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Neurotrophic effects of conditioned media of astrocytes isolated from different brain regions on hippocampal and cortical neurons

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Abstract. The present study was designed to reveal whether astroglial cells from different brain regions produce diffusible factors that differentially support the survival of neurons and the establishment of neuronal morphology. For this purpose, astrocyte conditioned media (ACM) were prepared by conditioning chemically-defined medium with type I astrocyte culture dissociated from cerebral cortex, hippocampus and hypothalamus. Hippocampal and cortical neurons were cultured in ACM or in non-conditioned medium. ACM derived from three brain regions all supported the survival of hippocampal and cortical neurons better than non-conditioned control medium. Of these, hypothalamic ACM was the most effective in supporting the survival of cortical neurons. The ACM also potentiated the elongation of the longest neurite of hippocampal and cortical neurons. However, there were no significant differences in the promoting effects on neurite elongation among ACM from three brain regions.
Key words. Astroglia; neurotrophic factor; cerebral cortex; hippocampus; hypothalamus.

Circuit formation in the central nervous system during development depends on neuronal survival and the establishment of the shapes of the individual neurons. Astroglial cells are closely associated with developing neurons, and the interaction between neurons and astroglia is important for neuronal survival, axonal guidance, neurite outgrowth and neuronal polarity[1,2]. These astrocyte effects can be exerted by direct contact of their cell surface membranes with neurons, and/or diffusible factors released from them[3-6].

Several lines of evidence have suggested that astroglia from different brain regions show heterogeneity, for example, in the expression of receptors and responses to several kinds of agonists[7,8]. Furthermore, there may be regional specificity of neuron-glia interactions[9-11]. In this study, we have attempted to determine whether diffusible factors produced by astroglial cells isolated from different brain regions can show differences in their effect on neuronal survival and the formation of neuritic processes. We prepared astrocyte conditioned media (ACM) from the cerebral cortex, hippocampus and hypothalamus, and tested the effects of these ACM on the survival and neurite elongation of hippocampal and cortical neurons.

Materials and methods

Preparation of ACM. Cell dissociation was performed according to the methods described previously[12] with some modifications. Briefly, whole brains were isolated from postnatal day 0 Wistar rats and the hippocampus, cerebral cortex and hypothalamus further dissected out. The tissue was cut into pieces and incubated twice with 0.25% trypsin and deoxyribonuclease 1 at 37 °C for 30 min. At the end of the incubation, the tissue was centrifuged at 1200 rpm for 5 min. The pellet was resuspended in modified Eagle's medium[12] containing 10% fetal bovine serum (Cansera), and single cells were dissociated by gently passing the suspension through a plastic tip. After passing through nylon-net to remove cell lumps, the suspension was diluted and plated on 25 cm^2 culture flasks (Falcon) at a density of 3×10^5 cells/cm^2. About 12 days after plating, when the primary culture of glial cells became confluent, cultures were shaken at 150 rpm for 24 h[13]. The cells remaining adherent after this treatment (mostly type I astrocytes, see 'Results') were detached by Ca^{2+}-free, 0.1% trypsin solution containing 5 mM EDTA, and were plated at a density of 5×10^4 cells/cm^2. Serum-free modified Eagle's medium with N2 supplements[14] was conditioned by the astrocyte culture for 24 h and collected. Conditioned media were centrifuged at 1200 rpm for 2 min and the supernatant were filtered through 0.22 μm membrane filter (Millipore) and stored at −80 °C.

Immunochemical staining of the glial culture was performed by the avidin-biotin-peroxidase complex method (Vectastain ABC Kit) after fixation with 4% paraformaldehyde.

Determination of neuronal survival and neurite elongation. Hippocampal and cortical neurons from embryonic day 18 Wistar rats were dissociated by the same methods as above, suspended in modified Eagle's medium containing 10% fetal bovine serum, and plated on 35 mm plastic dishes to give a final density of 2500 cells/cm^2 (ref. 15). They were cultured at 37 °C for 24 h in a humidified 5% CO_2-95% air atmosphere. The medium was changed 24 h after plating to serum-free modified Eagle's medium.

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After 24 h, the neurons that had neurites longer than their soma diameters were selected and their locations recorded using ACAS 470 work station (Meridian, Michigan, USA). These cells were photographed, and then the medium was changed to ACM or non-conditioned medium (day 0). One and two days later (day 1 and day 2, respectively), photomicrographs of the same cells were taken. The numbers of surviving neurons were counted from the photomicrographs on each day, and survival rate was calculated by setting the number on day 0 as 100%. The length of the longest neurite was measured from the photomicrographs with a digitizing tablet and appropriate software. The neurons that survived day 2 and made no contact with other cells were incorporated in the data for the measurement of neurite length.

