximal response in the survival assay. Yield: 0.005%; purification: ×1,650.

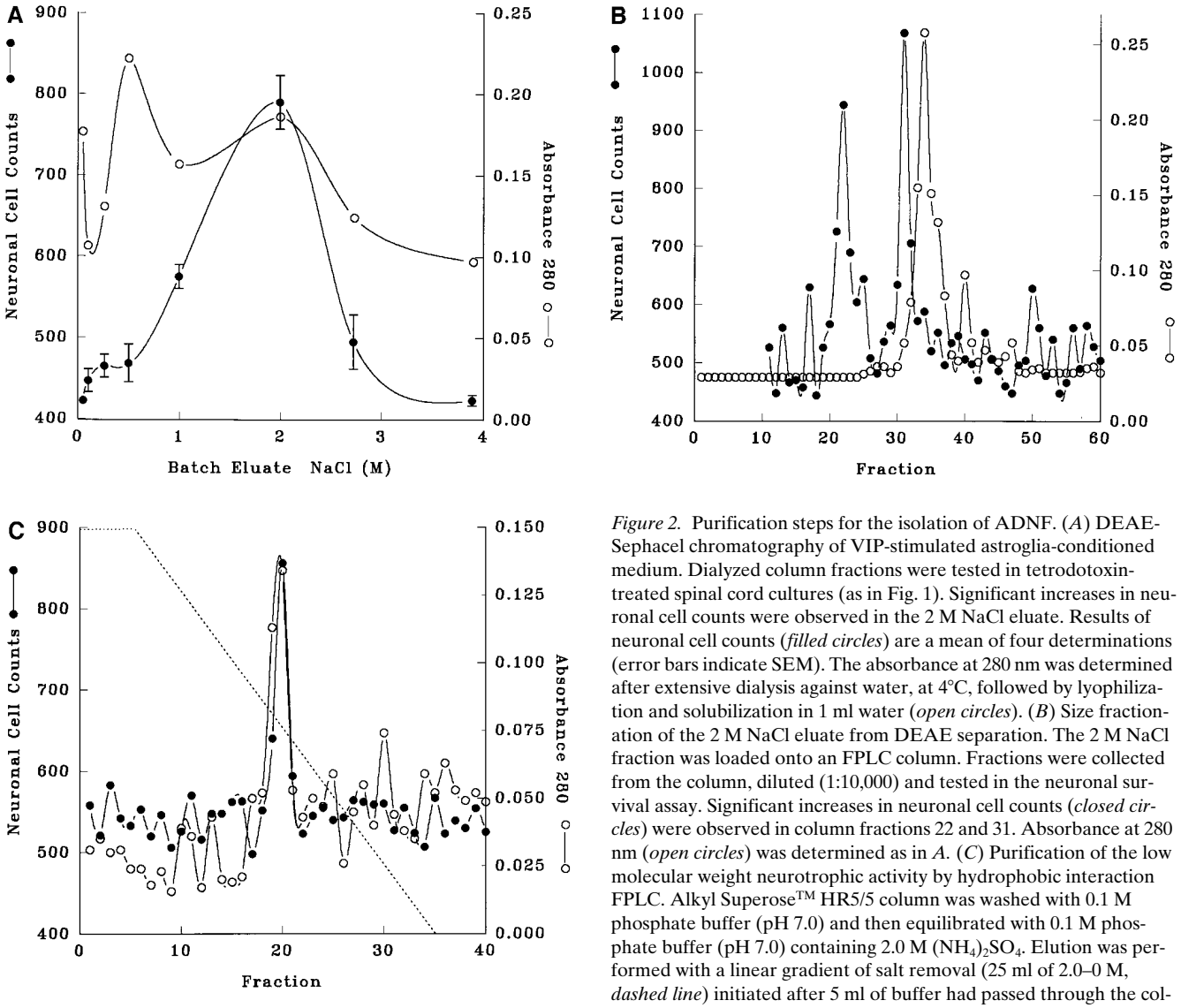
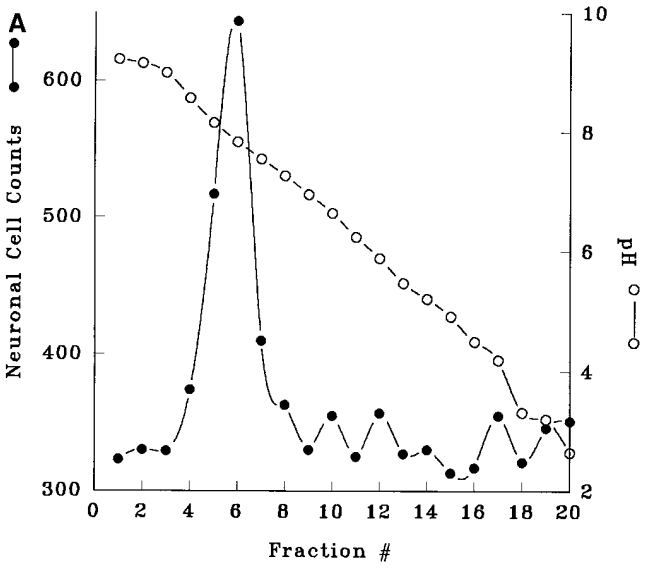
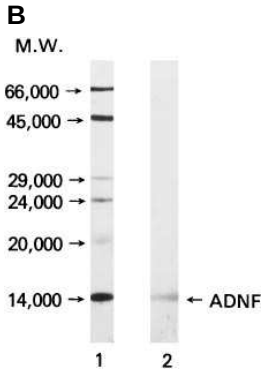


