1, 421 (1988)] is nearly identical to Shaker B (*19*). Oligonucleotide-directed mutagenesis was performed with the *dut*⁻ *ung*⁻ selection scheme of T. A. Kunkel [*Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985)], with the Mutagene kit (Bio-Rad) and oligonucleotides (18 to 23 bp) with up to three mismatched bases (Amber Incorporated, Guilford, CT). After confirmation of the mutation by dideoxy sequencing of the mutated region [F. Sanger, S. Nicklen, A. R. Coulson, *ibid.* **74**, 5463 (1977)] (Sequenase, U.S. Biochemical), RNA was prepared from the mutant DNA by linearization with Hind III (NEB) followed by in vitro transcription with T7 polymerase (Promega). *Xenopus* oocytes were prepared and injected as described previously [R. MacKinnon, P. Reinhart, M. M. White, *Neuron* **1**, 997 (1988)]. To avoid misinterpretation resulting from stray mutations that might occur during the mutagenesis process, we confirmed results (that differed from wild-type properties) with two independently isolated mutant complementary DNAs.

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Neurotrophic and Neurotoxic Effects of Amyloid β Protein: Reversal by Tachykinin Neuropeptides

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The amyloid β protein is deposited in the brains of patients with Alzheimer's disease but its pathogenic role is unknown. In culture, the amyloid β protein was neurotrophic to undifferentiated hippocampal neurons at low concentrations and neurotoxic to mature neurons at higher concentrations. In differentiated neurons, amyloid β protein caused dendritic and axonal retraction followed by neuronal death. A portion of the amyloid β protein (amino acids 25 to 35) mediated both the trophic and toxic effects and was homologous to the tachykinin neuropeptide family. The effects of the amyloid β protein were mimicked by tachykinin antagonists and completely reversed by specific tachykinin agonists. Thus, the amyloid β protein could function as a neurotrophic factor for differentiating neurons, but at high concentrations in mature neurons, as in Alzheimer's disease, could cause neuronal degeneration.

THE AMYLOID β PROTEIN IS A 39– TO 43–amino acid polypeptide that is the primary constituent of senile plaques and cerebrovascular deposits in Alzheimer's disease and Down syndrome (*1*). Although this protein has been well characterized biochemically, its primary biological function and role in the pathogenesis of Alzheimer's disease are unknown (*2*). Two seemingly disparate functions of amyloid β protein have been reported. A recombinant fragment of the amyloid precursor protein containing the amyloid β protein was neurotoxic to hippocampal neurons in culture (*3*). In another study, a peptide homologous to the amyloid β protein increased the survival of cultured hippocampal neurons (*4*). We now show that the amyloid β protein has both neurotrophic and neurotoxic effects that depend on neuronal age and the concentration of β protein.

We examined the effects of the amyloid β protein on neuronal viability in cultures of hippocampal cells from rats at embryonic day 18 (E18). These cultures initially consist of undifferentiated round cells, 80 to 90% of which are neuronal precursors (*5, 6*). After 2 to 3 days in culture, 68 to 86% of these cells die and over 90% of the remaining cells begin to elaborate processes and acquire neuronal characteristics. Addition of a polypeptide corresponding to the first 40 amino acids of amyloid β protein (β1-40) (*7*) to hippocampal cultures at the time of cell plating resulted in a significant increase in neuronal survival during the first 2 days when compared to control cultures (Fig. 1A). After 3 days in culture, however, β1-40–treated cultures showed a marked decline in neuronal survival and by 4 to 5 days after plating the number of pyramidal neurons in β1-40–treated cultures was significantly less than in control cultures (Fig. 1A).

