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Effect of Insulin on Differention of Chick Embryonic Neuroblasts Cultured *in vitro*

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To examine the effect of insulin on neuronal differentiation, telencephalic neuroblasts from chick embryonic brains were cultured in a serum-free medium. Indirect immunofluorescence microscopic studies revealed that the specific protein, MAP-2, was localized in both cell bodies and neurites of developing neuroblasts. Furthermore, treatment of increasing concentration of insulin promoted the MAP-2 synthesis as well as the neurite outgrowth activity. Thus, the enhancement of the morphological and biochemical parameters for neuronal differentiation appears to be closely correlated, and the neurotrophic effect of insulin may play a crucial role in neuronal process formation.

KEY WORDS: **Telencephalon, Insulin, Neurite outgrowth, MAP-2**

It has generally been accepted that the growth of neuron in culture requires the presence of serum in medium (Sensenbrenner *et al.*, 1980; Raizada, 1983). The use of serum, however, has hampered the precise analysis of specific serum component(s) that regulates the neuronal development because of its complexed composition. A number of defined media have therefore been developed, and these media typically contain insulin and transferrin in place of serum (Muller *et al.*, 1984; Aizenman *et al.*, 1986).

Although the central nervous system was considered insulin-independent due to the limited penetration of pancreatic insulin into brain, emerging evidences have suggest that insulin and insulin-like growth factor II (IGF-II) have neurotrophic properties. It has been found that the IGF-II gene is expressed in fetal rat brain and the binding sites for the factor are present in brain homogenates (Havrankova and Roth, 1978). It has also been reported that many neurons contain specific insulin binding sites (Raizada, 1983) and the occupation of these sites by insulin leads to an increase in the level of tubulin mRNA in human neuroblastoma (Mill *et al.*, 1985). Thus, it appears clear that insulin influences the growth and development of fetal nervous tissues, although the detailed mechanism of action remains totally unknown.

When dissociated neuroblasts are cultured under a proper condition, they stop dividing and undergo neuronal differentiation that is manifested by extention of long and branching neurites. These morphological changes seem to be tightly associated with the assembly of microtubules since the treatment of a drug causing depolymerization of microtubule results in a rapid retraction from neurite outgrowth (Yamada *et al.*, 1971; Daniels, 1972; Solomon and Magendantz, 1981). However, relatively little is known about the biochemical changes underlying the neurite's growth in developing neurons.

Microtubules prepared *in vitro* consist mainly of tubulin and additional protein components, which are collectively called as microtubule associated proteins (MAPs)(Borisy *et al.*, 1975; Kirschner, 1978). High molecular weight MAPs can be separated on polyacrylamide gels into a single band

designated as MAP-1 (Mr. of 330-350 kDa) and faster migrating protein doublet, MAP-2 (300 kDa)(Sloboda *et al.*, 1975). MAP-2 is of particular interest because of its well-charaterized capacity to stimulated the assembly of tubulin into microtubule (Murphy and Borisy, 1975; Sloboda *et al.*, 1976; Herzog and Weber, 1978). It is a heat-stable phosphoprotein and maintains a constant stoichiometry to tubulin through several cycles of temperature-dependent assembly and disassembly (Kim *et al.*, 1979; Sloboda *et al.*, 1975). Thus, MAP-2 is considered to be a potential regulator of the formation of nerve cell process in developing brains.

As an attempt to define the role of insulin in the neuronal development, we investigated the effect of insulin on the expression of the MAP-2 using the telencephalic neuroblasts from 6-day-old chick embryonic brains. These cell preparations are particularly useful because they are composed of a nearly pure population of neuronal cells and because they are in progress from a dividing to a post-mitotic stage (Tsai *et al.*, 1981; Aizenman *et al.*, 1986). In addition, we established a serum-free culture system for neuronal cells that can be used for the further analysis of specific serum component(s) regulating the growth and development of the cells.

Materials and Methods

Materials

Nitrocellulose membrane filters (0.45 μm) were purchased from Millipore. ^{125}I-protein A (70 uCi/μg) was obtained from New England Nuclear (NEN) and goat anti-rabbit IgG (150 U/mg) from Scripps Lab. All other agents were purchased from Sigma.