Figure 2. Purification steps for the isolation of ADNF. (*A*) DEAE-Sephacel chromatography of VIP-stimulated astroglia-conditioned medium. Dialyzed column fractions were tested in tetrodotoxin-treated spinal cord cultures (as in Fig. 1). Significant increases in neuronal cell counts were observed in the 2 M NaCl eluate. Results of neuronal cell counts (*filled circles*) are a mean of four determinations (error bars indicate SEM). The absorbance at 280 nm was determined after extensive dialysis against water, at 4°C, followed by lyophilization and solubilization in 1 ml water (*open circles*). (*B*) Size fractionation of the 2 M NaCl eluate from DEAE separation. The 2 M NaCl fraction was loaded onto an FPLC column. Fractions were collected from the column, diluted (1:10,000) and tested in the neuronal survival assay. Significant increases in neuronal cell counts (*closed circles*) were observed in column fractions 22 and 31. Absorbance at 280 nm (*open circles*) was determined as in *A*. (*C*) Purification of the low molecular weight neurotrophic activity by hydrophobic interaction FPLC. Alkyl Superose™ HR5/5 column was washed with 0.1 M phosphate buffer (pH 7.0) and then equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 2.0 M $(NH_4)_2SO_4$. Elution was performed with a linear gradient of salt removal (25 ml of 2.0–0 M, *dashed line*) initiated after 5 ml of buffer had passed through the column. Fractions of 1 ml were collected. The solvent flow was 0.5 ml/min. Results indicate that fraction 20 contained the neurotrophic activity. (*Closed circles*) Neuronal cell counts; (*open circles*) absorbance at 280.

ADNF identified as an extracellular heat shock protein 60 (hsp 60)-like protein. Once purified, the fundamental issue was the determination of the amino acid sequence of ADNF. When purified ADNF was analyzed by Edman degradation, NH_2-terminal blockade was observed. ADNF was then digested with V8 protease and the resulting peptides separated by HPLC (see Methods). Four overlapping peptides were sequenced, all of which showed sequence homology (but not identity) to rat hsp 60 (44). An alignment of the obtained sequences is exhibited in Fig. 3 *C*, representing the known structure for ADNF. Sizing analysis of the neurotrophic activity present in the conditioned medium gave no indication of survival-promoting activity in the 40–70-kD range. Furthermore, incubation of recombinant human hsp 60 (10^{-14}–10^{-9} M) with rat cerebral cortical cultures treated with tetrodotoxin revealed no apparent protection from the neuronal cell death. These data suggested that an hsp 60–like fragment and not hsp 60 accounted for the survival-promoting activity secreted by VIP-stimulated astroglia.