Results

First, we characterized the glial culture by specific immunostaining with antibody against glial fibrillary acidic protein (GFAP). After shaking the primary glial culture vigorously for 24 h, the cells remaining adherent to the culture flasks had a flat morphology. They were stained positively by anti-GFAP antibody, which suggested that type I astrocytes were predominant in these cultures. Accurate counting of the cells was difficult because of the flattened morphology and ill-defined boundary between cells. In the case of cortical glial cultures, where cell identification was relatively easy, more than 95% of the cells were GFAP positive. Neuronal cultures used for determination of neuronal survival and neurite elongation consisted primarily at least 90% of neuronal cells.

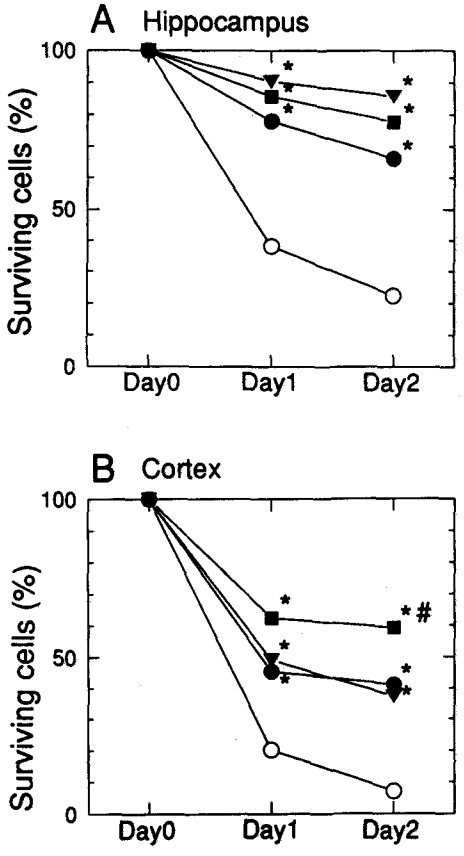

Figure 1. Effects of ACM on the survival of the *A* hippocampal and *B* cortical neurons. ○, control (n = 76, 84 in *A* and *B*, respectively); ●, cortical ACM (n = 59, 68); ▼, hippocampal ACM (n = 63, 69); ■, hypothalamic ACM (n = 63, 64). Survival rate is expressed as percentage of the number of surviving cells just after the change of medium. (Statistical significance of the differences among the percentage values on each day was evaluated by χ^2-the Kruskal-Wallis test *$p < 0.01$ vs. control, #$p < 0.01$ vs. cortical and hippocampal ACM).

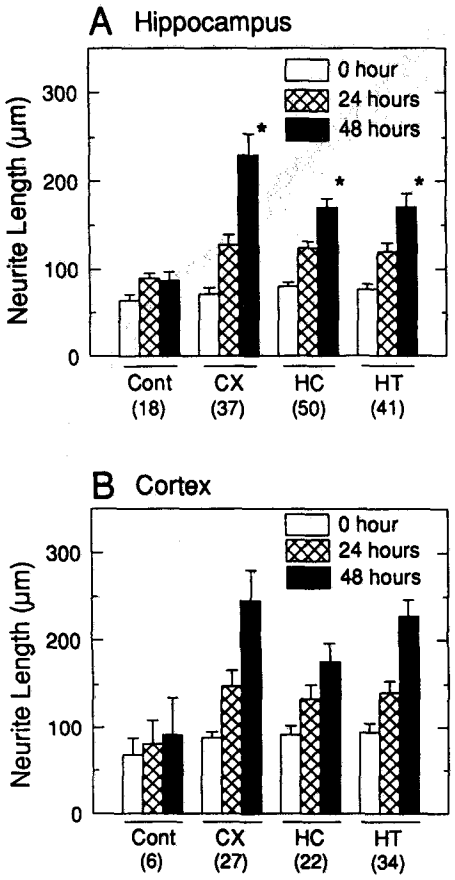

Figure 2. Effects of cortical (CX), hippocampal (HC) and hypothalamic (HT) ACM on neurite elongation. The length of the longest neurite of each *A* hippocampal or *B* cortical neuron was measured. Values represent mean ± S.E. of neurons which survived day 2. Numbers of neurons are given in parentheses. *$p < 0.01$ vs. control, Dunnett-C test.

Figure 1 shows the effect of ACM on the survival of hippocampal and cortical neurons. The number of surviving neurons markedly decreased with time in the control medium not conditoned by astroglia. Only 22% (17/76) and 7% (6/84) of hippocampal and cortical neurons, respectively, survived day 2. All of the ACM from three brain regions markedly and significantly supported the survival of both hippocampal and cortical neurons. The effects of ACM from three brain regions on the survival of hippocampal neurons were

Table. Effects of ACM on the number of processes.