Cell Culture

Telencephalons were obtained from 6-day-old chick embryonic brains and minced in 1:1 mixture of Dulbecco's modified Eagle's medium and neutrient mixture F-12 (DMEM/F-12). The tissues were then passed through 200 μm stainless filter and centrifuged for 6 min at 700 $\times$ g. The pellet containing neuroblasts were resuspended in a fresh DMEM/F-12 medium and passed through 2 layers of lens paper. The dissociated cells were plated at a density of about 5 $\times$ 10^5 cells/ml of DMEM/F-12 supplemented with proper amounts of insulin and/or transferrin. Culture dishes were precoated with 4 μg/ml of poly-L-lysine. The cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2.

Microscopic Observation

The morphological differentiation of neuroblasts was assayed by determining the extent of neurite outgrowth. After appropriate time of culture, the cells were washed with phosphate buffered saline (PBS) and immediately fixed using a mixture of 95% ethanol, 40% formaldehyde and acetic acid (20:2:1 by volume). The cells bearing neurites longer than 2 cell-diameter were scored under a phase-contrast microscope. At least 10 fields were chosen for cell-counting, and the values obtained were averaged.

Immunochemical Analysis

MAP-2 was purified to apparant homogeneity from the extract of 3-day-old chick brain as described by Sloboda *et al.* (1975). Anti-MAP-2 antiserum was prepared by injecting albino rabbits with the purified MAP-2 as described elsewhere. Dot-immunobinding assay was performed by following the method of John *et al.* (1984), and immunoblot analysis was carried out as described previously (Blake *et al.*, 1984).

To localize MAP-2 in cultured neuroblasts, indirect immunocytochemistry was performed as described below. Neuroblasts grown in DMEM/F-12 containing 10 μg/ml of insulin for 72 hrs were washed with calcium-free Hank's blanced solution (Ca^{2+}-free HBSS) and then twice with the same solution but containing 5 mM piperazin-N,N′-bis [2-ethane-sulfonic acid](PIPES), 2 mM $MgSO_4$ and 2 mM ethylenglycol-bis-(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA)(pH 6.4). The cells were fixed with 2.5% glutaldehyde for 15 min and treated once with 0.5% Triton X-100 for 10 min and three times with 0.5 mg/ml of $NaBH_4$ in Ca^{2+}-free HBSS for 5 min each. After rinsing twice

with PBS, the cell samples were incubated with the following agents in order: 4% bovine serum albumin for 2 hrs; rabbit anti-MAP-2 antiserum for 1 hr; FITC-conjugated goat anti-rabbit IgG for 1 hr. The cells were then observed under a fluorescence microscope.

Results

Effect of Insulin on Neuronal Differentiation

Telencephalic neuroblasts obtained from 6-day-old chick embryo were cultured for 5 days in DMEM/F-12 medium in the presence and absence of insulin (Fig. 1). The cells cultured without insulin remained in spherical shape and were not viable. In contrast, the cells cultured in the presence of insulin settled rapidly from the medium and attached to substratum, and majority of the attached cells beared long and branched neurites.

To determine more precisely the dose-dependent effect of insulin on neuronal differentiation, the cells bearing neurites longer than 2-cell diameter were counted. As shown in Fig. 2, the extent of neurite outgrowth rised as increasing the concentration of insulin and reached maximal at 10 μg/ml. And yet, insulin showed no apparent trophic effect on cell proliferation as determined by counting the cell number per culture dish. These results clearly demonstrate that the supple-

