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# Neurotrophic effects of hippocampal extracts on medial septal nucleus *in vitro*

(hippocampus/tissue culture/nerve development/cholinergic activity)

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ABSTRACT **Within peripheral sympathetic and sensory systems, target tissue provides diffusible factors such as nerve growth factor that influence neuronal survival, growth, and differentiation. To determine whether target tissue may exert retrograde effects within the central nervous system, we have used hippocampus as a source of neurotrophic factors and cultured medial septal nucleus explants as a source of neurons that may respond to such factors. Soluble extracts from rat hippocampus enhance cholinergic activity (choline acetyltransferase, choline uptake, acetylcholine synthesis) of the rat medial septal nucleus cultured in serum-free defined medium. The enhancement is dose dependent, relatively specific for hippocampus, and varies with age, reaching a peak in 2- to 3-week-old rat hippocampus. The enhancing activity of the hippocampal extract appears to be mediated by protease-sensitive polypeptide(s) that differ from nerve growth factor in biological and chemical characteristics.**

Observations in developmental neurobiology have demonstrated the importance of target cells for the survival, growth, and differentiation of innervating neurons. For example, the loss of a wing bud in the developing chicken leads to a loss of spinal neurons that normally would have projected to the wing (1). The addition of a limb leads to the enhanced survival of spinal neurons (2–4). Furthermore, in the same system, cell loss can be influenced by agents that act at the neuromuscular junction (4). A similar process is observed in other neural centers. Extirpation of the optic vesicle in chicken embryos results in progressive hypoplasia in the ciliary ganglion and secondary or retrograde transneuronal degeneration in the accessory oculomotor nucleus (5). Although the specific mechanism whereby target tissues exert their effect is not resolved, diffusible trophic factors released from such target cells appear to play a permanent role in these developmental processes (6).

Nerve growth factor (NGF) is the best studied example of a diffusible molecule that exerts retrograde trophic effects. It influences neuronal viability and differentiation in the sympathetic and sensory systems *in vitro* and *in vivo* (7, 8). Similar trophic factors have been postulated for other systems. In the motor system, cultured skeletal muscle (9), medium conditioned on skeletal muscle (10), or extracts of skeletal muscle (11) increase survival and differentiation of cholinergic neurons of ventral spinal cord *in vitro*. Furthermore, proteins extracted from intraocular tissue promote the survival of cultured ciliary ganglionic neurons (12). In both systems, molecules other than NGF appear responsible for the effects of the target tissue.

The phenomenon of retrograde degeneration (13) suggests that neurons within the central nervous system (CNS) may also be influenced by CNS target-derived diffusible factors. However, within the CNS, less is known about the retrograde phenomena and the biochemical characteristics of diffusible factors that may mediate such effects. In preliminary studies, we have used cultured CNS tissues as an assay for CNS target-derived trophic factors (14). Explants of substantia nigra demonstrated enhanced dopaminergic activity with the addition of target tissue (striatal) extracts. We have selected the septohippocampal system for detailed analysis because it has been extensively studied with respect to its anatomical relations (15, 16), developmental neurogenesis (17–19), neurotransmitter distribution (15, 17, 18, 20), and capacity for regeneration (21, 22). We report here that extracts of the target rat hippocampus enhance neurite outgrowth as well as cholinergic activity of rat medial septal nucleus cultured in serum-free defined medium. The ability of hippocampal extract to enhance septal cholinergic activity is mediated by protease-sensitive polypeptides, is dose dependent, is relatively specific for hippocampus, and varies with age.

