Purification and Characterization of Neurotrophic Factor for Retinal Cholinergic Neurons Derived from Cultured Hippocampal Neurons

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ABSTRACT—A neurotrophic factor that supports the development of cholinergic retinal neurons was purified from media conditioned by a primary culture of embryonic hippocampal neurons. Retinal choline acetyltransferase (ChAT), which is located exclusively in amacrine cells, served as a marker for the development of retinal cholinergic neurons. In a serum-free control culture, retinal neurons from 17-day-old rat fetus displayed little increase in the enzyme activity and a low proportion of neurite-bearing cells (15–20%) within 7 days. The conditioned media, when added to the retinal neuron culture, dose-dependently increased ChAT activity and the number of neurite-bearing cells (40–60%), the maximum ChAT activity being sixfold higher than that in the control. The fraction with these stimulatory activities was purified by Sephadex G-15 column chromatography and two times reverse-phase HPLC. The final fraction showed ~3,000-fold higher purification as compared with that in the Sephadex G-15 fraction. Gas-phase protein sequencing analysis of the final fraction yielded a peptide sequence: Tyr-Leu-Leu-Pro-Ala-Gln-Val-Asn-Ile-Asp. A synthetic peptide with this sequence dose-dependently stimulated ChAT activity in the retinal cell culture and dissociated cell culture of the septal nucleus. These findings suggest that the developing hippocampal neurons produce a neurotrophic peptide that stimulates the development of cholinergic neurons.

Keywords: Hippocampus, Neurotrophic factor, Choline acetyltransferase, Amacrine cell (retina), Peptide

It has become clear that neuronal development and maintenance of function are regulated by neurotrophic factors not only in the peripheral nervous system but also in the central nervous system. Neutrophins, nerve growth factor (NGF) and NGF-related factors (1–7), are the most fully characterized trophic factors that are target-derived and are retrogradely transported molecules (8, 9) with a primary structure of ~120 amino acids. The trophic activities of such molecules are limited to subsets of nervous system neurons. For example, NGF in the central nervous system affects some, but not all cholinergic systems, such as the septohippocampal and nucleus basalis-neocortical systems (10). Thus, members of the neutrophins appear to be involved in part of the formation and maintenance of the synapses in the central nervous system.

Putative neurotrophic factors other than NGF have been detected in hippocampal cell extracts, as evidenced by the acceleration of neurite extension from parasympathetic neurons (11), the increase in choline acetyltransferase (ChAT) activity of medial septal nucleus (12) and the survival supporting effect on neuronal cells in cerebral hemispheres (13). Furthermore, Hsiang et al. (14) and Harada et al. (15) demonstrated that cocultivation of hippocampal cells with the septal nucleus influenced the development of septal cholinergic neurons. However, the biochemical properties or structural relationship with known trophic factors of these putative hippocampal factors have not been elucidated in detail, except for a factor recently characterized to be a peptide of 11 amino acids, the hippocampal cholinergic neurostimulating peptide (HCNP) (12, 16).

The retina offers some advantages in the investigation of neurotrophic factors. While it is a central nervous tissue, it is also peripherally located in the eye and is easily identified and dissected from surrounding tissues. In addition, a wide variety of both morphologically and functionally distinct neuron types have been well characterized in this tissue. Some of these cells can be identified in dissociated retinal cultures with cell specific marker substances. ChAT has been localized only in a subpopulation of amacrine cells and displaced amacrine cells (17–19).
In the present study, a new neurotrophic factor that supports the development of cholinergic amacrine cells in culture was identified in conditioned media derived from embryonic hippocampal neurons by monitoring ChAT activity as a marker for the development. The factor was purified and found to be a peptide composed of 10 amino acids.

MATERIALS AND METHODS

Materials

Trypsin inhibitor, bovine serum albumin, laminin, poly l-lysine, N-(2-hydroxyethyl)piperazine-N'-[2-(ethanesulfonic acid) (HEPES), N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), transferrin, insulin, progesterone, hydrocortisone, sodium selenite and cytosine arabinoside (AraC) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was from Nissui Seiyaku (Tokyo). Acetonitrile (grade for chromatography) and trifluoroacetic acid (TFA) were from Wako Pure Chem. Ltd. (Osaka). Trypsin solution [0.25%, in Ca t+ and Mg2+-free phosphate buffered saline (PBS)] was obtained from Nacalai Tesque Co., Ltd. (Kyoto). Penicillin and streptomycin were obtained from Meiji Seika Co., Ltd. (Osaka). Fetal calf serum was from General Scientific Laboratories (Lenexa, KS, USA), and horse serum was from Flow Laboratories (Osaka). \[^{[Acetyl-1-14C]}\text{Acetyl coenzyme A} \text{ (4.0 mCi/mmol)}\] was obtained from Du Pont-New England Nuclear (Boston, MA, USA).

