Complete Sequence of a Novel Protein Containing a Femtomolar-Activity-Dependent Neuroprotective Peptide


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Abstract: The vulnerability of neurons and the irreversibility of loss make discoveries of neuroprotective compounds fundamentally important. Here, the complete coding sequence of a novel protein (828 amino acids, pl 5.99), derived from mouse neuroglial cells, is revealed. The sequence contained (1) a neuroprotective peptide, NAPVSIPQ, sharing structural and immunological homologies with the previously reported, activity-dependent neurotrophic factor; (2) a glutaredoxin active site; and (3) a zinc binding domain. Gene expression was enriched in the mouse hippocampus and cerebellum and augmented in the presence of the neuropeptide vasoactive intestinal peptide, in cerebral cortical astrocytes. In mixed neuron–astrocyte cultures, NAPVSIPQ provided neuroprotection at subfemtomolar concentrations against toxicity associated with tetrodotoxin (electrical blockade), the β-amyloid peptide (the Alzheimer’s disease neurotoxin), N-methyl-D-aspartate (excitotoxicity), and the human immunodeficiency virus envelope protein. Daily NAPVSIPQ injections to newborn apolipoprotein E-deficient mice accelerated the acquisition of developmental reflexes and prevented short-term memory deficits. Comparative studies suggested that NAPVSIPQ was more efficacious than other neuroprotective peptides in the apolipoprotein E-deficiency model. A potential basis for rational drug design against neurodegeneration is suggested with NAPVSIPQ as a lead compound. The relative enrichment of the novel mRNA transcripts in the brain and the increases found in the presence of vasoactive intestinal peptide, an established neuroprotective substance, imply a role for the cloned protein in neuronal function. Key Words: Vasoactive intestinal peptide—Apolipoprotein E—Learning and memory—Neuronal survival—Molecular cloning—mRNA.


Since the discovery of nerve growth factor (Hamburger and Levi-Montalcini, 1949), a consensus has emerged that neurotrophic proteins (Patterson, 1992; Maness et al., 1994) and neuropeptides (Gozes and Brenneman, 1993) have important regulatory functions during development and after nerve injury (see Brenneman et al., 1988; Gozes et al., 1996, 1997b). Among the known neuroprotective peptides, vasoactive intestinal peptide (VIP; Said, 1996; Brenneman et al., 1998b) has been linked with electrical activity and neuronal survival (Brenneman and Eiden, 1986). Blockade of spontaneous electrical activity in spinal cord cell cultures results in the death of ~50% of the neurons during a critical period of development. VIP prevents the cell death associated with electrical blockade by releasing glia-derived, survival-promoting substances (Brenneman and Gozes, 1996). Previous studies have identified several components within the neurotrophic milieu produced by VIP-stimulated astroglia, the most potent being activity-dependent neurotrophic factor (ADNF; Brenneman and Gozes, 1996; Gozes and Brenneman, 1996; Gozes et al., 1997a; Brenneman et al., 1998a). Comparative studies with other recognized growth factors indicated that at femtomolar concentration only ADNF prevented neuronal cell death associated with electrical blockade in rat cerebral cortical cultures (Gozes et al., 1997a). A 14-amino acid peptide derived from ADNF (ADNF-14, VLGGGSALLRSIPA) has been reported that protects cultured neurons from multiple neurotoxins (Brenneman and Gozes, 1996). This ADNF-derived peptide exhibited a remarkable structural similarity to the stress-induced chaperonin, heat shock protein 60 (hsp60), containing the highly conserved sequence VLGGSALLRCIPA.
(Gozes and Brenneman, 1996). Structure–activity relationships of peptides related to ADNF-14 revealed a nine-amino acid core peptide (ADNF-9, SALLRSIPA) with greater potency and broader effective concentration range (10⁻¹⁶ to 10⁻¹³ M) than ADNF or ADNF-14 in preventing neuronal death associated with tetrodotoxin treatment (Brenneman et al., 1998a). Antibodies prepared against ADNF specifically recognized the active peptides and induced apoptosis in cerebral cortical cultures (Gozes et al., 1997a). Here, antibodies prepared against ADNF-14 and affinity purified to recognize ADNF-9 were used to screen a cDNA expression library of neuroglial origin. A novel ADNF-14/9-like active peptide (NAPVSIPQ or NAP) with a greater in vivo neuroprotective efficacy, compared with ADNF-9, is disclosed that constitutes a part of a new protein. As the cloned protein contained the potent peptide that prevented neuronal death associated with electrical blockade, it was named the activity-dependent neuroprotective protein (ADNP).