## MATERIALS AND METHODS

**Tissue Culture.** Medial septal nucleus tissue was obtained from the brains of 16-day albino Sprague–Dawley rat embryos by removing the dorsal, caudal, and lateral tissue from whole septal region fragments. The remaining medial septal tissue was dissected into 0.3- to 0.4-mm-diameter pieces, and 20–30 pieces were explanted onto poly(L-lysine) (Sigma)-coated 35-mm Falcon plastic culture dishes. In control studies, 14-day embryonic upper cervical sympathetic ganglia or dorsal root ganglia were dissected and explanted. The culture was maintained with 2 ml of modified N2 defined medium (23) containing 22 mM D-glucose, 350 nM vitamin B-12, 3 mM L-glutamine, and 0.1 mg of gentamicin and was incubated in 7% $CO_2$/93% air at 36°C. The medium was changed every 2 or 3 days.

**Tissue Extract.** Hippocampus (including dentate gyrus), cerebral cortex, cerebellum, striatum, spinal cord, muscle, and liver from different ages of Sprague–Dawley rats were dissected in ice-cold sodium/potassium phosphate-buffered saline (140 mM NaCl/2.6 mM KCl/1.4 mM $KH_2PO_4$/1.2 mM $NaHPO_4$, pH 7.2) ($P_i$/NaCl/KCl). Hippocampal tissue (0.65 g wet weight) was able to be obtained from 10 2-week-old rats. The tissue was stored at −80°C for 1–2 weeks prior to use. When the extract was made, the tissue was thawed and homogenized with a glass/Teflon homogenizer (Wheaton; clearance, 0.12 mm) in 6–7 volumes of $P_i$/NaCl/KCl. The supernatant was collected after centrifugation at 4°C for 90 min at 100,000 × *g*, yielding a protein concentration of 4–5 mg/ml by the method of Lowry *et al.* (24). The extract was applied to cultures on days 3 and 5.

**Cholinergic Activity.** Acetylcholine (AcCho) synthesis was measured by the method of Johnson and Pilar (25). The cultured explant was washed with 10 mM Tris·HCl-containing Tyrode's solution, pH 7.4 (normal Tyrode's solution), prein-

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Abbreviations: NGF, nerve growth factor; CNS, central nervous system; AcCho, acetylcholine; CATase, choline acetyltransferase.

cubated with high-potassium Tyrode's solution (98 mM NaCl/55 mM KCl/3 mM $CaCl_2$/1 mM $MgCl_2$/12.2 mM glucose/10 mM Tris·HCl, pH 7.4) for 10 min at 37°C, and then incubated with 100 nM [$^3$H]choline chloride (15 Ci/mmol, Amersham; 1 Ci = 37 GBq) in normal Tyrode's solution for 30 min at 37°C. After being washed with ice-cold normal Tyrode's solution, the tissue was harvested in 0.5 ml of 1 M formic acid/acetone, 15:85 (vol/vol), containing 3.0 nM [$^{14}$C]AcCho (58.7 mCi/mmol; Amersham). Free [$^3$H]choline was converted to phosphocholine with choline kinase (Sigma), and both [$^3$H]AcCho and [$^{14}$C]AcCho were extracted with sodium tetraphenylboron in acetonitrile. For the measurement of total [$^3$H]choline uptake, the phosphorylation step was omitted. The radioactivity in 35-$\mu$l aliquots of harvested solution is directly counted. Net radioactivity was calculated by subtracting the dpm in dishes without tissue from the dpm in dishes with explant tissue. AcCho synthesis and choline uptake were expressed as fmol of [$^3$H]AcCho or [$^3$H]choline per explanted tissue or fmol of [$^3$H]AcCho or [$^3$H]choline per $\mu$g of protein.

Choline acetyltransferase (CATase) activity was measured by a microprocedure based on the incorporation of [1-$^{14}$C]acetyl-CoA into AcCho (26). The culture explants were washed with $P_i$/NaCl/KCl and collected by centrifugation. The tissue was sonicated in 50 $\mu$l of 10 mM sodium phosphate buffer (pH 7.4). The tissue homogenate (10 $\mu$l) was incubated with 10 $\mu$l of incubation mixture containing 0.4 mM [1-$^{14}$C]acetyl-CoA (50 mCi/mmol; New England Nuclear), 600 mM NaCl, 20 mM EDTA, 20 mM choline chloride, 20 mM NaCN, 0.3 mM physostigmine, 10 $\mu$g of albumin, and 100 mM sodium phosphate buffer (pH 7.4) with or without 3 units of acetylcholinesterase (Sigma) at 37°C for 30 min. After the reaction was stopped with 4 ml of ice-cold 10 mM sodium phosphate buffer (pH 7.4) containing 0.2 mM AcCho, the $^{14}$C-labeled AcCho was extracted with tetraphenylboron in 2-heptanone, and radioactivity was assayed. CATase activity was expressed as fmol per specimen or fmol per $\mu$g of protein.