hsp 60–like sequences exhibit neuroprotection. To further investigate the relationship between ADNF and hsp 60, neutralizing antiserum to hsp 60 was added to rat cerebral cortical cultures (Fig. 4 *A*). These studies revealed that the anti–hsp 60 produced a reduction in the number of surviving neurons (30% of control). In contrast, cotreatment with ADNF prevented the neuronal cell death associated with anti–hsp 60 (EC_{50}, 10 fM, Fig. 4 *B*). These experiments indicated that ADNF is related to hsp 60. Furthermore, this hsp 60–like molecule (ADNF) provided neuroprotection at unprecedented concentrations. Other known neurotrophic factors (nerve growth factor, EGF, PDGF, basic fibroblast growth factor, and IGF I) were tested in spinal cord cultures electrically blocked with tetrodotoxin. Over a broad range of concentrations (1 pM to 1 μM), no neuroprotective activity against tetrodotoxin toxicity was detected for any of these compounds (data not shown). In contrast, the EC_{50} for VIP in this paradigm was 30 pM, with an efficacy equal to that of ADNF.





C

...*Val*-Leu-*Gly*-*Gly*-*Gly*-SER-Ala-Leu-Leu-Arg-SER-Ile-Pro-Ala-

Leu-Asp-Ser-Leu-Lys-Pro-Ala-Asn-Glu-Asp-Gln-Lys-Ile-Gly-

Ile-Glu-Ile-Ile-Lys-Arg-*Ala*-Leu...

Figure 3. Biochemical characteristics of purified ADNF. (*A*) Purified ADNF (0.4 μg) was analyzed by isoelectric focusing using the MinipHor™ apparatus. Eluted fractions were assayed for survival-promoting activity after dilution 1:1,000 with PBS. Each value is the mean of two closely agreeing (< 10%) samples. Neuronal cell counts are depicted by closed circles and pH by open circles. (*B*) SDS-polyacrylamide gel electrophoresis of purified ADNF. Before electrophoresis, ADNF was tested for purity by reverse phase HPLC chromatography. ADNF then was subjected to electrophoresis on a 12% polyacrylamide SDS gel containing 0.1% SDS. The 14-kD band was eluted, tested for biological activity, resubjected to electrophoresis, and detected with silver stain. (*C*) Amino acid structure of ADNF. The amino acid sequence of ADNF was determined by V8 protease digest as outlined in Methods. As detailed in Results, four overlapping peptides were analyzed and aligned. Because the amino acid sequence obtained exhibited a marked similarity to hsp 60, the alignment was deduced from the hsp 60 sequence. Two differences from hsp60 are depicted in capital letters. (Amino acids that were missing in the original sequence are in italics and represent the corresponding hsp 60 residues). The partial known sequence is shown in the figure. The first 14 amino acids (line one in the figure) are ADNF-14.