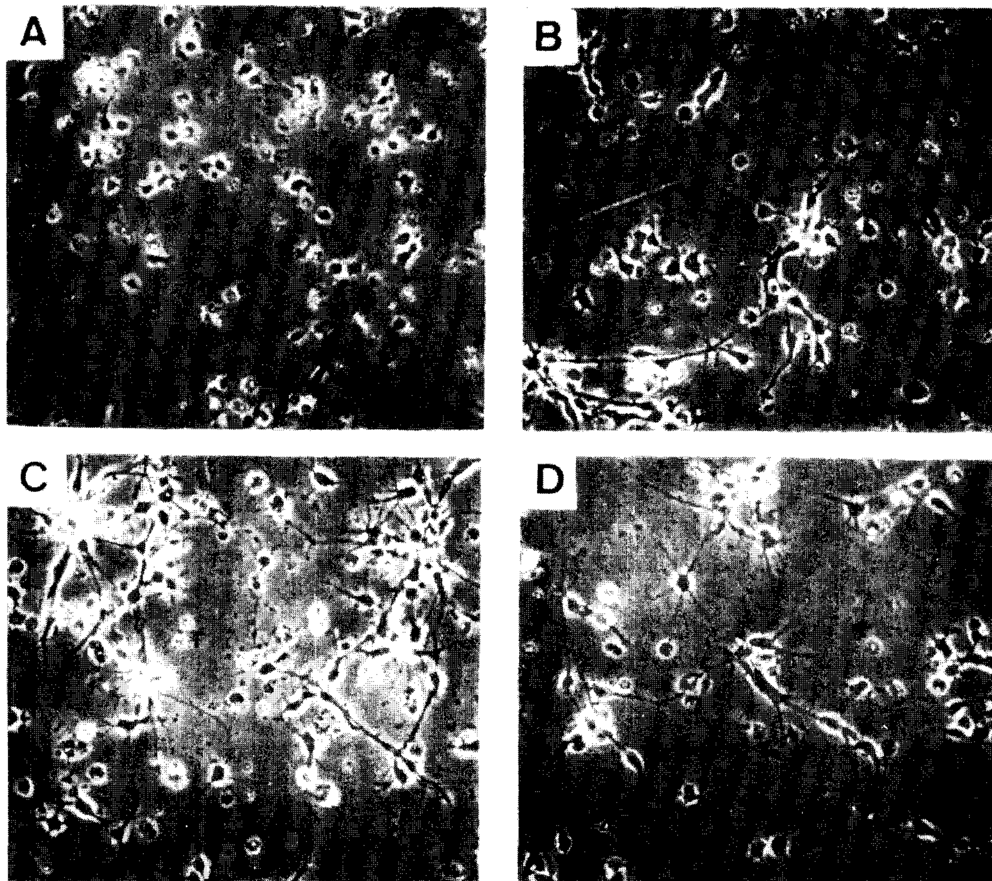

Fig. 1. Effect of insulin on differentiation of cultured neuroblasts. Neuroblasts obtained from telencephalons of 6 day embryonic chick brains were cultured for 5 days in DMEM/F-12 in the absence (A) and presence of 1 (B), 10 (C) and 100 μg/ml (D) of insulin. After the culture, the cells were photographed under a phase-contrast microscope.

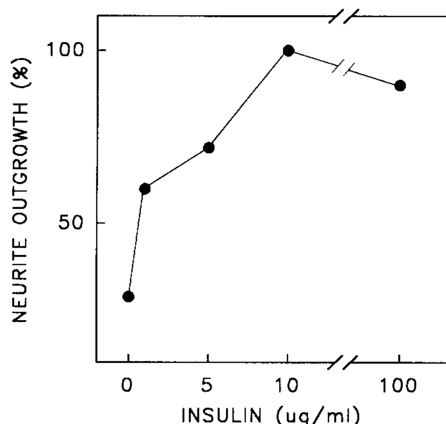

Fig. 2. Dose-response effect of insulin on neurite outgrowth activity of cultured neuroblasts. After culturing for 5 days as in Fig. 1, the cells bearing neurites longer than 2-cell diameter were scored under a microscope. The neurite outgrowth activity was expressed as percent of the cell number with neurites against total number of cells.

mentation of insulin to DMEM/F-12 is sufficient for the survival and development of telencephalic neurons at least for a week. Therefore, the neuroblasts were routinely cultured in DMEM/F-12 medium containing 10 μg/ml of insulin in addition to 10 μg/ml of transferrin unless otherwise indicated.