**Protease Digestion.** Hippocampal extract in Hank's balanced salt solution (GIBCO) was incubated with or without 0.1% trypsin (Sigma) at 37°C for 30 min, and the reaction was terminated with 0.15% soybean trypsin inhibitor (Sigma). Hippocampal extract also was incubated with 6 units of insoluble nonspecific protease (*Streptomyces griseus*; Sigma) at 25°C for 5 min or 60 min or with 5 units of insoluble papain (Boehringer Mannheim) at 30°C for 5 min or 90 min. The enzyme was removed from the extract by centrifuging and passing through Millex GV filter (pore size, 0.2 $\mu$m) (Millipore).

**Other Chemicals.** NGF and anti-NGF antibody were obtained from Collaborative Research (Waltham, MA).

## RESULTS AND DISCUSSION

Neuronal tissue grown under these conditions becomes attached to the culture dish surface within 1 hr and begins to extend processes within 24 hr after explantation. Processes

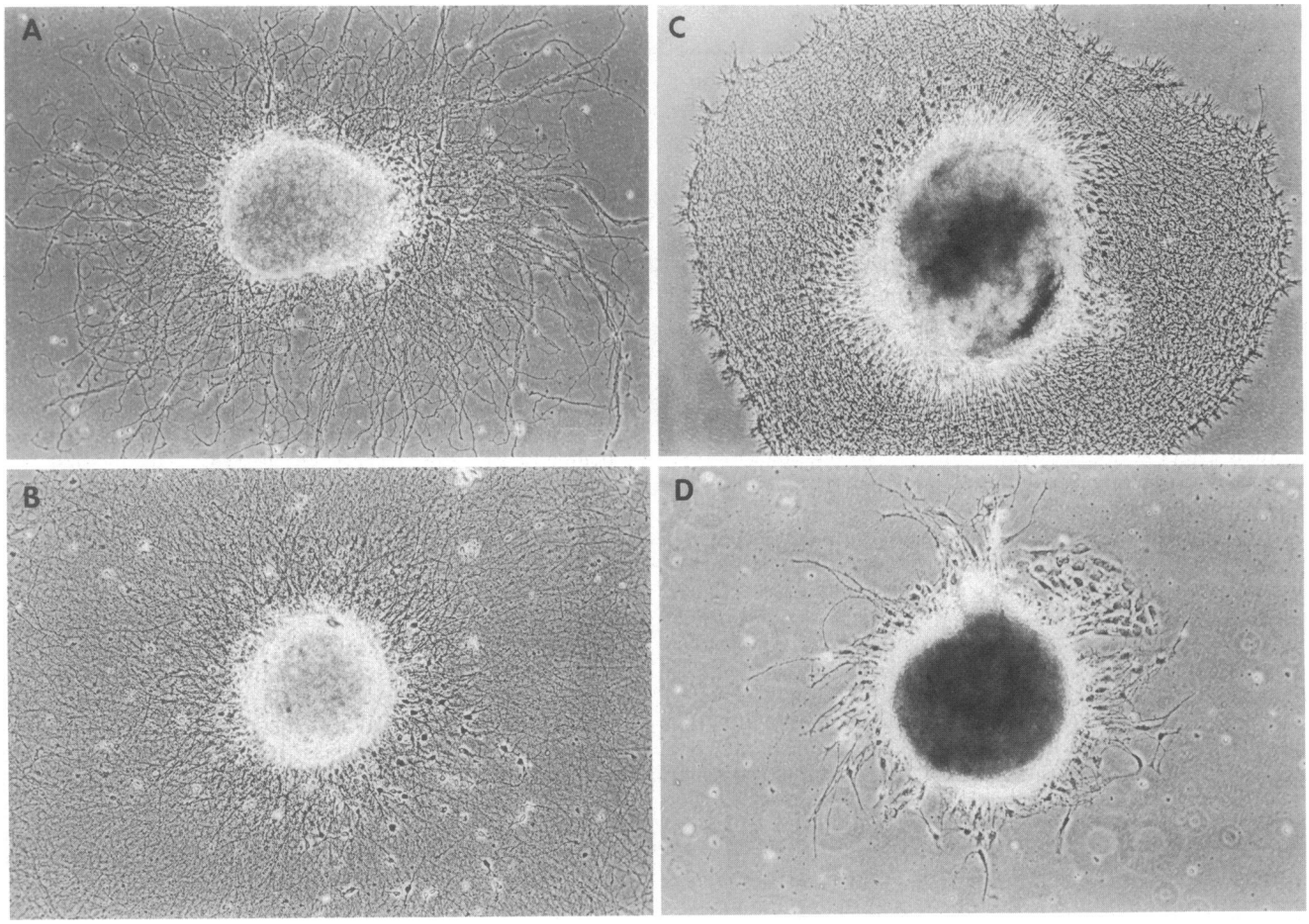


FIG. 1. The medial septal nucleus tissue (*A* and *B*) and dorsal root ganglia (*C* and *D*) from day 16 and day 15 embryonic rat brain, respectively, were cultured on poly(L-lysine)-coated 35-mm Falcon plastic dishes with defined medium. For the medial septal nucleus culture, at days 3 and 5 *in vitro*, cultures were supplemented with $P_i$/NaCl/KCl or with hippocampal extract (40 $\mu$g of protein per ml) from 2-week-old rat brain. (*A*) Septal nucleus—8 days *in vitro* treated with $P_i$/NaCl/KCl. (*B*) Septal nucleus—8 days *in vitro* with hippocampal extract. For the dorsal root ganglia culture, NGF [60 units (7.5 ng) per ml] or hippocampal extract (40 $\mu$g of protein per ml) was applied 1 hr after explantation. (*C*) Dorsal root ganglia—24 hr *in vitro* treated with NGF. (*D*) Dorsal root ganglia—24 hr *in vitro* treated with hippocampal extract. (×70.)