When β1-40 was added at the time of cell plating (day 0) there was a significant increase in neuronal survival 24 hours later relative to control cultures (Fig. 1, B and C). This trophic effect became progressively less apparent when β1-40 was added during the next 2 days in culture. If β1-40 was added to older cultures (3 days or more), it had a toxic effect on the neurons and caused a decline in neuronal survival 24 hours after addition relative to controls (Fig. 1, B and C). After the initial period of cell death, control cultures showed only a small change in neuronal number from 2 to 5 days in culture. Thus, β1-40 is neurotrophic during the early period of neuronal differentiation (days 0 to 2) when neuronal death and axonal outgrowth are occurring [stages 1 to 3 (*8*)]. Since mitosis of undifferentiated neuronal precursors is almost complete by E18 when the dissection is performed (*8*), the neurotrophic effect of β1-40 is probably due to a decrease in neuronal death. In contrast, β1-40 is neurotoxic to older, more differentiated neurons (days 3 to 5) that are undergoing dendritic growth and synaptogenesis [stages 4 and 5 (*8*)]. At this later stage of neuronal development, β1-40 caused the almost complete collapse of the dendritic arbor, axonal retraction and thickening, and the appearance of vacuolar inclusions in the somatodendritic region (Fig. 1C). Glial cell number and morphology did not significantly change after addition of β1-40 (*9*).

When β1-40 was added to hippocampal cultures at a low concentration (0.1 nM), the early neurotrophic effect was observed but there was no neurotoxic effect, even after 3 days. We measured the β1-40 concentration dependence of the neurotrophic and neurotoxic effects by adding β1-40 at day 0 and day 4, respectively, and then determining neuronal survival after 24 hours (Fig. 2) (*10*). The half-maximal concentration for the trophic response was 0.06 nM, whereas the toxic response was first detected at 40 nM β1-40 and the half-maximal concentration was about 100 nM. Thus, the trophic and toxic responses to β1-40 depend on both the stage of neuronal differentiation and the concentration of β1-40.

We determined the primary sequence of the β1-40 domain responsible for the neurotrophic and neurotoxic effects by assaying overlapping peptides spanning the entire amyloid β protein sequence (Fig. 3 and Table 1). At 20 μm, β1-38 elicited the same activity as β1-40. β1-28 showed some activity but was much less potent than β1-40 (Table 1). β1-16 and β17-28 showed no trophic or toxic activity at 20 μm. β17-28 showed similar activity to β1-28 at higher concentrations (not shown). The β25-35 peptide showed the same early neurotrophic and late neurotoxic activities as β1-40. β34-42 was inactive. A peptide corresponding to the COOH-terminal 20 amino acids of the amyloid precursor protein (APP 676-695) (*11*) and glucagon, a 28–amino acid peptide possessing β-pleated sheet structure similar to that of the amyloid β protein (*12*), were both inactive. Thus, the functional domain

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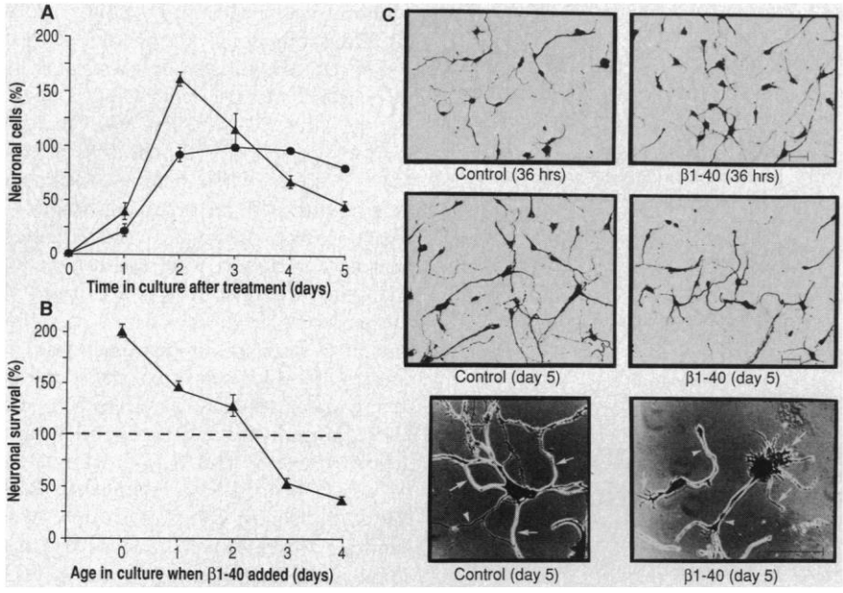