Detection of MAP-2 in Neuroblast Culture

To test the specificity of anti-MAP-2 antiserum produced in the present study, extracts were prepared from embryonic brain tissues and from neonatal brain and breast muscle. Proteins in the extracts were separated by polyacrylamide gel electrophoresis in the presence of SDS and then subjected to immunoblot analysis using the anti-MAP-2 antiserum. As shown in Fig. 3, the antiserum strongly interacted with MAP-2 but not with other proteins in both neonatal and embryonic brain extracts. Moreover, no immunoreactivity was evident in the muscle extract of new-born chick. These results clearly indicate that the antiserum prepared in this study is specific to MAP-2.

We then carried out immunocytochemical staining of the neuroblasts that had been cultured for 3 days. Fig. 4 shows that nearly all of the cells have one or more branched neurites and thier cell bodies as well as neurites are intensely stained with anti-MAP-2 antiserum. These results indicate

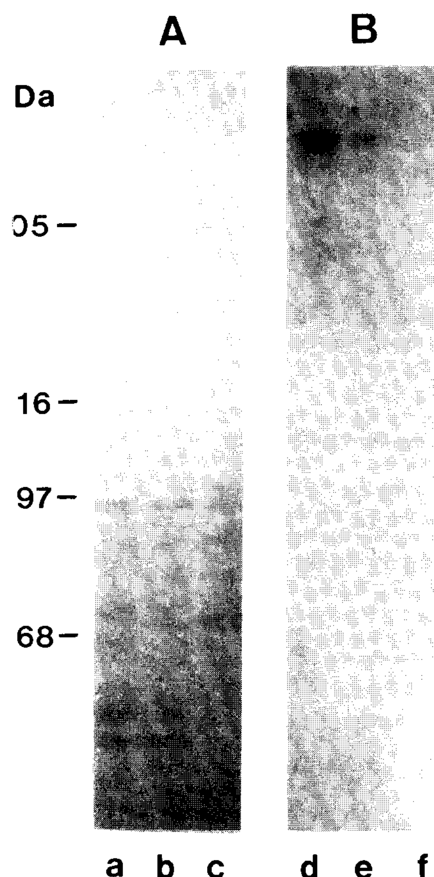

Fig. 3. Immunochemical detection of MAP-2 in brain and muscle extracts. Extracts were prepared from brains of new-born (lanes a and d) and 12-day embryonic chick (b and e) and from breast muscles of new born chick (c and f). Typically, the cultured cells or tissues were disrupted using a glass homogenizer in 0.1 M PIPES buffer (pH 6.4) containing 1 mM $MgSO_4$, 2 mM EGTA and 0.5 mM phenylmethanesulphonyl fluoride (PMSF). Proteins in the extracts were separated by electrophoresis in 5-14% polyacrylamide gradient gels containing 0.1% SDS. (A) One of the gels run in duplicate was stained with Coomassie R 250. (B) The other gel was electro-transferred onto a nitrocellulose sheet, incubated with anti-MAP-2 antiserum and then with ^{125}I-protein A, and autoradiographed.

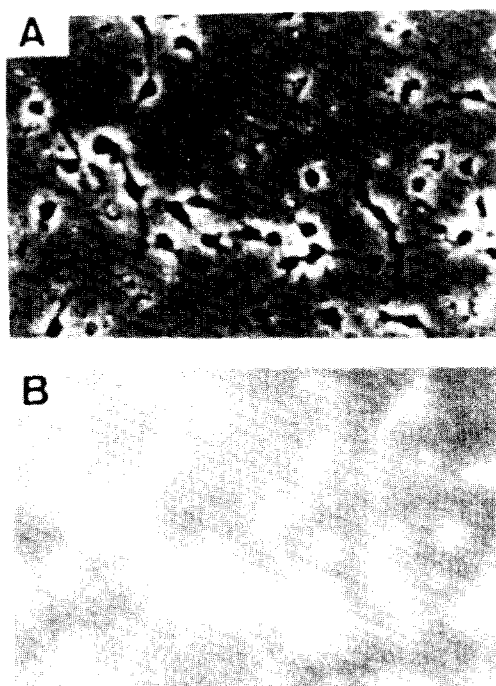

Fig. 4. Immunocytochemical detection of MAP-2 in cultured neuroblasts. The cells cultured for 3 days in DMEM /F-12 containing 10 μg/ml of insulin were fixed and immunostained as described in Materials and Methods. (A) A typical field of the neuronal culture. (B) The same field but visualized by immunostaining.

that MAP-2 is present in all developing neuroblasts.