increase in number and in length over at least 6–8 days *in vitro*. During this period, cell viability is maintained with minimal degeneration. To avoid monitoring effects on initial attachment, the hippocampal tissue extract was first applied at day 3 *in vitro* when the septal explants were fully attached. The addition of extract results in a gradual increase in the length and density of fiber outgrowth compared to control cultures. This enhancement in density of neuritic outgrowth in the presence of hippocampal extract becomes most prominent by days 6–8 *in vitro* (Fig. 1). In the absence of extract, control cultures begin to degenerate by day 8.

Because many different cell types may contribute to the outgrowth pattern *in vitro*, neither the neuronal origin of the outgrowth nor its quantitation can be made with precision. To document the requirement for neurons in this outgrowth process, biochemical parameters were used. The medial septal nucleus connections to the hippocampus are predominantly cholinergic (15, 17, 18, 20); therefore, high-affinity choline uptake (20, 27), the extent of AcCho synthesis (25), and CATase (26) activity can be used as an index of medial septal neuron metabolic activity. The addition of hippocampal extract enhanced both choline uptake and AcCho synthesis compared with the control (Table 1). The conversion ratio of total [$^3$H]choline uptake to [$^3$H]AcCho was ≈20% for control cultures and 30% for the extract-treated group. The addition of hippocampal extract also enhanced CATase activity (Table 1). Thus, enhancement of AcCho synthesis in explants by hippocampal extract results from increases of both choline uptake and CATase activity.