MATERIALS AND METHODS

Antibody preparation

Antibodies were elicited in rabbits after covalent linkage of ADNF-14 (CVLGGGSALLRSIPA) to keyhole limpet hemocyanin through the attached N-terminal cysteine moiety. In a parallel experiment, the carrier protein was bovine serum albumin instead of keyhole limpet hemocyanin. Affinity chromatography was performed on Sepharose columns with conjugated CVLGGGSALLRSIPA or CSALLRSIPA peptides (both conjugated through the cysteine residue). Antibodies were bound in phosphate-buffered saline, washed with 0.5 M NaCl in phosphate-buffered saline and eluted in 0.1 M glycine-HCl, pH 2.5. Eluted fractions were neutralized with 0.1 volume of 2 M Tris (pH 8.0). Antibodies were first purified against VLGGSALLRSIPA and then against SALLRSIPA.

Molecular cloning

Complementary DNA [oligo(dT)-primed] derived from the P19 carcinoma cells differentiated (in the presence of retinoic acid) into neuronal cells was ligated into the vector Uni-Zap XR (Stratagene, La Jolla, CA, U.S.A.). The resulting expression library (a gift from Dr. Dayao Zhao, Pfizer, Groton, CT, U.S.A.; Zhao et al., 1996) contained 2 x 10⁶ plaque-forming units (pfu) and 2 x 10¹⁰ pfu after the first amplification in E. coli XLI-Blue. Cloning was performed according to standard methods (Sambrook et al., 1989). Expressed clones (protein transferred to nitrocellulose filters in the presence of isopropyl-β-D-thiogalactopyranoside) were further reacted with ADNF-specific antibodies [SALLRSIPA antibodies (1:250)] followed by goat anti-rabbit IgG-peroxidase conjugate (Sigma Immuno Chemicals, Holon, Israel; 1:30,000) and the ECL nonradioactive detection kit (Amersham, Little Chalfont, U.K.). Antibodies were elicited in rabbits after covalent linkage of ADNF-14,9 to keyhole limpet hemocyanin through the attached N-terminal cysteine residue. Antibodies were obtained in vivo from the AZAP vector, using the EXAssist helper phage system (Stratagene). After transformation into XL1-Blue cells, ampicillin-resistant clones were collected. Phagemid preparation used the Wizard Midi-Prep DNA Puriﬁcation System (Promega, Madison, WI, U.S.A.).

Preparation of the expressed protein and western blotting

Bacteria carrying the phagemid clone were incubated with isopropyl-β-D-thiogalactopyranoside and proteins were extracted in 4 M guanidine-HCl, 100 mM KCl, 50 mM Tris, pH 8.0, 1 mM EDTA, 12.5 mM MgCl₂, 0.1% Nonidet P-40, and a mixture of protease inhibitors, i.e., phenylmethylsulfonyl fluoride, aprotonin, and leupeptin. Protein solutions (1 mg/ml) were stored at −80°C. Bacterial extracts were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis followed by western blot analysis (Sambrook et al., 1989).

Laboratory animals

The experimental protocols were approved by the appropriate institutional review committee and meet the guidelines of the responsible governmental agency.

RNA preparation

Rat cerebral cortical astrocytes were prepared as before (Gozes et al., 1991; Brenneman and Gozes, 1996). Astrocytes were incubated in the presence of 1 mM VIP in phosphate-buffered saline for 3 h. RNA was prepared by using RNAeasy (Qiagen, Hilden, Germany). For tissue RNA extraction, RNAzol B solution (Tel-Test, Friendwood, TX, U.S.A.) was used.

Northern blot hybridization

RNA (10–12 μg) was subjected to electrophoresis followed by northern blot hybridization (Gozes et al., 1987; Sambrook et al., 1989) on Nytran 0.45 filters (Schleicher and Schuell, Dassel, Germany). For probe labeling, the cDNA was subjected to PCR, using AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ, U.S.A.) and [α-³²P]dCTP (Amersham; 3,000 Ci/mmol). The primers used for amplification of ADNP cDNA were base pairs 137–156 and 503–520, sense and antisense, respectively, for the mouse ADNP cDNA (see Fig. 1). Actin mRNA (Gozes et al., 1987) or the 28S ribosomal RNA (Barbu and Dautry, 1989; Glazer and Gozes, 1994) was used as an internal standard.