Cell cultures

The isolation of retinas and preparation of retinal cell culture were carried out essentially according to the procedures described by Politi et al. (20). Rat fetuses (Wistar) obtained on the 17th embryonic day were used for the culture preparation. The neural retinas were carefully dissected free of pigment epithelium, cut into small pieces and incubated in 0.25% trypsin solution at 37°C for 17 min. The tissue was rinsed twice in 0.25% soybean trypsin inhibitor in DMEM, further rinsed twice in DMEM containing 1% BSA and triturated with a Pasteur pipette. Medium I consisted of DMEM supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES, 10 mM TES, 1.28 mg/l cytidine 5'-diphosphoethanolamine, 2.56 mg/l cytidine 5'-diphosphocholine, 100 mM hydrocortisone and the modified N, supplement, i.e., 5 mg/l insulin, 10 mg/l transferrin, 40 mM progesterone, 200 μM putrescine and 60 mM sodium selenite.

For the experimental cultures used for ChAT determinations, 2 × 10^5 cells in 800 μl medium were seeded per well (16-mm diameter) in a multiwell plate (NUNC) that had been pretreated overnight with poly l-lysine (5 μg/ml), rinsed three times in Hank’s solution and then incubated at 37°C for 3–4 hr in DMEM containing 20 μg/ml laminin. The conditioned medium or factor preparation to be tested was added at cell-seeding. Cultures were grown for 7 days at 37°C under humidified 95% air–5% CO₂. Half of the culture medium was replaced 3 days after seeding by fresh medium containing 0.6 μM AraC and each original concentration of additives. Since, AraC kills postmitotic neurons at a dose higher than 10 μM (21), it was added at the minimum concentration that could almost completely inhibit the proliferation of non-neuronal flat cells (22).

For the dissociated cell culture of medial septal nuclei, the tissue was dissected from 17-day-old rat fetuses. The procedures for separation of cells and culture were essentially the same as those described above for the retinal cell culture except that the culture medium was changed with serum-free DMEM supplemented with the N₂ component as described by Bottenstein and Sato (23).

Choline acetyltransferase (ChAT) activity

Cultures from retina or septal nucleus were washed three times with cold PBS and solubilized with 120 μl of 10 mM sodium phosphate buffer, pH 7.4, containing 0.5% (w/v) Triton X-100 and 10 mM EDTA. After centrifugation of the cell extracts at 10,000 × g for 10 min, aliquots of the supernatant were assayed for ChAT activity as described by Fonnum (24). The protein content was measured according to the method of Lowry et al. (25).

Microscopic analysis

Live cultures were periodically observed by phase contrast microscopy (IMT-2; Olympus Co., Ltd., Tokyo). A cell that has one or more processes longer than one cell body diameter in length was defined as a process-bearing cell.

Hippocampus-conditioned media (HCM)

The neuronal cell culture was prepared from the hippocampus of 17-day-old rat fetuses (Wistar). The tissues were cut into small pieces and triturated with a Pasteur pipette in Ca²⁺- and Mg²⁺-free Hank’s solution. The resulting suspension was centrifuged at 450 × g for 5 min, and the cells were suspended in DMEM supplemented with 10 mM TES, 10 mM HEPES, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10% horse serum and 10% fetal calf serum (Medium II). The cells were seeded in plastic dishes precoated with poly-l-lysine at a density of 3 × 10^5 cells/cm² and incubated at 37°C under humidified 95% air–5% CO₂. After incubation for 3 days, the cells were exposed to 20 μM AraC in Medium II for 24 hr and then washed three times with serum-free Medium I. The
medium was replaced by Medium I and changed every 4–6 days. The conditioned Medium I was collected 2 times after replacing the Medium II, centrifuged at 3000×g for 20 min, and the supernatant obtained was used as HCM.

**Molecular sieve chromatography**

HCM (ca. 13 ml) were applied on a Sephadex G-15 column (2×100 cm), which was equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl and eluted with the same buffer. Each fraction was measured for absorbances at 230 nm, and an aliquot of each fraction was assayed for neurite-promoting and ChAT-stimulating activities in retinal cell cultures.