Fig. 1. Neurotrophic and neurotoxic effects of β1-40 on hippocampal neurons. (**A**) β1-40 (20 μM) was added to the cultured cells at plating and the number of neurons was scored as in (*5*). ●, Control; ▲, β1-40. Data are expressed as percent of maximal neuronal cell number in untreated control cultures (±SEM). In untreated control cultures, the maximal number of cells that were scored as neurons was achieved by day 2 to 3 and is represented as 100%. Values of neuronal cell number greater than 100% represent increased neuronal survival relative to controls after initial cell death. (**B**) β1-40 (20 μM) was added to hippocampal neurons at different times after plating and neuronal survival was determined 24 hours later. Data are expressed as percent of the mean number of neurons in untreated control cultures. Determinations of viable neurons were performed as in (*5*). Each value is the mean ± SEM, n = 12 to 30 from four experiments. Error bars were omitted where they would be smaller than the symbol. The β1-40–induced increase in neuronal survival at day 2 and decrease at day 5 were both statistically different from controls. Analysis of variance (ANOVA): day 2, $F(1,13) = 8.8$, $P = 0.012$; day 5, $F(1,27) = 20.7$, $P < 0.001$. (**C**) Neurons showing the early trophic and late toxic effects of β1-40. Hippocampal neurons were treated with β1-40 at an early stage (36 hours) or at a later stage (day 5) of differentiation and were stained with a monoclonal antibody (Tu-J1) to the neuron-specific Class III β tubulin isotype (*6*). Differentiated neurons (day 5) treated with β1-40 show collapse of dendrites (arrows) and retraction of axons (arrowheads). Dendritic localization in day 5 neurons was confirmed with a monoclonal antibody to MAP-2 (*6*). Scale bar, 30 μm.

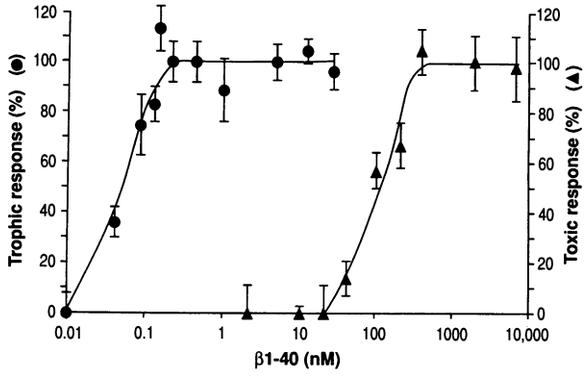

Fig. 2. Effect of β1-40 concentration on the early neurotrophic and late neurotoxic responses. The trophic response was determined by adding β1-40 at the indicated concentrations to neurons at the time of cell plating (day 0), and the toxic response was determined by adding β1-40 to neurons at day 4 in culture. Values were normalized to the maximum β1-40 trophic and toxic responses (100% response) (*10*). Values represent the mean ± SEM, n = 10 to 20, from three experiments.

Fig. 3. Primary sequences of the amyloid β protein (β1-42) (*1, 11*) and tachykinin neuropeptides. The biologically active sequence β25-35 is underlined. Residues that are identical or represent conservative changes are bold in the amyloid β protein and the tachykinin sequences (*13*).

of amyloid β protein required for both the trophic and toxic effects is contained in the β25-35 sequence. The dose response relation for β1-40 in Fig. 2 was also observed for β25-35.

A search for sequences homologous to β25-35 revealed similarity to the tachykinin family of neuropeptides. β25-35 is 73% homologous to eledoisin including conservative changes and at least 56% homologous to the other tachykinins (Fig. 3) (*13*). The region of greatest homology is in the COOH-terminal amino acids of the tachykinin sequence, which are required for high affinity receptor binding and biological activity of the tachykinins (*14*). Tachykinins were tested for their effects on hippocampal neuronal survival. Exogenous substance P, eledoisin, and physalaemin had no effect on early or late neuronal survival (Table 1). We also tested tachykinin antagonists. The potent tachykinin antagonists [D-Pro^2, D-Trp^{7,9}] substance P and [D-Arg^1, D-Trp^{7,9}, Leu^{11}] substance P (spantide) (*15*) showed early neurotrophic and late neurotoxic effects that could be reversed by the addition of substance P (Table 1). The effects of tachykinin antagonists closely mimicked those of β1-40 with respect to the time course and magnitude of changes in neuronal survival (Table 1).