Cell cultures and neuronal survival

Cerebral cortical cultures derived from newborn rats (Gozes et al., 1996; Brenneman et al., 1998a) were used for the neuron survival assays. For mixed neuroglial cultures, dissociated cerebral cortical tissue was seeded on a confluent layer of astroglial cultures derived from rat cerebral cortex. Three hundred thousand cells were placed into a 35-mm dish in a volume of 1.5 ml. The mixed cultures were maintained in medium consisting of 5% horse serum in minimal essential medium supplemented with defined medium components and 5'-fluoro-2-deoxyuridine (15 μg/ml) plus uridine (3 μg/ml). Four days after neuronal plating, cultures were given their respective treatment (once) and assayed for neuronal survival after an additional 5-day incubation period. Neuronal cell counts were conducted as before (Brenneman and Gozes, 1996; Gozes et al., 1996), using antisera against neuron-specific enolase (NSE) to identify surviving neurons (Schmechel et al., 1978). In parallel experiments, neuronal cell counts were conducted, in sister cultures, after fixation with glutaraldehyde (Brenneman et al., 1987) and neuronal identity was established by morphological criteria. Performing the experiments in such a manner excluded the possibility of measuring increases in the expression of NSE in a subset population of neurons. Mitogenic effects in this cell culture system are also essentially excluded, because we are dealing with postmitotic neurons (Rappoport and Fritz, 1972).
The apolipoprotein E (ApoE)-deficient mouse model
ApoE knockout mice and normal controls were a gift from the late Prof. Shlomo Eisenberg of Tel Aviv University, originally provided by Dr. J. L. Breslow (Plump et al., 1992). The ApoE knockout mice were obtained from embryonic stem cells OLA 129 in C57B6 × FVB mice (Plump et al., 1992). Age-matched inbred C57B6 mice were also tested. Groups of mouse pups were treated from birth until the age of 14 days. Daily subcutaneous injections included 20 μl of saline (days 1–4), 40 μl of saline (days 5–10), and 80 μl of saline (days 11–14). NAP and ADNF-9 (synthesized as before; Gozes et al., 1996, 1997b; Brenneman et al., 1998a) were diluted to a final concentration of 25 μg/ml before administration, ADNF-14 was diluted to a final concentration of 50 μg/ml. To obtain homogeneous solutions of peptides, initial solubilization was performed in dimethyl sulfoxide (1 mg/30 μl) followed by serial dilutions in saline. Controls received saline. The vehicle (dimethyl sulfoxide diluted in saline) did not have any effect, and results obtained with vehicle were similar to the results obtained with saline alone.

Measurements of cholinergic activity
Choline acetyltransferase (Chat) activity was measured according to published procedures (Fonnum, 1975). Enzyme activity was determined by measurements of the rate of synthesis of [14C]acetylcholine from choline and [14C]acetyl-CoA. Nonspecific background was measured in the absence of choline. Brains from 21-day-old normal (control) and ApoE-deficient mice were assessed by measuring their Chat activity. Each brain (300–400 mg) was homogenized in a Teflon homogenizer, with 10 volumes of 50 μM EDTA, and 0.5% Triton. The homogenates were centrifuged at 12,000 g for 15 min, and 10 ml of the supernatant (in triplicates) was mixed with 10 ml of a solution containing 14 μM [14C]acetyl-CoA (56 mCi/mmol, NEN), 20 mM acetylcholine, 1.6 mM choline chloride, 0.25 mM eserine, and phosphate buffer. Incubation was performed at 37°C for 15 min. The reaction was terminated by adding 50 μl of 15 mg/ml tetraphenylboron (prepared in 3-hexanone) and vigorous mixing for 30 s. Twenty microliters of the organic phase was collected (2 min, 10,000 g), mixed with scintillation liquid, and radioactivity was measured in a β-counter. Protein concentrations were determined by using the Bio-Rad (München, Germany) kit for protein assay.

Measurements of developmental milestones
At least three dams (five to eight pups each) were used for every experimental point. All observations were made between 1200 and 1600 h. From day 1 through day 6, mice were weighed and tested 60 min after the daily injection. The following behavioral parameters were assessed: (1) time (in seconds) to surface right (test animal is placed on its back and the time measured until it turns over); (2) time (in seconds) to perform negative geotaxis (animal, facing down, is placed on a screen held at angle of 45°, and the time required to turn around and start to climb up the screen is measured); (3) time (in seconds) to perform a placing behavior (placing responses are measured as time elapsing between contact of the foot against the edge of an object and its placing on the top of the same object); and (4) cliff avoidance: the pup is placed at the edge of a cliff (or a table top) with forepaws and face over the edge, and the time is measured for turning away from the edge.

Assessment of short-term spatial memory in a water maze
Three-week-old mice were subjected to two daily tests in a water maze, including a hidden platform (Morris, 1984; Gordon et al., 1995; Gozes et al., 1996, 1997b). Every day for the first test, both the platform and the animal were situated in a new location with regard to the pool (with the pool being immobile). The experiment was performed as follows: The animal was positioned on the platform for 0.5 min, then placed in the water. The time required to reach the platform (indicative of learning and intact reference memory) was measured (first test). After 0.5 min on the platform, the animal was placed back in the water (in the previous position) for an additional second test and search for the hidden platform (retained in the previous position). The time required to reach the platform in the second trial was recorded, indicative of short-term (working) memory.

Statistical analysis
ANOVA with Student–Newman–Keuls multiple comparison of means test was used to assess the results.