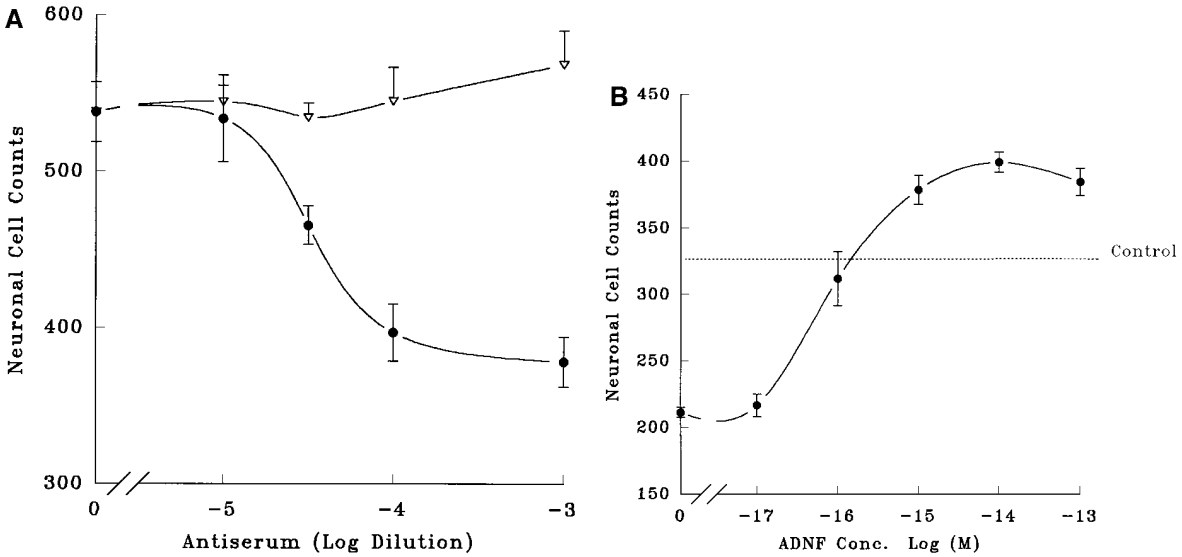


Figure 4. (A) Antisera to hsp 60 produced neuronal cell death. Antiserum to hsp 60 (rabbit anti–hsp60, SPA-804; StressGen Biotechnologies Corp., Victoria Canada) (*closed circles*) is compared to normal rabbit serum (*open triangles*). Antisera was added to 9-d-old rat cerebral cortical cultures. 5 d after the addition of the antiserum, surviving neurons were counted as above. Significant decreases in cell counts were observed at 1:10,000 fold dilution of the anti–hsp 60 ($P < 0.001$). Normal rabbit serum produced no changes in cell counts as compared to controls. (*B*) ADNF prevents hsp 60 antisera–induced death. Antiserum to hsp 60 (1:10,000 dilution) was added to 7-d-old rat cerebral cultures along with increasing concentrations of ADNF. In comparison to cultures treated with antibody alone, significant increases in cell counts were observed in cultures treated with ADNF at concentrations $\geq 10^{-16}$ M ($P < 0.01$). The dotted line illustrates the number of cells in cultures not treated by the anti–hsp 60 antibodies.

A femtomolar-acting neuroprotective peptide: ADNF-14 identified as an active site for neuroprotection. Based on the sequence analyses and the recognized homology to hsp 60, peptides were synthesized and tested for neuroprotective activity in tetrodotoxin-treated cerebral cortical cultures. The synthesized peptides were chosen to include regions of observed sequence similarity with minor differences between hsp 60 and ADNF, with the rationale being that ADNF would have functions distinct from that of hsp 60. As shown in Fig. 5, a 14-amino acid peptide (ADNF-14, comprised of the following sequence, VLGGGSALLRSIPA) increased neuronal survival with an EC_{50} of 0.3 fM, a potency identical to that of intact ADNF in this culture system. ADNF-14 was active over a wider range of concentrations than that observed for the intact protein (see Fig. 5 *A*). Importantly, the homologous peptide from hsp 60 (VLGGGCALLRCIPA) was also tested and shown to be 100,000-fold less potent and ~ 50% less efficacious than ADNF-14 or intact ADNF. These data indicated that the two amino acid residues that were different between ADNF-14 and the hsp 60 peptide (two Cys residues replaced by two Ser residues) were critical for the survival-promoting activity for CNS neurons. Furthermore, these studies established the molecular identity of an active site for ADNF, confirming the structure and extraordinary potency of this new molecule.

ADNF-14 prevents neuronal cell death from clinically relevant toxins. Since VIP (45), a secretagogue for ADNF, had been shown earlier to protect hippocampal neurons from