Increases in AcCho synthesis, choline uptake, and CATase activity by hippocampal extract is time dependent. In the control group, changes in AcCho synthesis parallel changes in morphology. Both AcCho synthesis and fiber outgrowth increase until day 6 *in vitro* and then decline. After application of hippocampal extract at day 3 *in vitro*, AcCho synthesis increases significantly and by day 6 is almost twice the control value whether calculated on a specimen or a protein basis (Fig. 2). Between days 6 and 8, *in vitro* AcCho synthesis continues to increase in culture treated with hippocampal extract, whereas it declines in the control group. Some of the effects of hippocampal extract by day 8 are due to its ability to enhance neuronal survival. However, the effects noted between days 3 and 6 *in vitro* suggest that hippocampal extract enhances both morphologic outgrowth and AcCho synthesis, with minimal changes in cell degeneration or survival. Thus, from day 3 to day 8, the effects of the extract appear to represent a combination of increased survival and increased AcCho synthesis. The effect of this hippocampal extract on cholinergic development increases in a linear fashion with protein concentration and saturates at 50 μg/ml (Fig. 3).

The age of the animal from which the hippocampal extract was derived is an important variable. When equal amounts of protein extracted from newborn, 2- to 3-week-old, 1-month-old, and >4-month-old rat hippocampal tissue are compared, the increase in cholinergic activity 6 days after the addition of the extract is respectively 2.4, 3.5, 1.7, and 1.5 times the control value. Thus, the highest specific activity of extract is noted in 2- to 3-week-old rat hippocampus. In the rat, the development of the septohippocampal pathway and CATase activity in the hippocampal tissue increase from birth to days 16–17 *in vivo* (17, 18). The fact that the highest specific activity of hippocampal extract *in vitro* coincides with this time of increasing synaptogenesis of the septohippocampal pathway *in vivo* supports the potential relevance of target-derived factors for development of presynaptic septal neurons.

The enhancement of cholinergic activity by hippocampal extracts is independent of species because calf, rabbit, and human hippocampal extracts demonstrate effects on rat sep-

Table 1. The effect of hippocampal (hippo) extract on cholinergic differentiation of medial septal nucleus (MSN) culture *in vitro*

| Cholinergic markers | MSN culture treatment | Enhancement of cholinergic markers: fmol per specimen | Enhancement of cholinergic markers: fmol/μg of protein |
|---|---|---|---|
| CATase | Control | 190.0 ± 60.0 | 540.0 ± 21.0 |
| | Hippo extract | 470.0 ± 10.0 | 1210.0 ± 22.0 |
| Choline uptake | Control | 11.2 ± 1.4 | 21.5 ± 2.7 |
| | Hippo extract | 26.6 ± 4.2 | 45.1 ± 7.1 |
| AcCho synthesis | Control | 2.2 ± 0.7 | 4.1 ± 1.3 |
| | Hippo extract | 8.6 ± 1.0 | 14.8 ± 1.7 |

Hippocampal extract (Hippo) from 2-week-old rat brain (50 μg of protein per ml) was applied at days 3 and 6, and CATase, choline uptake, and AcCho synthesis were measured at day 9. The activity represents the mean of four cultures ± SEM.

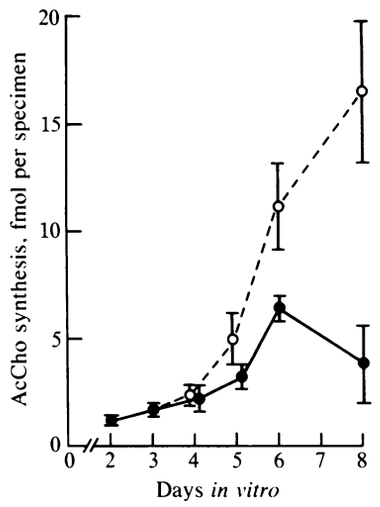


FIG. 2. AcCho synthesis in the medial septal nucleus culture *in vitro* with (---) and without (—) hippocampal extract (100 μg of protein per ml) from 2-week-old rat brain. Medium change and application of extract were carried out at days 3 and 5. AcCho synthesis was measured by the method of Johnson and Pilar (25). Each point represents the mean synthesized AcCho (fmol per specimen) of four dishes, and the vertical bar represents the SEM.