RESULTS
Molecular cloning of a new protein immunologically related to ADNF
As SALLRSIPA (ADNF-9) and VLGGSALLRSIPA (ADNF-14) provided neuroprotection at unprecedented femtomolar concentrations, we have decided to identify potential new molecules with similar potencies, using an immunomolecular approach. A cDNA expression library derived from a mouse embryonic carcinoma (P19) induced to differentiate into glia and neurons by retinoic acid was screened with a specific antibody elicited against ADNF-14 and affinity-purified against ADNF-9. Here we report on a positive clone (clone 25) detected in 50 plates (90 mm) containing 2 × 10⁴ plaques. The immunopositive cDNA included 2,484 bp of open reading frame, encoding 828 amino acids with a calculated molecular mass of 92,062 kDa (Fig. 1). The cloned sequence was compared with cloned sequences obtained from public databases (i.e., GenBank and EMBL) and was shown to be novel, except for random short expressed sequence tags (ESTs) that were identified.

When analyzing the novel cDNA sequence of clone 25, a 10-amino acid sequence (LLGNAPVSIP) exhibited a 70% structural similarity to ADNF-14 (VLGGSALLRSIPA; Brenneman and Gozes, 1996; Gozes and Brenneman, 1996). Further comparative sequence analysis revealed a zinc finger domain (Fig. 1, boldface; Rosenfeld and Margalit, 1993). Within this sequence, a homology to the active site of glutaredoxin (a thiol transferase) was also observed (Fig. 1; Johnson et al., 1991). Overall analysis, using the prediction of Chou and Fasman (1978), indicated that the protein was a flexible hydrophilic molecule with multiple antigenic sites and mixed α-helices and β-sheets. Nine potential glycosylation sites suggested a protein that was membrane associated or secreted. The hydrophilic nature was consistent with a secreted protein. A putative signal peptide of 15 amino acids (starting at the N-terminal Arg; Fig. 1) comprising hydrophobic, polar, and basic amino acids without acidic groups was identified at the
N-terminal of the molecule (net charge +2). The long stretch of glutamic acid residues at the C-terminal region of the molecule (Fig. 1) could mediate interactions with extracellular basic molecules, such as polyamines (Chang et al., 1987), or serve as a site for proteolytic cleavage (Cheshtukhin et al., 1997). Other potential processing sites found in the molecule are:  

FIG. 1. The complete coding sequence of mouse ADNP. A positive clone 25 was detected among 1013 plaques screened. Sequencing of clone 25, encoding ADNP, was automated (Applied Biosystems, The Weizmann Institute of Science Core Facilities, Rehovot, Israel). Synthetic oligodeoxynucleotides were used in conjunction with the Erase-a-Base Kit (Promega, Madison, WI, U.S.A.), which generates fragments with overlapping deletions. To precisely map the initiation codon, the mouse gene was also cloned in a BAC system (Genome Systems, St. Louis, MO, U.S.A.), and the initiation codon AUG was chosen as the first one appearing 640 bases downstream of a termination codon in the gene sequence. The initiator AUG was the first one containing the consensus initiation codon including a G immediately after the AUG codon and a G three bases before. To further verify expression of the upstream noncoding sequence, the gene sequence was also compared with EST sequences, obtained by Blast search against the translated EST databases, maintained at the National Center for Biotechnology Information (NCBI) at Bethesda, MD, U.S.A. The EST sequence (\textit{Mus musculus} cDNA clone, gene bank accession no. W12764) showed 98.88% identity to 363 bases, 13 bases upstream of the presumptive initiation codon, suggesting at least 376 bases of noncoding region at the 5' end of the cloned mRNA. Motifs in the DNA and translated protein sequences were determined by using the GCG programs (Wisconsin package version 8.1 UNIX, August 1995). GenBank submission no. bankit 198634/AF068198.

Single Underline - homologies to HSP60 of ADNP  
Double Underline - Glycosylation site of ADNP (amino acid no. 118-120, 205-207, 393-395, 426-428, 576-578, 606-608, 756-758, 775-777, 792-794)  
Bold & Italic - represents two motifs:  
1. Glutaredoxin active site (amino acid no. 233-243)  
2. Zinc finger C2H2 type, domain (amino acid no. 233-254)  
Bold & Underline - putative signal peptide
The antibody used for the screening detected the antigen SALLRSIPQA and NAPVSIPQ (NAP, clone 25 sequence) but not LGGGS (Fig. 2, right panel, dot blot). This antibody specifically identified the bacterially expressed clone 25 protein by western blotting (~90 kDa, Fig. 2, p25). Transformed bacterial extract with the carrier phagemid devoid of the cloned insert was used as a negative control [Fig. 2, plasmid blue script (pBS)]. An additional protein band (~60 kDa) was identified by the antibody, in both p25 and pBS, suggesting that this immunoreactive band was not specific for the cloned protein. The ~90-kDa protein band probably represented the expressed protein, as expected from the size of the cDNA open reading frame.

After cloning the cDNA, studies were directed toward two different routes: (1) mRNA expression analysis and (2) neuroprotective functions of NAPVSIPQ. As NAPVSIPQ provided potent neuroprotection against electrical blockade, tetrodotoxin, and excitotoxicity provided potent neuroprotection against electrical blockade, neuroprotective functions of NAPVSIPQ. As NAPVSIPQ two different routes: (1) mRNA expression analysis and (2) cDNA open reading frame.