**Reverse-phase HPLC**

The active fractions on a Sephadex G-15 column were collected, desalted by a Sep-Pak C₁₈ cartridge (Waters, Millipore Corp., Bedford, MA, USA) and lyophilized. The sample was then suspended in 2 ml of 10 mM sodium phosphate buffer, pH 7.4 and purified on a reverse-phase column (C₁₈, 10×100 mm; Applied Biosystems, Foster, CA, USA), with a gradient (0–40%) of acetonitrile at a flow rate of 1 ml/min in the presence of 0.04% TFA. The absorbance of the eluate at 230 nm was recorded. An aliquot of each fraction was lyophilized and assayed for neurotrophic activities. The active fractions were further purified on a second reverse-phase column (CAPCELL PAK C₁₈, 4.6×250 mm; Shiseido Research Center, Yokohama) with an acetonitrile gradient of 0–12% for 10 min, followed by a gradient of 12–20% for 40 min, both at a flow rate of 1 ml/min in the presence of 0.04% TFA. An aliquot of each fraction was lyophilized and assayed for the neurotrophic activities.

**Amino acid sequence analysis and peptide synthesis**

The final peak fraction on the second reverse-phase HPLC was lyophilized and analyzed by a gas-phase protein sequencer (Model 470A Protein Sequencer, Applied Biosystems). Peptides were synthesized by a solid phase peptide synthesizer (Model 430A Peptide Synthesizer, Applied Biosystems).

**RESULTS**

**Retinal cultures used to assess neurotrophic effect**

In the retina, the onset of differentiation (the time of final mitosis) is different among individual specialized types of cells. Amacrine cells are one of the cell types that cease division earliest, almost within the embryonic stage (26–28). Therefore, the embryonic day 17 rat retina was preferred for preparation of the cell culture enriched with amacrine cells. To confirm the enrichment, 4,6-diamino-2-phenylindol (DAPI), which is known to be selectively incorporated in cholinergic amacrine cells in the retina (17), was added to a 6-hr culture of embryonic day 17 retinas in serum-free control media. Approximately 60–70% of cells were stained with the fluorescent marker (data not shown), suggesting that the retinal culture contained cholinergic amacrine cells in this proportion. Phase-contrast microscopic examination of the retinal culture on the 6th culture day revealed that the culture contained some non-neuronal cells. Less than 5% of the total cells were a population of small dark cells, possibly representing immature photoreceptors. However, another population of non-neuronal flat cells was observed only in a very low frequency (1–2%), because of the cultivation in the presence of AraC. Therefore, in the present culture, it is unlikely that the neurotrophic factors stimulate proliferative non-neuronal cells, which in turn could promote differentiation of cholinergic neurons.

**Effects of HCM on the development of retinal cells**

To identify neurotrophic factors derived from developing neurons in the central nervous system, media conditioned by central neurons were screened for the trophic effect on retinal cells. The cerebral of 17-day-old embryonic rat was divided into two parts, hippocampus and the remainder, and the media conditioned by a dissociated cell culture of those parts were added to the retinal cell cultures. Table 1 shows the percentages of process-bearing cells and ChAT activities of retinal cells grown for 7 days in the absence or presence of conditioned media. Both media exhibited supporting activity for the development of retinal cholinergic cells, and HCM showed more

| Conditioned medium | Process bearing cells (%) | Choline acetyltransferase (nmol/min/mg) |
|---------------------|--------------------------|---------------------------------------|
| None                | 15.9±1.9                 | 0.17±0.04                             |
| Cerebrum (-hippocampus) | 31.7±4.0*                 | 0.35±0.04*                            |
| Hippocampus         | 45.5±5.9*                 | 0.47±0.04*                            |

The rat cerebral on the 17th embryonic day was divided into two parts, the hippocampus and the remainder; and the dissociated cells from each part were grown in Medium II for 3 days. After exposure to AraC, both cells were grown in serum-free medium for 4 days, and the resulting conditioned media were added to the retinal cell culture at a final concentration of 200 µl/ml. After 7 days in culture, the cells were analyzed for neurite-outgrowth and ChAT activity. Each value represents the mean of 4 measurements±S.E.M. *The unpaired Student's t-test was used for statistical analysis with P<0.001 compared with the culture without conditioned medium (None).
potent activity. As shown in Fig. 1, HCM dose-dependently increased ChAT activity, numbers of process-bearing cells and protein contents per dish, the maximum ChAT activity being approximately sixfold higher than the activity in control serum-free media. NGF, which in the brain has been shown to be synthesized in hippocampal, cortical or striatum neurons (10, 29) and to promote development of cholinergic neurons in rat septal and striatal cultures (10), had no effect on ChAT activity or neurite-outgrowth in rat retinal cultures (data not shown), as reported in chicken retinal cultures (30). These findings suggest that neurotrophic factors other than NGF derived from hippocampal neurons support the development of cholinergic retinal neurons.