Synthesis of MAP-2 during Neuronal Differentiation

To examine if the expression of the MAP-2 changed in the process of neuronal differentiation, the levels of MAP-2 in the extracts of cultured neuroblasts and of embryonic brain tissues were estimated by dot-immunobinding assay. As shown in Fig. 5, the level of MAP-2 in the cultured neuroblasts almost linearly increased as the culture period prolonged. At the time of 5th day of culture, the cell's content of MAP-2 was double as compared to that in first day. Thus, it appears likely that the increased synthesis of MAP-2 may occur in parallel with neuronal differentiation which event is manifested by the outgrowth of neurites.

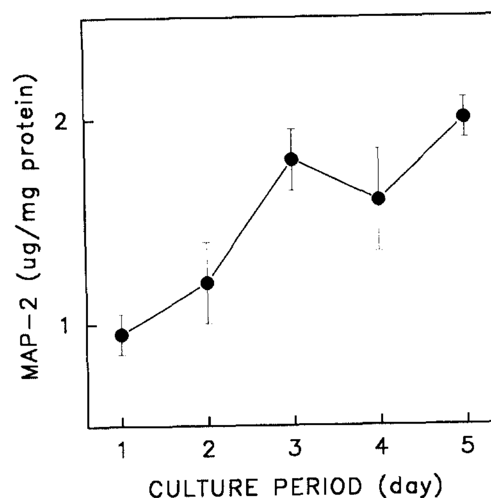

Fig. 5. Changes in the level of MAP-2 during differentiation of cultured neuroblasts. Extracts were prepared from the cells that had been cultured for the indicated periods in the presence of 10 μg/ml of insulin. The content of MAP-2 in the cell extracts were then determined by dot-immunobinding assay method.

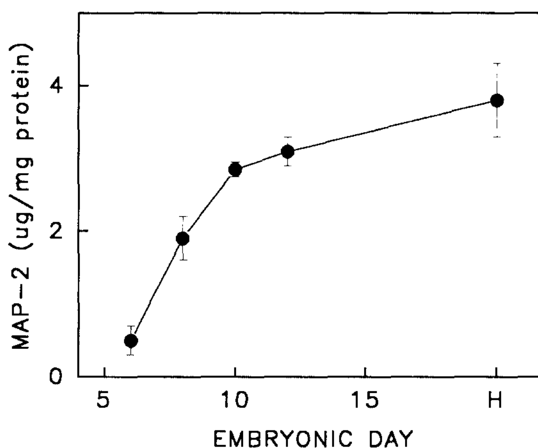

Fig. 6. Changes in the level of MAP-2 during the development of embryonic brains. Extracts were prepared from the embryonic brains at the indicated time of development, and their contents of MAP-2 were determined by dot-immunobinding method. The symbol H indicates the time of hatch.

The content of MAP-2 in developing chick brains also increased as illustrated in Fig. 6. This change in MAP-2 level is particularly featured by a dramatic 5-7 fold increase during the early de-

velopment of brain. These results again suggest that the increase in the MAP-2 gene expression is a requisite for neuronal differentiation in a developing brain.

Effect of Insulin on the Accumulation of MAP-2

Since insulin appears to stimulate neuronal differentiation, we tested if it also affects the synthesis of MAP-2. Extracts were prepared from the neuroblasts cultured for 5 days in the presence of increasing concentration of insulin and subjected to dot-immunobinding assay. Fig. 7 shows that the cells treated with increasing concentration of insulin contained higher amounts of MAP-2. In addition, it appears that the dose-dependent effect of insulin on neurite outgrowth is closely correlated with the effect on the cell's content of MAP-2. Therefore, it seems possible that insulin may enhance the expression of the MAP-2 which, in part, involved in promotion of neuronal differentiation.