The expressed protein, as expected from the size of the protein. The immunoreactive band was not specific for the cloned antibody, in both p25 and pBS, suggesting that this macromolecular interactions (Taira et al., 1993).

Further hybridizations have identified ADNP mRNA in rat astrocytes (Fig. 3B). The major ADNP mRNA in the rat astrocytes exhibited the same size as the mouse mRNA (Fig. 3A and B). However, in the rat, an additional slightly higher molecular weight band was observed, which is related to ADNP. The ADNP mRNA content was increased two- to threefold after a short exposure (3 h) to the neuropeptide VIP (Fig. 3B). Hybridization with rRNA (28S) was used as an internal control (Barbu and Dautry, 1989; Glazer and Gozes, 1994) and densitometric scanning indicated at least 1.7-fold increase in ADNP mRNA also after normalization with the internal standard.

**A neuroprotective eight-amino acid peptide (NAP) derived from ADNP**

As NAPVSIPQ (NAP) exhibited structural and immunological similarity to the active ADNF-9 (Figs. 1 and 2), it was further tested for biological activity. The number of surviving (protected) neurons was assessed in cerebral cortical cultures derived from newborn rats (Gozes et al., 1996) using two toxins: (1) tetrodotoxin, a blocker of electrical activity (Brenneman and Eiden, 1986), enhancing apoptosis in 30–50% of the neurons, including the cholinergic population (Gozes et al., 1996), enhancing apoptosis in 30–50% of the neurons, including the cholinergic population (Gozes et al., 1996) and (2) a fragment of the β-amyloid peptide, an Alzheimer’s disease-associated toxin (Selkoe, 1993), providing a 50–70% reduction in neuronal cell counts (Gozes et al., 1996). NAP (10⁻¹⁶ and 10⁻¹⁵ M) protected against neurotoxicity associated with the β-amyloid peptide (p < 0.01) and, at concentrations ranging from 10⁻¹⁸ to 10⁻¹⁴ M, NAP protected against electrical blockade (p < 0.01; Fig. 4A). The cell counts totaled >100% of control, because the treatment may have prevented neuronal cell death that occurred naturally in the cultures (Brenneman and Gozes, 1996; Gozes et al., 1997a) as follows below. The number of neurons plated is 300,000 per plate; the number that survives the disso-
The association and plating procedure is ~88%; and the number that survives at the termination of the experiment is 75% (without any additional toxins). As evidenced by Fig. 4B, at $10^{-10} \ M$ NAP concentration (without the addition of toxins), the number of neurons was >100% observed in untreated cultures at the termination of the experiment. Alternative interpretations are discussed below.

Considerable breadth of activity was evident in that NAP also protected neurons against toxicity associated with gp120, the envelope protein from the human immunodeficiency virus (Brenneman et al., 1988) and from NMDA (Lipton et al., 1991). The range of neuroprotective concentrations against gp120, $10^{-15}$ to $10^{-10} \ M$ (Fig. 4B), was quite broad and was surpassed by the range of neuroprotective concentrations against NMDA, $10^{-16}$ to $10^{-8} \ M$ ($p < 0.01$), representing an unusual wide limit of efficacy (Fig. 4B).

**NAP protects against deficits in ApoE-deficient mice**

The in vivo efficacy of NAP was assessed in ApoE-deficient (knockout) homozygous mice (Plump et al., 1992; Gordon et al., 1995; Masliah et al., 1995; Gozes et al., 1997b; Oitzl et al., 1997; Chapman and Michaelson, 1998; Fisher et al., 1998), a useful model system for studies of neurodegeneration and neuroprotection. Brain ApoE coordinates the mobilization and redistribution of cholesterol in association with repair, growth, maintenance, and plasticity (Weisgraber et al., 1994). One of the three common alleles of ApoE, the ApoE4 allele, was identified as a major susceptibility gene for Alzheimer’s disease. ApoE4 promotes the assembly of the $\beta$-amyloid peptide into toxic filaments (Ma et al., 1994), whereas ApoE2 inhibits $\beta$-amyloid peptide toxic aggregation (Ma et al., 1996). Previous studies have identified neuronal destruction (Masliah et al., 1995) and memory impair-

**FIG. 3.** A: Northern blot, identification of ADNP mRNA. RNA was extracted from various tissues and brain sections of 18-day-old inbred C57B6 mice. RNA was subjected to northern blot hybridization with a PCR-labeled ADNP ([6-32P]dCTP, Amersham, 3,000 Ci/mmol)-specific probe in comparison with an actin-specific probe as before (Gozes et al., 1987). B: ADNP mRNA is increased after VIP treatment. RNA was extracted from rat astrocytes treated and untreated with VIP for 3 h and subjected to northern blot hybridization as above.