The possibility that amyloid β protein may act as a tachykinin antagonist was tested by determining whether tachykinins could reverse either the early neurotrophic or late neurotoxic responses to β1-40. Tachykinin peptides were added together with β1-40 to hippocampal neurons at the time of plating to assay the trophic effect or after 4 days in culture to assay the toxic effect. Substance P and physalaemin completely reversed both the early neurotrophic and late neurotoxic effects of β1-40 in a dose dependent manner (Fig. 4, A and B). Neurokinin B partially reversed the activity of β1-40 but was less potent than substance P and physalaemin. Neurokinin A, eledoisin, and kassinin did not show significant effects in the concentration range tested. Thus, the effects of amyloid β protein are selectively reversed by specific tachykinin neuropeptides.

We have demonstrated that the amyloid β protein has both neurotrophic and neurotoxic effects in culture. The neurotrophic activity of amyloid β protein is selective for early differentiating neurons and occurs at 10^{-11} to 10^{-10} M, suggesting that β1-40 acts via a high affinity receptor. The neurotrophic activity of peptides homologous to amyloid β protein was previously reported to require very high concentrations (*4, 16*). By effective solubilization of amyloid β protein, we have been able to demonstrate biological activity at very low concentra-

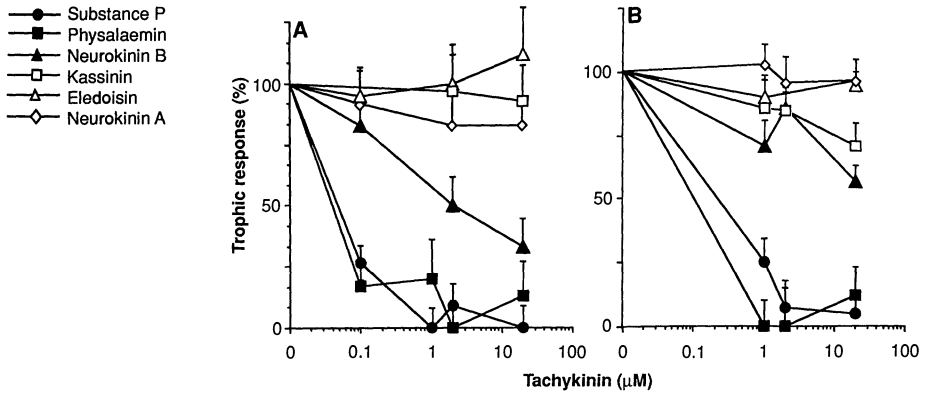

Fig. 4. Effect of tachykinins on the trophic and toxic responses to β1-40. (**A**) Tachykinins were added at increasing concentrations with β1-40 (20 μM) at the time of cell plating, and the trophic response was determined 1 day later. (**B**) Tachykinins were added with β1-40 (20 μM) in 4-day-old cultures, and the toxic response was determined 1 day later. Values were normalized to the day 1 trophic and day 5 toxic responses to β1-40 alone (100%) (*10*) and are the mean ± SEM, n = 10 to 30 from two to three experiments for each peptide.

Table 1. Trophic and toxic effects of amyloid β protein and related peptides. Hippocampal neurons were treated at the time of cell plating or at 4 days in culture with the indicated peptides (Fig. 3) to measure early trophic and late toxic responses, respectively, 1 day later. Values were normalized to the β1-40 trophic and toxic effects (designated 100% response) (*10*). Peptide concentrations were 20 μM except where indicated otherwise and were added directly to the cultures (*5, 7*). Values represent the mean ± SEM, n = 10 to 20 (each assay was performed at least twice).