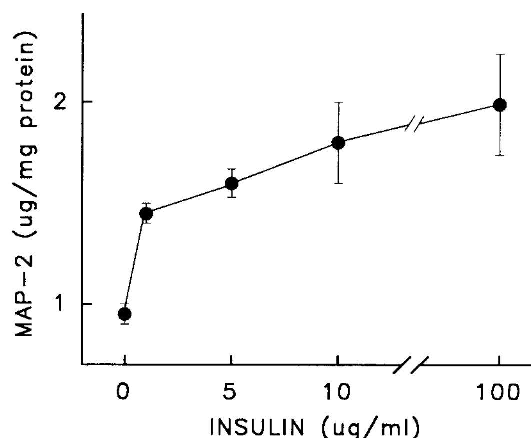

Fig. 7. Effect of insulin on the level of MAP-2 in cultured neuroblasts. Extracts were prepared from the cells that had been cultured for 5 days in the presence of increasing concentration of insulin, and their contents of MAP-2 were estimated by dot-immunobinding assay.

Discussion

In the present study, we developed a serum-free culture system, in which the supplementation of a single hormone, insulin, was sufficient for the survival and development of telencephalic neuroblasts. This defined medium also contained transferrin, which was reported to show a stimulatory effect on the differentiation of several cell lines and primary cell cultures perhaps through its iron-binding property (Barnes and Sato, 1980; Landschultz *et al.*, 1984). Under these conditions used, the neuronal culture displayed a typical developmental phenomenon of neurite outgrowth (Figs. 1 and 2). In addition, the cells accumulated MAP-2 in their neurites as well as cell bodies (Fig. 4).

Using a monoclonal antibody against MAP-2, Izant and McIntoch (1980) have reported that MAP-2 is predominantly localized to dendrites unlikely to our findings. It is possible that our anti-MAP-2 antiserum has reacted with newly synthesized MAP-2 in cytoplasm of neurites that have not yet established any distinguishable axons and dendrites. However, it is quite unlikely that he immunostaining of cell bodies is due to non-- pecific cross-reactivity since the antiserum ineracted neither with other proteins in brain omogenates nor with any protein in embryonic nuscle extracts (Fig. 3).

Immunochemical studies revealed that the level of MAP-2 in cultured neuroblasts increased as differentiation proceeded upon proloning the culture period (Fig. 5). This increase in the cell's content of MAP-2 may be a prerequisite for outgrowth of neurites because MAP-2 is known to promote tubulin polymerization and the assembly of microtubule is an absolute requirement of growth and maintenance of neuronal processes. Of additional note-worth is the finding that the MAP-2 level in the extracts of embryonic brain tissues dramatically increase during the early period of development (Fig. 6). Thus, MAP-2 can be considered as a potential biochemical marker for neuronal differentiation in accord with the suggestion by an earlier report of Izant and McIntoch (1980).

Of particular interest was the finding that insulin promoted both the neurite outgrowth activity and MAP-2 synthesis in the cultured neuroblasts. The activity of neurite outgrowth sharply increased by adding to the culture as little as 1 μg/ml of insulin

and approached to a maximal level at 10 μg/ml. Furthermore, this dose-dependent effect by insulin appears to be in parallel with changes in the MAP-2 level by insulin (see Figs. 2 and 7). Perhaps, insulin triggers or promotes the expression of the MAP-2 through an unknown but presumably specific route(s) of signal transduction. The increased production of MAP-2 may then facilitate the assembly of microtubules and consequently enhance the neurite outgrowth activity. However, the precise cellular events by which insulin brings about the neuronal differentiation remains to be resolved.

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References

Aizenman, Y., M. E. Weichsel, and J. D. Vellis, 1986. Changes in insulin and transferrin requirements of pure brain neuronal cultures during embryonic development. *Proc. Nat'l. Acad. Sci.* USA. **83:** 2263-2266.

Barnes, D. and G. Sato, 1980. Method for growth of cultured cells in serum-free medium. *Anal. Biochem.* **102:** 255-270.

Blake, M. S., K. H. Johnston, G. J. Russel-Jones, and E. C. Gotschlich, 1984. A rapid, sensitive method for detection of alkalin phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* **136:** 175-179.

Borisy, G. G., J. M. Marcum, J. B. Olmsted, D. B. Murphy, and K. A. Johnson, 1975. Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly in vitro. *Ann. N.Y. Acad. Sci.* **253:** 101-132.