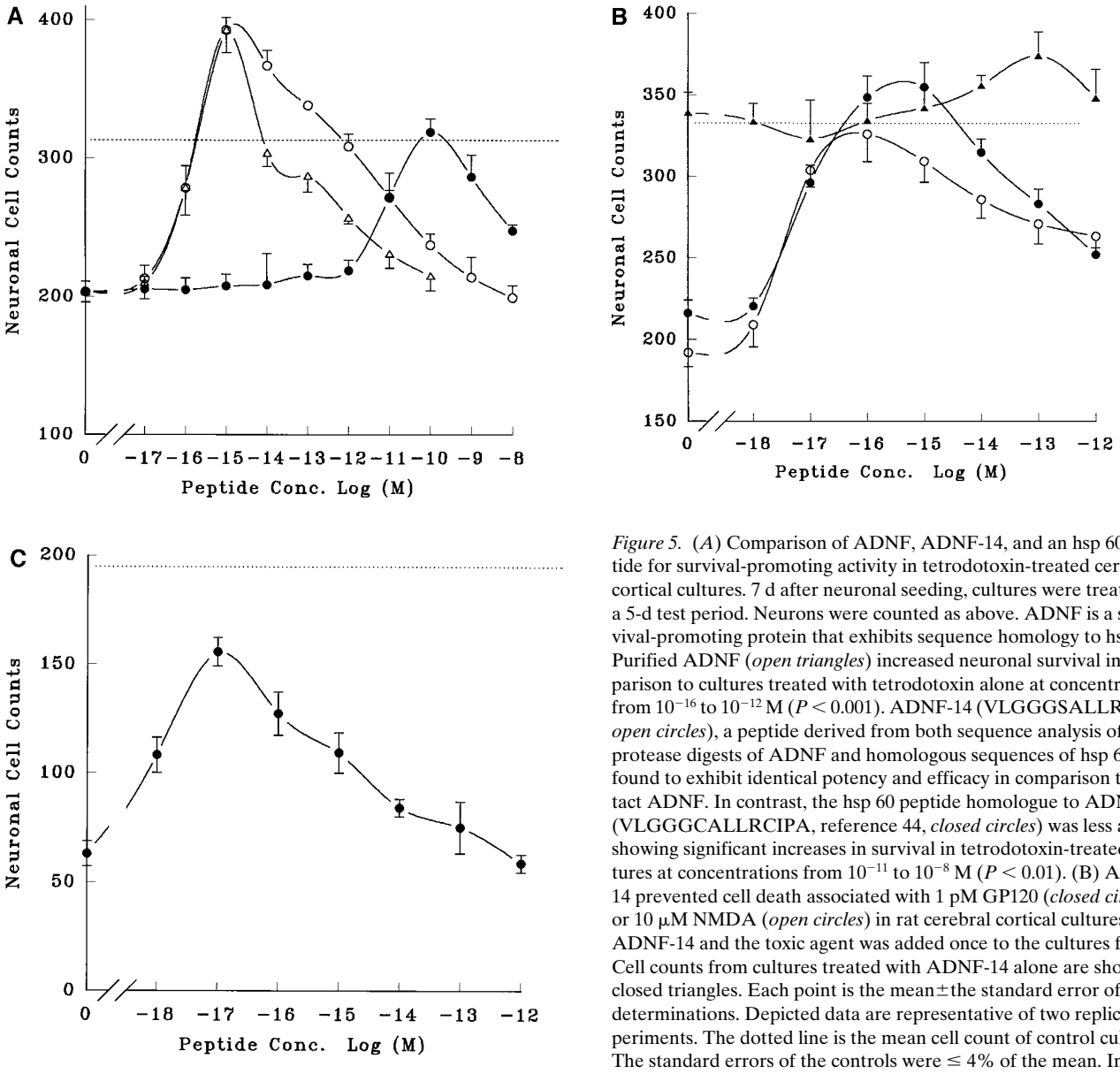


Figure 5. (*A*) Comparison of ADNF, ADNF-14, and an hsp 60 peptide for survival-promoting activity in tetrodotoxin-treated cerebral cortical cultures. 7 d after neuronal seeding, cultures were treated for a 5-d test period. Neurons were counted as above. ADNF is a survival-promoting protein that exhibits sequence homology to hsp 60. Purified ADNF (*open triangles*) increased neuronal survival in comparison to cultures treated with tetrodotoxin alone at concentrations from 10^{-16} to 10^{-12} M ($P < 0.001$). ADNF-14 (VLGGGSALLRSIPA, *open circles*), a peptide derived from both sequence analysis of V8 protease digests of ADNF and homologous sequences of hsp 60, was found to exhibit identical potency and efficacy in comparison to intact ADNF. In contrast, the hsp 60 peptide homologue to ADNF-14 (VLGGGCALLRCIPA, reference 44, *closed circles*) was less active, showing significant increases in survival in tetrodotoxin-treated cultures at concentrations from 10^{-11} to 10^{-8} M ($P < 0.01$). (B) ADNF-14 prevented cell death associated with 1 pM GP120 (*closed circles*) or 10 μM NMDA (*open circles*) in rat cerebral cortical cultures. ADNF-14 and the toxic agent was added once to the cultures for 5 d. Cell counts from cultures treated with ADNF-14 alone are shown as closed triangles. Each point is the mean±the standard error of three determinations. Depicted data are representative of two replicate experiments. The dotted line is the mean cell count of control cultures. The standard errors of the controls were ≤ 4% of the mean. In cultures cotreated with GP120 and ADNF-14, significant increases in neuronal cell counts were observed at all concentrations of ANDF-14 ≥ 10^{-17} M ($P < 0.01$) in comparison to cultures treated with GP120 alone. Similarly, cultures treated with 10 μM NMDA and ADNF-14 had cell counts significantly greater than those treated with NMDA alone at concentrations of ADNF ≥ 10^{-17} M ($P < 0.01$). Treatment with ADNF-14 alone had no detectable effect on neuronal survival in cerebral cortical cultures (*closed triangles*). (*C*) ADNF-14 attenuates the neuronal cell death produced by 25 μM beta amyloid peptide (amino acids 25–35) (46). Significant increases in cell counts were observed in cultures treated with 10^{-18} to 10^{-15} M ADNF-14 ($P < 0.05$) in comparison to cultures treated with beta amyloid peptide alone.