Peptide	Trophic response (%)	Toxic response (%)
β1-40	100 ± 6**	100 ± 7**
β1-38	109 ± 10**	97 ± 9**
β1-28, 20 μM	0 ± 5	0 ± 6
β1-28, 100 μM	29 ± 10	55 ± 11*
β1-16	0 ± 8	0 ± 10
β17-28	0 ± 4	0 ± 8
β25-35	114 ± 17**	94 ± 10**
β34-42	0 ± 10	0 ± 7
APP676-695	0 ± 4	0 ± 7
Glucagon	0 ± 3	0 ± 11
Substance P	16 ± 8	0 ± 7
Physalaemin	0 ± 4	0 ± 7
Eledoisin	0 ± 11	0 ± 6
[D-Pro^2, D-Trp^{7,9}]-substance P	125 ± 11**	117 ± 13**
[D-Arg^1, D-Trp^{7,9},Leu^1]-substance P	116 ± 8**	118 ± 9**
[D-Arg^1, D-Trp^{7,9},Leu^1]-substance P plus substance P	0 ± 8	26 ± 7

*P < 0.01, **P < 0.001, by ANOVA prior to normalization of data; peptide-treated cultures compared to cultures in the absence of peptide.

tions. Amyloid β protein is neurotoxic to older, more differentiated hippocampal neurons at higher concentrations than required for the neurotrophic response. Nevertheless, the neurotoxic potency of amyloid β protein is considerable. On a molar basis, amyloid β protein is about a thousand times more neurotoxic than glutamate (*17*). Amyloid β protein neurotoxicity was characterized by initial dendritic and axonal retraction, which suggests an inhibitory effect on neuronal adhesion.

The possibility that amyloid β protein interacts with the tachykinin neuropeptide system is suggested by several observations. The biologically active domain of amyloid β protein, β25-35, is homologous to a conserved region in peptides of the tachykinin family. However, tachykinin antagonists rather than tachykinins themselves exhibit the same early neurotrophic and late neurotoxic effects as amyloid β protein in culture. Moreover, tachykinin agonists completely reverse both the trophic and toxic effects of amyloid β protein. Substance P and the related non-mammalian tachykinin physalaemin were the most potent inhibitors of the action of amyloid β protein, which suggests the involvement of the substance P receptor (*18*). However, it is not known whether amyloid β protein and substance P interact directly at a common receptor site or indirectly at different binding sites. Our results imply that endogenous tachykinins may have neuronal effects opposite to those of amyloid β protein, that is, tachykinins may have toxic effects on early undifferentiated neurons and trophic effects on mature differentiated neurons. There are receptors for substance P and other tachykinins in the rat hippocampus (*19*). Substance P–containing neurons are more abundant in the human neocortex and hippocampus than in the rat (*20*). Significant depletion of substance P–containing neurons has been demonstrated in the hippocampus of patients with Alzheimer's disease (*21*).

Our results suggest biologic and pathologic roles of amyloid β protein in the nervous system. The amyloid β protein could normally function at low concentrations as a neurotrophic factor for immature differentiating neurons in the central nervous system. However, accumulation of high concentrations of amyloid β protein in the mature differentiated central nervous system, as occurs in Alzheimer's disease, could lead to neuronal degeneration. If amyloid β protein neurotoxicity proves to be relevant to the pathogenesis of Alzheimer's disease, the reversal of neurotoxicity by tachykinins could have clinical import.