**FIG. 4.** A: An eight-amino acid peptide (NAPVSIPQ or NAP) provides neuroprotection. Filled circles, protection against tetrodotoxin; open circles, protection against $\beta$-amyloid. B: Examples of neuroprotection by NAP against 1 pM gp120 (RFII isolate; Brenneman et al., 1988; Brenneman and Gozes, 1996), NMDA (10 $\mu$M), and naturally occurring cell death. Toxins were added to 9-day-old cultures and incubated for an additional 5 days. The respective peptides were added together with the toxins at indicated dilutions. Experiments were repeated at least three times. The number of neurons counted in the control cultures was $387 \pm 20$ in A and $313 \pm 11$ in B.
ments in certain strains of ApoE-deficient mice (Gordon et al., 1995; Gozes et al., 1997b; Masliah et al., 1997; Oitzl et al., 1997) that are associated with dysfunctions of adult basal forebrain cholinergic neurons and that, perhaps, mimic in part the ApoE4 genotype in humans (Gordon et al., 1995; Chapman and Michaelson, 1998; Fisher et al., 1998).

During postnatal development, ApoE-deficient mice also exhibited a significant decrease (~25%) in brain ChAT activity in comparison with age-matched (21-day-old) inbred C57B6 mice (Gozes et al., 1997b). Daily subcutaneous injections of ApoE-deficient mice with NAP (from birth to 14 days of age) resulted in brain ChAT activity (at 21 days of age) that was not significantly different from inbred C57B6 mice (Fig. 5A) and was significantly improved compared with vehicle-treated or untreated ApoE-deficient mice (p < 0.009). In contrast, similar treatment with ADNF-14, or ADNF-9, did not improve cholinergic activity significantly, in this model system (Fig. 5A).

FIG. 5. ApoE-deficient mice exhibit impairments that are ameliorated by prophylactic NAP treatment. A: ApoE-deficient mice exhibit a reduction in ChAT activity. The graph depicts incorporation of radiolabeled choline into acetylcholine. Experiments were conducted as described in the text. ApoE-deficient mice are designated ApoE; 100% activity in the control (C57B6) mice indicated 669–758.4 pmol/mg of protein/min. As the experiments were repeated three to five times, the results were standardized against the control calibrated at 100% per each experiment. ApoE-deficient mice injected daily with peptides are designated ADNF-14, ADNF-9, and NAP, respectively. B: ApoE-deficient mice exhibit developmental retardation, protection by ADNF peptides. Animals (15–37 per experimental group and five to eight animals per litter) were submitted to placing response assays daily after injection of (1) saline (open circles: C57B6 mice = control, n = 37; filled circles: ApoE-deficient = ApoE, n = 31); (2) ADNF-14 (filled triangles: 15 ApoE-deficient animals); (3) ADNF-9 (filled squares; ApoE-deficient animals, n = 32); (4) NAP (open rectangles: ApoE-deficient animals, n = 22). Experiments were repeated three times. Scores are as follows: 0 = no reaction; 1 = time to acquire a response of <15 s; 2 = time to acquire a response of <10 s; and 3 = time to acquire a response of <5 s. The age of the tested animals is in days. Results are mean ± SEM values. C and D: Two daily water maze trials were performed on 3-week-old animals. Groups tested were (1) control animals (C57B6 mice) injected with vehicle (saline) for the first 2 weeks of life [35 animals of six different litters (five to seven animals from each litter), open circles]; (2) ApoE-deficient animals injected with vehicle for the first 2 weeks of life [18 animals derived from three different litters (five to seven pups per litter), filled circles]; (3) control animals chronically treated with NAP for the first 2 weeks of life (14 animals derived from three different litters, open triangles); (4) ApoE-deficient mice chronically treated with NAP for the first 2 weeks of life (19 animals derived from three different litters, open rectangles). The panels depict latency [of the first (C) and the second (D) daily trials] measured in seconds, to reach the hidden platform 0.5 min after being on it. Tests were performed over 5 consecutive days, and then with a 2-day delay tested for 1 additional day. ApoE, ApoE-deficient animals. There were no differences between animals treated with vehicle and untreated animals (data not shown). Statistical comparisons shown (ANOVA) were made between all groups (C and D) as follows: (1) between ApoE-deficient and controls; (2) between ApoE-treated with NAP and ApoE-treated with vehicle; and (3) between control, treated with NAP, and control, treated with vehicle (*p < 0.001; **p < 0.03; ***p < 0.002).
Further measurements in the ApoE-deficient mouse included assessments of the time at onset of developmental milestones of behavior. As previously demonstrated, major differences were found in the acquisition patterns of placing and cliff avoidance responses between ApoE-deficient animals and age-matched inbred C57B6 mice (Fig. 5B; Gozes et al., 1997b). However, daily injection to the deficient mice with ADNF-14, ADNF-9, or NAP showed acceleration of the acquisition of the placing response and the cliff avoidance response. As an example, the placing response is demonstrated in Fig. 5B. A significant difference was observed among the treatment groups on the first day of testing \((p < 0.0001)\), with inbred C57B6 mice developing faster than the ApoE-deficient animals. Injection of NAP and ADNF-9, but not ADNF-14, resulted in marked improvement even at the first day of treatment, suggesting that an hour exposure to the short peptide was enough to elicit a response. NAP-treated animals were the fastest. From postnatal day 2 onward, the best performers were the NAP- and ADNF-9-treated ApoE-deficient animals \((p < 0.0001)\). In contrast to ApoE-deficient animals treated with NAP or ADNF-9, the deficient animals treated with ADNF-14 did not develop as fast and a significant difference between treated and untreated began to appear only on postnatal day 3 \((p < 0.05)\). Furthermore, although ADNF-14 administration improved the development of the placing response in ApoE-deficient animals (see Fig. 5B, days 5 and 6, \(p < 0.05\)), the treated mice never reached the level of performance of the NAP- or ADNF-9-treated animals.