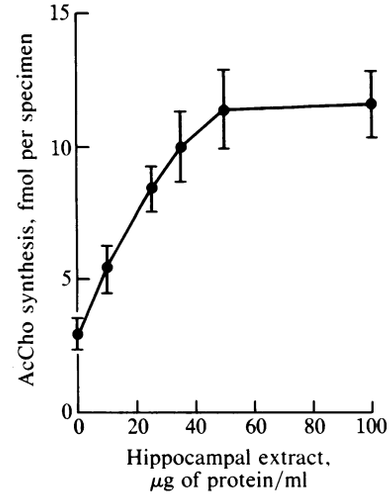


FIG. 3. Dose–response of hippocampal extract on the AcCho synthesis of medial septal nucleus cultures. Medium change and application of hippocampal extract were carried out at days 3 and 6, and AcCho synthesis was measured at day 9 *in vitro*. Each point represents the synthesized mean fmol of AcCho per specimen of four dishes, and each vertical bar represents the SEM.

tum comparable to rat hippocampal extracts. The enhanced AcCho synthesis is relatively specific for hippocampal extracts. To test the effects of different tissue extracts on AcCho synthesis by medial septal nucleus culture, equivalent amounts of tissue extracted from 2-week-old rats were applied and assayed at day 8 *in vitro*. Results of two typical experiments are shown in Table 2. For all tissues except hippocampus, the saturation of the enhancing effect occurred at 25 μg with crude extracts, whereas saturation occurred at 50 μg with hippocampal extracts. The AcCho synthesized in the presence of hippocampal extract was significantly increased compared to control ($P < 0.001$) and other tissue extract groups ($P < 0.002$). Some tissues other than hippocampus also increased AcCho synthesis significantly compared to controls (cerebellum, $P < 0.02$; spinal cord, $P < 0.001$; muscle, $P < 0.01$; by Student's $t$ test). Enhancement with tissue other than hippocampus is not unexpected because our explants are maintained in defined medium, and nonspecific factors present in many tissues may enhance survival and secondarily influence neuritic outgrowth and cholinergic activity. Alternatively, specific factors that are trophic for medial septal nucleus cells may be present in lesser amounts in different organs. Further tests with factors purified from different tissues or specific antibody against the factors may be needed to differentiate between these possibilities.

NGF has been reported to enhance cortical cholinergic activity (28) and may represent the active constituent within hippocampal extract that enhances survival and the rate of neuritic outgrowth of cultured ciliary neurons and lumbar sympathetic ganglia (29). However, effects of hippocampal extract upon medial septal nucleus explants are not due to NGF for the following reasons. First, 2.5S NGF in doses from 60 to 180 units produces neither morphologic nor cholinergic effects on cultured medial septal tissue, whereas the same doses enhance neuritic outgrowth in rat dorsal root ganglia and superior cervical ganglia. Second, hippocampal extracts produce no morphological effects on rat dorsal root ganglia or on superior cervical ganglia cultured on either collagen or poly(L-lysine) (Fig. 1). Third, treatment of hippocampal extract with anti-NGF-antibody does not modify the effects of hippocampal extract on medial septal nucleus, although it does block the effects of NGF on dorsal root ganglia and superior cervical ganglia. Thus, although NGF is active on sensory and sympathetic ganglia, it does not influence the morphologic or cholinergic activity on the medial septal nucleus and, therefore, is not the active factor in our hippocampal extract. Our results differ from two other reports where NGF was found to influence cultures of dissociated telencephalon (28) and ciliary ganglia (29). The differences may be related to the cell source of the culture system, the use of dissociated cells versus explants, or, in one study, (29), the use of a morphological assay for survival versus our biochemical assay of neuronal function.