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soma (absence of vacuolar inclusions and exclusion of 0.25% trypan blue). Determinations of the number of viable pyramidal neurons were performed in triplicate 16-mm tissue culture wells; five 1.5-mm^2 fields were scored per well. At least 10 neurons per field or 50 neurons per well were counted in control cultures.
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7. Amyloid peptides were synthesized by the solid-phase fluorenylmethoxycarbonyl method on a Milligen peptide synthesizer and purified by reversed-phase high-pressure liquid chromatography on C18 columns. The sequences were confirmed with an Applied Biosystems Model 470 sequencer. The β1-16 and APP676-695 peptides were from Multiple Peptide Systems (San Diego, CA); purity and primary sequences were confirmed. Purified tachykinin peptides were from Bachem, Inc. (Torrance, CA). Lyophilized amyloid peptides were solubilized in 35% acetonitrile, 0.1% trifluoroacetic acid. In this solution, β1-40 did not exhibit aggregation on elution from a C18 column or on 10 to 20% SDS-polyacrylamide gels. Peptide solutions were diluted at least 1:50 directly into culture medium. At this dilution, the vehicle had no detectable effects on neuronal viability or process outgrowth.
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In Vitro Reconstruction of the Respiratory Central Pattern Generator of the Mollusk *Lymnaea*

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Most rhythmic behaviors such as respiration, locomotion, and feeding are under the control of networks of neurons in the central nervous system known as central pattern generators (CPGs). The respiratory rhythm of the pond snail *Lymnaea stagnalis* is a relatively simple, CPG-based behavior for which the underlying neural elements have been identified. A three-neuron network capable of generating the respiratory rhythm of this air-breathing mollusk has been reconstructed in culture. The intrinsic and network properties of this neural ensemble have been studied, and the mechanism of postinhibitory rebound excitation was found to be important for the rhythm generation. This in vitro model system enables a better understanding of the neural basis of rhythm generation.

DETAILED KNOWLEDGE OF THE neurons that make up CPGs is critical to understanding the neural basis of rhythm generation in both vertebrates and invertebrates. Although in situ recordings can identify specific neurons involved in a behavior, an ideal approach for elucidating the intrinsic and network properties of a neural ensemble is to attempt to reconstruct the circuit in culture. Such a feat is probably impossible for higher animals but might be feasible for adult invertebrates. For instance, mollusks have large identifiable neurons that can be removed from ganglia and maintained in vitro, where they exhibit neurite outgrowth and synapse formation (*1*). Some partial circuits of neurons have been reconstructed in culture (*2–4*), but none of these were involved in rhythm generation.

The aim of this study is to develop an in vitro model system for testing the necessity, appropriateness, and sufficiency of individual components of a respiratory neuronal network in the freshwater pond snail *Lymnaea stagnalis*. Understanding the neural basis of respiratory behavior in this snail may help to determine how similar behaviors are controlled in vertebrates, particularly in diving mammals. *Lymnaea* is a pulmonate mollusk, which, like diving mammals, makes periodic visits to the water surface in order to replenish its air supply. Unlike the opisthobranch mollusks (for example, *Aplysia*) that respire via gills, the pulmonates have lungs. A respiratory orifice termed the pneumostome links the lung cavity with the external environment. Upon reaching the water surface, the pneumostome opens (expiration) and closes (inspiration) several times before the animal resubmerges (*5*). In isolated and semi-intact preparations of *Lymnaea*, this respiratory rhythm is thought to be under the control of two identified interneurons, Input 3 (I.P3.I) and Visceral Dorsal 4 (V.D4) (Fig. 1, A and B), controlling expiration and inspiration, respectively. These respiratory interneurons have reciprocal inhibitory connections with each other and synapse with the appropriate motor neurons that control pneumostome opening and closing (*6, 7*). A third interneuron, the giant dopamine cell of the right pedal ganglion (R.Pe.D1) (Fig. 1, A and B), also appears to be involved in the circuit since it causes the excitation of I.P3.I by postinhibitory rebound (PIR) excitation (*8–10*). Activation of I.P3.I in turn excites the giant dopamine cell while inhibiting V.D4 (*11*). Upon release from I.P3.I inhibition, V.D4 fires and a cycle of alternating bursts is initiated. The giant dopamine cell and V.D4 also have reciprocal inhibitory connections (Figs. 1B and 3A). In this study we show that these three interneurons are sufficient to account for the respiratory rhythms in vitro (Fig. 3B).

The circuitry underlying rhythmic respiratory behavior in *Lymnaea* (Fig. 1B) has features in common with the "half center model" whereby two groups of neurons

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