A week after cessation of treatment, cognitive functions were assessed in the Morris water maze. ADNF-14- or ADNF-9-treated ApoE-deficient animals did not exhibit any improvement in short-term memory after chronic peptide treatment (data not shown). In contrast, marked improvements of reference and short-term memory were observed a week after cessation of the 2-week daily NAP treatment, i.e., in 21-day-old mice exposed to an 8-day training protocol (Fig. 5C and D). Reference memory was assessed by performance in the water maze, measuring the time required to find a hidden platform in the first of two daily trials (Fig. 5C). Short-term memory processes were examined by performance in the water maze, measuring the time required to find the hidden platform in the second of two daily trials (Fig. 5D; Gordon et al., 1995; Gozes et al., 1997b). The platform location and the starting point in which the animal was placed in the water were held constant within each pair of daily trials, but both locations were changed every day. Before the first test the animal was positioned on the platform for 0.5 min, then placed in the water. Examining the performance of the mice in the first of the two daily tests revealed a difference between the ApoE-deficient mice and all the other treatment groups, with the ApoE-deficient animals exhibiting significant increases in the time required for finding the hidden platform (Fig. 5C). The difference was already apparent on the first daily test \((p < 0.001)\). Previous experiments (Gordon et al., 1995) have suggested that the difference between the control and the ApoE-deficient mice was not due to motor or visual impairments in the deficient mice. Furthermore, the slope of the curve of the first daily test for the control in comparison with the ApoE-deficient mice differed in that there was no improvement with the ApoE-deficient mice in day 2 of testing, whereas a significant improvement was observed with the control mice (Fig. 5C; \(p < 0.03\)). ApoE-deficient mice were significantly delayed in performance compared with control mice, even after 5 training days (Figs. 5C and D). Unexpectedly, chronic treatment of C57B6 mice with NAP improved their performance (Fig. 5D). Furthermore, NAP-treated ApoE-deficient animals performed as well as NAP-treated ApoE-expressing (C57B6) animals on all test days (Fig. 5D).

**DISCUSSION**

The complete coding sequence of a novel neuroglial-derived cDNA is revealed. The expressed mRNA of ~5.5 kb is composed of the coding sequence (2,484 bp of open reading frame), with at least 400 bases of untranslated 5' sequence (Fig. 1, legend), a putative 3' noncoding sequence, and a poly(A) tail. The increased expression of the presumptive mRNA in the brain structures (Fig. 3A) implies a role in brain function, perhaps associated with learning and memory. In vivo administration of the active peptide derivative (NAP) accelerated learning and memory (Fig. 5C), suggesting a new lead compound for drug design against neurodegeneration. The quantitation of the in vitro effect was conducted by counting NSE-positive neurons and, in parallel, morphologically identified neurons to exclude bias of the peptide treatment increasing NSE in a subset population of neurons. The neuronal cells in the culture are probably postmitotic (Rappoport and Fritz, 1972). During the culture period there is some naturally occurring cell death (Brenneman and Gozes, 1996; Gozes et al., 1997a) and, thus, addition of peptide alone protected against this naturally occurring death (Fig. 4B). However, the possibility of a mitogenic effect of the peptide cannot be completely excluded and an apparent neuroprotective effect resulting from a combination of mitogenic and survival components is a subject for future research.