GP120-associated cell death (22), ADNF-14 was tested for its neuroprotective properties (Fig. 5 *B*). Neuronal cell death produced in cerebral cortical cultures treated with 1 pM GP120 (native RFII isolate) was completely and potently prevented by ADNF-14 (EC_{50}, 0.01 fM). Similarly, intact ADNF (1 fM) prevented GP120-induced death in dissociated hippocampal cultures derived from fetal mice (data not shown). Since toxicity associated with GP120 is believed to be associated with activation of a subtype of glutamate receptor (29), the neuroprotective action of ADNF-14 also was tested on cultures treated with NMDA, a specific agonist for a subtype of glutamate receptor. As shown in Fig. 5 *B*, ADNF-14 potently and completely prevented neuronal cell death associated with NMDA treatment. The neuroprotective effects of ADNF-14 against GP120 and NMDA were apparent over 3–4 orders of magnitude, although this action was attenuated at amounts > 0.01 pM. To further test the breadth and possible clinical significance of ADNF-14, cerebral cortical cultures were treated with beta amyloid peptide (amino acids 25–35), a substance that may contribute to the neurodegenerative process in Alzheimer's disease (33, 46). Although treatment with ADNF-14 alone had little effect on naturally occurring neuronal cell death in the cerebral cortical cultures, the peptide attenuated the neuronal cell death produced by the beta amyloid peptide with extraordinary potency (Fig. 5 *C*).

Discussion

We have described the isolation of a novel neuroprotective protein from conditioned medium of VIP-stimulated astroglia cultures derived from the rat cerebral cortex. ADNF displayed a molecular mass between 14 and 16 kD and a pI of 8.3 ± 0.25. The isolated molecule was extraordinarily potent in preventing neuronal cell death in electrically blocked spinal cord test cultures. The EC_{50} of these neuroprotective effects was in the femtomolar range. To our knowledge, there is no precedent for the neuroprotective actions of any recognized neurotrophic factor in this range of concentrations. We have also mapped an active peptide site for the newly isolated protein, ADNF-14. ADNF-14 exhibited neuroprotection against neurotoxins associated with HIV infection, electrical blockade, excitotoxicity, and Alzheimer's disease.