Fig. 1. Dose-dependent effects of hippocampus-conditioned media (HCM) on retinal cell culture. Retinal cells were grown in the presence of various amounts of HCM. After 7 days in culture, the cells were assayed for neurite-outgrowth (○), ChAT activity (●) and protein concentration (△).

Fig. 2. Sephadex G-15 column chromatography of hippocampus-conditioned media (HCM). Thirteen milliliters of HCM was fractionated on a Sephadex G-15 column (2 × 100 cm). Three milliliters of fractions were collected, and each fraction was added to the retinal cell cultures at the concentration of 250 μl/ml. After 7 days in culture, the number of process-bearing cells (○) and ChAT activity (●) were measured. The ChAT activity and process-bearing cells in the control culture were 0.17 nmol/min/mg and 0.54 cells/cm² (×10⁴), respectively.
Purification of hippocampal neurotrophic factor

HCM was applied to Sephadex G-15 column (2 x 100 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl. The fractionation with the same buffer yielded only one peak of activity to stimulate ChAT activity as well as neurite outgrowth at the elution position corresponding to the molecular weight of \( \sim 1,000 \) (Fig. 2). To test the heat stability of the trophic factor(s), the fractions were pooled, heat-treated at 90°C for 10 min and added to the retinal cultures. The culture supplemented with the heat-treated fraction showed a time-dependent increase in ChAT activities until the 7th culture day, but in the control culture, the activity increased only slightly (Fig. 3). These findings suggested that the neurotrophic factor(s) has a molecular weight of \( \sim 1,000 \) and is heat-stable. The active fraction was purified on a reverse-phase column (10 x 100 mm), and three major peaks of stimulatory activities for ChAT as well as neurite-outgrowth were obtained (Fig. 4, fractions I, II and III). The retinal cultures grown in the presence of each fraction were compared by phase-contrast microscopy. In the culture supplemented with fraction II, neurons most prominently developed a characteristic phenotype, including a relatively large cell body (1.5- to 2-fold in diameter as compared with cells in the control culture) (Fig. 5, A and B) and extensive neurite development, typically showing a circular meshwork of dendrites concentric to the cell body (Fig. 5C). Such a phenotype resembled that of cholinergic amacrine cells (31). On the other hand, the culture supplemented with fraction I or III contained more of other types of neurons with one or more long neurites in addition to cholinergic cell-like cells. Fraction II, therefore, was further purified by the second reverse-phase HPLC (4.6 x 250 mm) (Fig. 6). A peak fraction with stimulatory activity of ChAT and neurite-outgrowth was eluted at the position corresponding to one of the 230 nm-absorbance peaks. When the amount of each sample was designated in terms of optical density (O.D.) unit at 230 nm, this final fraction was about 3,000-fold more purified than the Sephadex G-15...
fraction (Table 2).

The number of surviving cells after 7 days in culture supplemented with the final fraction did not change as compared with that in the control culture (data not shown). Furthermore, the culture contained non-neuronal flat cells in a frequency as low as that in the control culture. Therefore, it is unlikely that the present factor supports the development of cholinergic neurons via survival promoting activity or a stimulatory effect on the proliferation of non-neuronal cells that may promote the neuronal development as reported in chicken retina (30).

Characterization of hippocampal neurotrophic factor

Automated gas-phase protein sequencing of the final fraction (Table 2).

The number of surviving cells after 7 days in culture supplemented with the final fraction did not change as compared with that in the control culture (data not shown). Furthermore, the culture contained non-neuronal flat cells in a frequency as low as that in the control culture. Therefore, it is unlikely that the present factor supports the development of cholinergic neurons via survival promoting activity or a stimulatory effect on the proliferation of non-neuronal cells that may promote the neuronal development as reported in chicken retina (30).

**Fig. 5.** Phase-contrast photomicrographs of rat retinal cells after 7 days in culture. A, cells in serum-free control culture; B and C, cells cultured in the presence of fraction II in Fig. 4. Magnification bars: A and B, 30 \( \mu \)m. C, 10 \( \mu \)m.