The in vitro neuroprotection bell-shaped dose-response curves, with an abrupt decline at increasing concentrations, is a pharmacological response of growth factors and neuropeptides in a wide variety of tissues (Brenneman and Eiden, 1986). However, NAP provided defense over six orders of magnitude of peptide concentration against NMDA-associated neurotoxicity. As NMDA toxicity may be a common pathway underlying neuronal death from many causes (Lipton et al., 1991), a broad application for NAP in neuroprotection is inferred. The broad range of active concentrations against NMDA surpassed that observed for ADNF-9 (Brenneman et al., 1998a), in agreement with the observed advantageous neuroprotective properties of NAP in mice (Fig. 5).
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NAP also protected against β-amyloid toxicity in vitro (Fig. 4A). However, in contrast to the broad range of effective concentrations against NMDA-associated toxicity, here only a narrow range of effective concentrations was observed. In contrast, ADNF-9 provided a broader range of protective concentrations against β-amyloid toxicity, $10^{-16}$ to $10^{-11}$ M (Brenneman et al., 1998a). Previous studies have suggested that multiple mechanisms may explain the β-amyloid toxicity. For example, the toxic effects vary with brain regions. In the cortex, superoxide dismutase activity and peroxide production increase after β-amyloid treatment (Cafe et al., 1996), whereas in the hippocampus, β-amyloid treatment did not induce the production of superoxide anions (Prehn et al., 1996). In human neurons, β-amyloid downregulated the expression of the anti-apoptotic protein Bcl-2 and increased the levels of Bax, a protein known to promote cell death (Paradis et al., 1996). β-Amyloid treatment has been shown to result in the generation of free radicals (Marksbery, 1997), leading to lipid peroxidation, impaired glucose transport (Mark et al., 1996), uncoupling G protein-linked receptors, and producing neuronal cell death. The generation of free radicals may also involve the production of mutations and mitochondrial dysfunction (Schapira, 1996). The accumulation of peroxide can be induced by fragments of the β-amyloid peptide in rat cortical neurons (amino acids 25–35; Cafe et al., 1996). In this respect, ADNF-9 may protect against peroxide accumulation (Glazner et al., 1997). As ADNP contains a glutaredoxin active site, coupled with a zinc binding domain (Fig. 1), future studies are aimed at the assessment of the biological activity of the entire protein in neuroprotection and its association with β-amyloid toxicity.

Studies on ADNF are a result and a continuation of our previous investigation on the major neuropeptide VIP. Earlier investigations have demonstrated that the neuroprotective actions of VIP require the presence of glial cells (Brenneman et al., 1987, 1990) expressing high-affinity VIP binding sites (Gozes et al., 1991). Interaction of VIP with the glial cells results in the secretion of proteins that can increase the survival of developing CNS neurons including interleukin-1 (Brenneman et al., 1992, 1995), protease nexin-1 (Festoff et al., 1996), and ADNF (Brenneman and Gozes, 1996; Gozes and Brenneman, 1996). ADNF and ADNP-derived peptides are the most potent neuroprotective substances derived from astroglia. Our working hypothesis is that VIP-associated neuroprotection is provided through glial factors, exhibiting rapid increases in cellular expression, after exposure to VIP, with ADNP being an important player (Fig. 3B). We have previously hypothesized that stress proteins may be secreted into the extracellular milieu of glial cells and protect protein structure as molecular chaperones (Bassan et al., 1998) and we would hypothesize now that ADNP may function in this capacity. The structure of the protein contains a putative signal peptide (i.e., a short sequence at the amino terminal, flanked by basic amino acids containing hydrophobic amino acids and exhibiting a relatively positive charge; Fig. 1). ADNP also contains basic residues (e.g., KRKK, amino acids 526–529), which may allow proteolytic processing. Future studies are aimed at the investigation of protein processing and trafficking of ADNP.

Peptide derivatives of growth factors and of neurotrophic factors have been shown before to capture some of the neuroprotective properties of the entire molecule (Clos and Dicou, 1997; Dicou et al., 1997). Furthermore, peptide fragments of stress proteins, formed after secretion or cellular damage, have been shown to possess clinical relevance (Bockova et al., 1997). In general, neuropeptides derived from large protein precursors have been shown to be involved in neuronal plasticity in development (Schwartz, 1998) and in behavioral tasks associated with memory (e.g., McDonald and Crawley, 1997). Here, ApoE-deficient animals treated with NAP developed faster than ApoE-expressing C57B6 mice (Fig. 5B). It is suggested that NAP (directly or indirectly) may strengthen synapse formation and activity, while protecting against cholinergic loss (Fig. 5A), reflected in faster acquisition of reflexes during development.

The difference in the slope of the learning curve (Fig. 5C) implies differences in cognitive functions between ApoE-deficient and control mice. ApoE-deficient animals treated with NAP exhibited cognitive functions indistinguishable from NAP-treated C57B6 mice, that were improved corresponding to untreated animals (Fig. 5D). By comparison, ApoE-deficient animals, treated with VIP superactive analogues (Gozes et al., 1997b), were retarded corresponding to VIP analogue-treated C57B6 mice (Gozes et al., 1997b), suggesting a broader efficacy for NAP over the superactive VIP analogue. Furthermore, the greater-than-expected difference at day 1 between control untreated and NAP treated (Fig. 5D) also suggested that chronic peptide treatment may be affecting additional processes other than enhancing synapse activity/number. Although the mechanism of femtomolar peptide-mediated neuroprotection remains an enigma, the discovery and molecular characterization of ADNP and NAP provides new impetus for the control of neuronal survival and novel drug design.

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