Table 2. The effect of tissue extracts on cholinergic activity of medial septal nucleus (MSN) culture *in vitro*

| Tissue source | MSN culture cholinergic activity,* fmol per specimen | |
|---|---|---|
| | Exp. 1 | Exp. 2 |
| Control | 3.8 ± 0.7 (100) | 2.5 ± 0.4 (100) |
| Hippocampus | 12.0 ± 2.2 (316) | 7.2 ± 0.8 (288) |
| Cortex | 5.6 ± 1.2 (147) | — |
| Striatum | — | 3.5 ± 0.5 (140) |
| Cerebellum | 5.7 ± 0.9 (150) | 4.0 ± 0.5 (160) |
| Spinal cord | 6.5 ± 0.5 (171) | 4.5 ± 0.4 (180) |
| Muscle | 5.5 ± 0.5 (145) | 4.1 ± 0.6 (164) |
| Liver | 5.6 ± 1.2 (147) | — |

In experiment 1, 40 μg of protein per ml of each tissue extract was used; in experiment 2, 25 μg of protein per ml was used. For each tissue extract tested, 4–6 cultures were used, and activity is presented as the mean ± SEM.

*Values in parentheses are percentages compared with the control value of 100%.

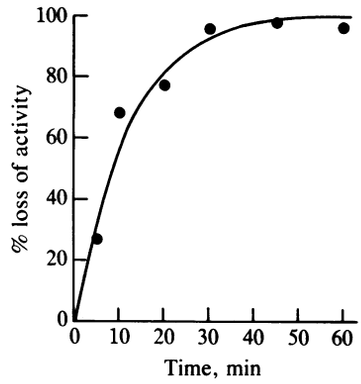


FIG. 4. Effect of heat on the ability of hippocampal extract to enhance cholinergic activity on medial septal nucleus. Hippocampal extract (0.3 ml) from 2-week-old rat brain was treated at 60°C for various times. Heat-treated samples were applied to medial septal nucleus culture at days 3 and 6 *in vitro*, and AcCho synthesis was measured at day 9. The experiment was performed in triplicate. By using the mean AcCho synthesized in each group, the percentage loss of activity was estimated as follows:

$$100\% - \left(\frac{\text{heat-treated group} - \text{control}}{\text{nontreated group} - \text{control}} \times 100\right) = \%\ \text{loss},$$

in which the control was $P_i$/NaCl/KCl.

The cholinergic activity of the crude extract is heat labile at neutral pH. At neutral pH, 50% of activity is lost after 8 min at 60°C (Fig. 4). In addition, cholinergic effects of crude hippocampal extract are inactivated by trypsin and nonspecific insoluble protease. The cholinergic enhancing activity of the crude extract can be recovered in the ultrafiltrate after Amicon YM5 filtration (cutoff, 5000 daltons). This activity is heat labile at pH 7.2 but heat stable at pH 2–3. Cholinergic activity is extractable in polar organic compounds such as methanol or acetonitrile. It is destroyed by insoluble papain. Thus, cholinergic activity in the crude fraction as well as in a partially purified fraction resides in polypeptides.

During development, the ability of neurons to survive, the definitive choice of the neurotransmitter they will use, and the achievement of a differentiated mature state can be influenced by target tissue and by other environmental cues. Hormonal influences (trophic factors), cell-surface interactions, and target activity are among the mechanisms that could explain such phenomena and could contribute to the "chemospecificity" by which a growing axon finds its appropriate target (30). The present experiments demonstrate that extracts of hippocampus enhance cholinergic activity of medial septal neurons, which normally innervate the hippocampus *in vivo*. The ability of polypeptides from hippocampus to regulate the growth and cholinergic activity of cultured medial septal neurons provides a convenient assay to study the role of neurotrophic factors in development, their specific mechanism of action, and their potential loss in disease.

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