**Fig. 6.** Second reverse-phase HPLC. Fraction II in Fig. 4 was lyophilized and further purified on a CAPSELL PAK C_{18} column (Shiseido, 4.6 × 250 mm). Fractions corresponding to the absorbance peak (---) at 230 nm were collected, and an equal aliquot of each fraction was lyophilized. Each sample was added to the retinal cell culture and assayed for its activity to promote ChAT activity (○) and neurite-outgrowth (○) after 6 days in culture. The active peak fraction was designated by a star. The ChAT activity and process-bearing cells in the control culture were 0.90 nmol/min/mg and 2.08 cells/cm² (×10⁴), respectively.
fraction showed that the neurotrophic factor is a peptide comprised of 10 amino acids (Table 3), from which a molecular weight of ~1,000 can be estimated, consistent with that observed on Sephadex G-15. According to the determined structure, a synthetic peptide was prepared and added to the retinal cultures. As shown in Fig. 7, the synthetic peptide dose-dependently stimulated ChAT activity. The maximal effect of the synthetic peptide on ChAT activity was approximately 2.6-fold over the control level. This effect is lower than that of the hippocampus-conditioned media (sixfold over the control level, Fig. 1), probably because the present factor was one of three neurotrophic factors contained in the conditioned medium (Fig. 4) and an interaction or additive effect of these factors is needed to yield the original effect. In the phase-contrast microscopy, the retinal culture supplemented with 5–20 ng/ml of the peptide was found to have the most prominent development of cholinergic amacrine cell-like cells, as shown in Fig. 5C, maximally increasing process bearing cells ~2.2-fold over the control level [maximum value, 7.09 cells/cm² ($\times 10^4$); basal value, 3.25 cells/cm² ($\times 10^4$)]. On the other hand, the synthetic peptide just devoid of the N-terminal amino acid also displayed a dose-dependent effect on ChAT activity, but the maximum effect was much lower than that of the entire peptide (Fig. 7). In addition, another synthetic peptide with glutamine substituted for N-terminal tyrosine did not show any stimulatory effect on ChAT activity or neurite-outgrowth (data not shown). These findings suggest that the neurotrophic effect of the factor is dependent on the amino acid sequence including the N-terminal amino acid. From these results, it is concluded that the hippocampal neurotrophic factor is a peptide with the following sequence: Tyr-Leu-Leu-Pro-Ala-Gln-Val-Asn-Ile-Asp.

Table 2. Purification of the hippocampal neurotrophic factor

| Fraction       | Total amount O.D. units at 230 nm | Biological activity O.D. units at 230 nm/μl | Purification (fold) | Yield (%) |
|----------------|-----------------------------------|---------------------------------------------|---------------------|-----------|
| Sephadex G-15  | 130.910                           | $166 \times 10^{-3}$                        | 1                   | 100       |
| Reverse-phase HPLC (10 × 100 mm) | 0.190                             | $1.30 \times 10^{-3}$                       | 128                 | 19        |
| Reverse-phase HPLC (4.6 × 250 mm) | 0.004                             | $0.05 \times 10^{-3}$                       | 3320               | 10        |

The amount of each sample was designated in terms of optical density (O.D.) units at 230 nm. Biological activity represents the amount of peptide in 1 ml of culture medium that enhances ChAT activity 50% over the control level, as estimated from the dose-response curves at each purification step.

Table 3. Sequencing of the hippocampal neurotrophic factor

| Edman cycle | PTH-aa | Yield (pmol) |
|-------------|--------|--------------|
| 1           | Tyr    | 103          |
| 2           | Leu    | 112          |
| 3           | Leu    | 107          |
| 4           | Pro    | 78           |
| 5           | Ala    | 57           |
| 6           | Gin    | 39           |
| 7           | Val    | 33           |
| 8           | Asn    | 25           |
| 9           | Ile    | 31           |
| 10          | Asp    | 40           |
| 11          | n.d.   | —            |

The peak fraction in Fig. 6 was lyophilized and analyzed by a gas-phase protein sequencer (Model 470A, Applied Biosystems). n.d., no PTH-aa detected.