Culture conditions	Hippocampal neurons	Cortical neurons
Control	5.0 ± 0.3 (18)	4.2 ± 0.8 (6)
Corical ACM	5.8 ± 0.2 (37)	4.9 ± 0.3 (27)
Hippocampal ACM	5.1 ± 0.2 (50)	4.3 ± 0.3 (22)
Hypothalamic ACM	5.2 ± 0.2 (41)	4.9 ± 0.2 (34)

The number of processes per neuron on day 2 are presented. Numbers of neurons examined are given in parentheses.

similar (fig. 1A). In the case of cortical neurons, however, hypothalamic ACM was more effective than hippocampal and cortical ACM (fig. 1B).

The culutred neurons extended several short processes that were roughly equal in length, and one long axonal process. Figure 2 illustrates the effects of ACM on the elongation of the longest neurite. ACM derived from three brain regions markedly and significantly enhanced the elongation of the longest neurite of hippocampal neurons, compared to the non-conditioned medium. However, there were no significant differences between the effects of ACM (fig. 2A). The neurite elongation of cortical neurons was also markedly enhanced in the presence of either ACM, although the difference from control did not reach significance because of the low number of surviving neurons in the control culture. Again, no significant differences were found among the effects of ACMs on cortical neurons (fig. 2B). The ACM had no effect on the number of processes per neuron, another parameter indicating neuronal morphology (table).

Discussion

In the present study, we have used ACM in order to clarify the effects of diffusible factors produced and released from type I astrocytes, and have clearly demonstrated that ACM promote the survival of cultured hippocampal and cortical neurons. Furthermore, we have shown that ACM from different brain regions can differentially affect neuronal survival. It has been reported using neuron-glia co-culture[16] that substantia nigra glial cells are more effective in supporting the survival of substantia nigra dopaminergic neurons, than glial cells from other brain region (striatum, cerebellum and hippocampus). In the present study, however, we could not obtain evidence for similar regional specificity of trophic effects, such as cortical ACM for cortical neurons, or hippocampal ACM for hippocampal neurons. Rather, hypothalamic ACM was more effective than cortical ACM in promoting the survival of cortical neurons. A possible explanation for this result is that hypothalamic ACM contains a larger amount of specific trophic factor(s) for cortical neurons, and the low survival rate of cortical neurons compared with hippocampal neurons clearly uncovered the trophic effect of hypothalamic ACM. Similar paradoxical results have been reported, using mesencephalic dopaminergic neurons that exhibit more extensive neurite outgrowth on glia derived from the olfactory bulb than on mesencephalon- or striatum (their target)-derived glia[17]. Although the physiological significance is not clear, our results confirmed that astrocytes from different brain regions differ in the ability to produce diffusible trophic factors. The molecular nature of the factors that are involved in region-specific effects on neuronal survival is presently unknown. Although further investigations will clearly be needed to reveal the molecular mechanisms of region-specific neuron-glia interactions, attention should be paid to the heterogeneity of astrocytes in their roles in neuronal survival in the central nervous system.

In the present study, postnatal day 0 rats were used for the preparation of astrocyte cultures, in order to obtain a purer astrocyte population. Chamak et al. have reported[18] that there are no differences between embryonic and postnatal glial cells in maintaining the number of neurites of co-cultured mesencephalic neurons. Nevertheless, further investigations are needed to clarify whether maturational stages of astroglial cells affect the outcome.

Our present data also demonstrate that ACM are effective in enhancing neurite elongation in hippocampal and cortical neurons. It is still controversial whether direct contact of the astrocyte cell membranes with neurons is necessary for the regulation of neuronal shape[4–6,10,11]. Our present results strongly suggest that astroglial influences can be exerted, at least in part, in the absence of direct contact with neurons. On the other hand, whether region-specific neuron-glia interactions need direct contact of neurons with glial cells is again a matter of controversy. Using coverslip co-culture, Qian et al.[11] have reported that, in the absence of direct neuron-glia contacts, target-derived astroglia regulate the axonal outgrowth of hippocampal and spinal neurons in a region-specific manner, which suggest that astrocyte-derived diffusible factors are responsible for the differential determination of neuronal shape. However, where neurons and astrocytes are present together in culture, we should take into consideration the possible influence of neuron-derived diffusible factors on astrocytes. Several lines of evidence have suggested that diffusible factors produced by neurons influence glial cell functions[19–21]. Walsh et al.[22] have demonstrated that cortical neurons cultured with, but in no direct contact with cortical astrocytes, form a dense neuronal network, whereas cortical neurons cultured in cortical ACM show poor neurite elongation and do not form any network. In the present study we have tested the effect of ACM, which contain diffusible factors produced by astrocytes in the absence of neurons, i.e.,

conditions where neuronal influences on astrocytes are eliminated. We could not obtain clear evidence that ACMs can exert region-specific effects on neurite elongation, that is, any significant differences in promoting neurite elongation of hippocampal and cortical neurons. Our present observations, with those of Walsh et al.[22], suggest the presence of reciprocal interactions between neurons and glial cells, via diffusible factors produced and released from each other. Such interactions may play an important role in the region-specific determination of neuronal morphology.

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