This is the first demonstration of a neuroprotective peptide with stress-protein–like sequences. Stress proteins, which are some of the most highly conserved and abundant proteins in nature, act as molecular chaperones, assisting in proper folding and the formation of protein multimolecular structures, essential for cell survival (47). Most molecular chaperones are induced by a wide variety of cellular stresses (including heat, infection, and inflammation) or during development (48). Expression of stress proteins before a toxic insult can confer neuroprotective effects (49). Although the family of stress proteins has recognized intracellular functions, recent work has identified chaperonin 10 as secreted early pregnancy factor, suggesting that this protein and others in the family may also have extracellular functions (50). Glial cells have been demonstrated to release several heat shock proteins, but not hsp 60 (51). However, hsp 60 immunoreactive material was found in the cerebrospinal fluid of patients with multiple sclerosis (52). Furthermore, changes in patterns of gene expression of intracellular stress proteins were found to be associated with neurodegeneration and neurodegenerative diseases (53). Our demonstration of a VIP-stimulated, secreted form of an hsp 60–like protein from glial cells provides a new orientation for stress proteins having an extracellular role in neuroprotection.

Molecular chaperones produce organized macromolecular structures (47, 48). It is the subject of future research to analyze the possible molecular interactions of ADNF with itself and with other proteins. It is interesting to note that the isoelectric point of ADNF was determined by two methods; both indicated a pI of 8.3 ± 2.5. However, the elution characteristics of ADNF after DEAE-Sephacel suggested an acidic molecule (i.e., at neutral pH, the molecule eluted at very high salt concentration). This elution pattern reflects perhaps another property of the molecule, such as hydrophobicity or the occurrence of highly charged microenvironments resulting from protein assembly into macromolecular structures, providing a different group of exposed amino acids for solvent interactions.

ADNF neuroprotection may be mediated via interaction to protect the structure or facilitate the action of extracellular substances that have regulatory roles during development or nerve injury (8, 21). Alternatively, ADNF may act directly to protect neurons without interacting with other extracellular mediators. The primary structure of ADNF contains an Ala-Leu-Leu sequence that has been found as a repetitive motif in a signal peptide that allows membrane transport of short peptides (54). Similarly, the di-leucine motif has been identified as a sorting signal-mediating endocytosis (55). It is possible that this sequence facilitates membrane association/penetration of ADNF and ADNF-14, allowing them to act with $<$ 100–1,000 molecules per cell.

The neurotoxic action of HIV envelope protein was first discovered in hippocampal cultures derived from fetal mice (22). This neurotoxic action was also observed in rat retinal ganglion cells and extended with the observation that GP120 produced increases in calcium which could be attenuated by NMDA antagonists or calcium channel blockers (29, 56). Previous studies indicated that VIP could protect against the neuronal cell-killing action of GP120 (22). The original hypothesis put forth by Pert et al. was that VIP and the HIV envelope protein had a common binding site, and that the protective role afforded by VIP was simply a competitive inhibition between VIP and GP120 (57). Alternatively, we hypothesize here that the neuroprotective effect of VIP is mediated through the release of ADNF, and it is ADNF which protects neurons from the deleterious effects of GP120 by modulating NMDA responses. The fact that ADNF-14 protects from both GP120 and NMDA toxicity supports this working hypothesis.

Furthermore, the neurotoxicity exhibited by the beta amyloid peptide (amino acids 25–35) may also involve the NMDA-responsive glutamate receptor in hippocampal neurons (58), in lateral septal neurons (59), and in cortical neurons (60). The beta amyloid peptides destabilize calcium homeostasis and hence render human cortical neurons vulnerable to excitotoxicity (60). Activation of the NMDA-responsive receptor (ionotropic glutamate receptor) may induce an increase in the amyloid precursor protein level (61). This increase, coupled with deficits in glutaminergic transmission (metabotropic glutamate receptors), which have been observed in Alzheimer's brains, may hinder the breakdown of amyloid precursor protein, which normally forms soluble, nonamyloidergic derivatives in vivo (62). It is possible that ADNF inhibits neurotoxicity by influencing Ca^{++} mobilization. Such a calcium-dependent mech-

anism could be a common denominator for protection against neurotoxicity.

The discovery of ADNF has provided additional knowledge on the neuroprotective action of VIP (45). The neurotrophic property captured in the ADNF peptide has significant implications for its therapeutic potential. The discovery that ADNF can be mimicked by a 14–amino acid peptide is predicted to facilitate innovative drug design for the treatment of the neurological symptoms associated with HIV infection, Alzheimer's disease, and, potentially, other prevalent neurodegenerative diseases.

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