Fig. 7. Dose-dependent effects of the synthetic peptides on retinal cell culture. Retinal cells were grown in the presence of various concentrations of the synthetic peptide (●) or the peptide just devoid of the N-terminal amino acid (○). After 7 days in culture, the ChAT activity of each culture was assayed. Each value represents the mean of 3 determinations ± S.E.M. Asterisks indicate the statistical comparison between cultures in the absence and presence of peptide, estimated by Student's t-test: *P < 0.05, **P < 0.01.
The main cholinergic neurons that project to the hippocampal neurons are the neurons of the septal nucleus. Therefore, the effect of the synthetic peptide on dissociated neurons of the septal nucleus from rats on the 17th embryonic day was analyzed (Fig. 8). The peptide showed a similar stimulatory effect on ChAT activity as observed in retinal cultures, suggesting that in the brain, the hippocampal factor supports the in vivo development of cholinergic cells as a target-derived factor in a specified developmental stage.

**DISCUSSION**

We identified and purified a hippocampal neurotrophic factor that stimulates the development of cholinergic amacrine cells. The structural analysis of the factor revealed that it is a peptide comprised of 10 amino acids (Table 3). This observation is supported by the following findings: On a molecular sieve column, the active fraction eluted at a position corresponding to the molecular weight of \( \sim 1,000 \) (Fig. 2). In addition, the synthetic peptide that was prepared according to the sequence analyzed showed a neurotrophic effect similar to that of the purified factor (Fig. 7). Moreover, the reduced effect or lack of effect of the synthetic peptide just devoid of the N-terminal amino acid or that with substitution in the same amino acid suggest that the peculiar amino acid sequence of the factor including the N-terminal amino acid is responsible for the neurotrophic effect. Thus, we concluded that the neurotrophic activity of the hippocampal factor is substantially attributable to its 10 amino acid sequence.

Such a sequence was not detected in proteins listed in the GENETYX sequence data base, excluding the possibility that the hippocampal factor is a cleaved segment of another known polypeptide with higher molecular weight. Similar to the factor in our study, a neurotrophic factor that supports the development of cholinergic neurons of septal nucleus was recently purified from rat hippocampal cell extracts, and its sequence was determined as a peptide with 11 amino acids by Ojika et al. (16). However, there was no sequence homology between this factor and the present hippocampal factor. In the purification procedure of the present factor, we found two other neurotrophic factors with a molecular weight of \( \sim 1,000 \) (Fig. 4). This observation together with the study by Ojika et al. (16) suggests that hippocampal cells produce several different neurotrophic peptides probably depending on the developmental state. This should be confirmed in the developing rat brain by immunohistochemistry or by in situ hybridization.

The proteinous factors, known or supposed to stimulate the growth of cholinergic neurons in the central nervous system, can be roughly divided into two groups, depending on the difference in structural characteristics, transport mode or receptor type: factors that belong to the NGF gene family, NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 and -5, and the others including ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6) and leukemia inhibitory factor (LIF). The former group has a primary structure of \( \sim 120 \) amino acids with 50–60% identity to each other (1–7), and BDNF, NT-3 as well as NGF were shown to be target-derived, retrogradely transported molecules (8, 9, 32). On the other hand, CNTF is not a target-derived factor but is a cytosolic protein with a 200 amino acid sequence. The present factor is much smaller than these known factors. In addition, none of these factors had any sequence stretches with significant homology with the present factor. The present factor, thus, hardly displayed any structural relation to these cholinergic neuron-stimulating factors.

The present factor appeared to be a neuron-derived but not glial cell-derived molecule, because the hippocampal factor was obtained from a culture treated with a sufficient concentration of AraC, and ChAT-stimulating activities corresponding to fraction II (Fig. 4) did not increase even when conditioned media were obtained from a culture in the absence of AraC in which non-neuronal flat cells were apparently observed (data not shown). In addition, the factor also supported the development of cholinergic neurons of the septal nucleus. Therefore, the
factor may be a target-derived molecule which in vivo plays an important role in the development of cholinergic neurons. The control retinal culture may contain target cells of cholinergic amacrine cells that produce the factor, but such a retinal factor did not seem to be produced at a substantial concentration to stimulate the development of amacrine cells in the culture at such a low concentration of cells ($2 - 3 \times 10^5$ cells/ml) as we used. Whether the factor is produced in the retina or not should be analyzed for each in vivo developmental stage by using an anti-peptide antibody and/or cDNA of the factor.

Thus, the present hippocampal factor appeared to be a new neuron-derived cholinergic neuron-stimulating peptide. Whether this peptide acts on non-cholinergic neurons or not remains to be clarified.

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