Daniels, M. P., 1972. Colchine inhibition of nerve fiber formation *in vitro*. *J. Cell Biol.* **53:** 164-176.

Havrankova, J. and J. Roth, 1978. Insulin receptors are widely distributed in the central nervous system of the rat. *Nature* **272:** 827-829.

Herzog, W. and K. Weber, 1978. Fractionation of brain microtubule-associated proteins. Isolation of two differential proteins which stimulate tubulin polymerization *in vitro*. *Eur. J. Biochem.* **92:** 1-8.

Izant, J. G. and J. R. McIntoch, 1980. Microtubule-associated proteins: a monoclonal antibody to MAP-2 binds to differentiated neurons. *Proc. Nat'l. Acad. Sci.* USA. **77:** 4741-4745.

John, R., W. Schiebler, and P. C. Greenegard, 1984. A quantitative dot-immunobinding assay for protein using nitrocellulose membrane filters. *Proc. Nat'l. Acad. Sci.* USA. **81:** 1684-1687.

Kim, H., L. I. Binder, and J. L. Rosenbaum. 1979. The periodic association of MAP-2 with brain microtubules *in vitro*. *J. Cell Biol.* **80:** 266-276.

Kirschner, M. W., 1978. Microtubule assembly and nucleation. *Int. Rev. Cytol.* **54:** 1-71.

Landschultz, W., I. Thesheff, and P. Ekblom, 1984. A liphophilic iron chelator can replace transferrin as a stimulator of cell proliferation and differentiation. *J. Cell Biol.* **98:** 596-601.

Mill, J. F., M. V. Chao, and D. M. Ishii, 1985. Insulin, insulin-like growth factor II, and nerve growth factor effects on tubulin mRNA levels and neurite formation. *Proc. Nat'l. Acad. Sci.* USA. **82:** 7126-7130.

Muller, H. W., S. Beckh, and W. Seifert, 1984. Neurotrophic factor for central neurons. *Proc. Nat'l. Acad. Sci.* USA. **81:** 1248-1252.

Murphy, D. B. and G. G. Borisy, 1975. Association of high molecular weight proteins with microtubules and their role in microtubule assembly *in vitro*. *Proc. Natl. Acad. Sci.* USA. **72:** 2696-2700.

Raizada, M. K., 1983. Localization of insulin-like immunoreactivity in the neurons from primary culture of rat brain. *Exp. Cell Res.* **143:** 351-357.

Sensenbrenner, M., K. Maderspach, L. Latzkovits, and G. G. Jaros, 1980. Neuronal cells from chick embryo cerebral hemispheres cultivated on polylysine-coated surfaces. *Develop. Neurosci.* **1:** 90-101.

Sloboda, R. D., W. L. Dentler, and J. L. Rosenbaum, 1976. Microtubule-associated proteins and the stimulation of tubulin assembly *in vitro*. *Biochemistry* **15:** 4497-4505.

Sloboda, R. D., S. A. Rudolph, J. L. Rosenbaum, and P. Greengard, 1975. Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Nat'l. Acad. Sci.* USA. **72:** 177-181.

Solomon, F. and M. Magendantz, 1981. Cytochalasin separates microtubule disassembly from loss of asymmetric morphology. *J. Cell Biol.* **89:** 157-161.

Tsai, H. M., B. B. Garber, and L. M. H. Larramend, 1981. ^{3}H-Thymidine autoradiographic analysis of histogenesis in the chick embryo. *J. Comp. Neur.* **198:** 275-306.

Yamada, K. M., B. S. Spooner, and N. K. Wessells, 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* **49:** 614-635.

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배양 계배 신경아세포의 분화에 미치는 Insulin의 영향

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신경세포의 분화에 미치는 insulin의 영향을 알아보기 위하여 계배의 난뇌 신경아세포를 serum-free defined medium에서 배양하였다. Immunofluorescence실험을 통하여 신경특이단백질인 MAP-2는 신경아세포의 세포체와 신경돌기에 존재하는 것으로 나타났다. 또한, 배양액내에 insulin의 농도를 증가하면 신경아세포의 신경돌기 형성이 증가할 뿐 아니라 MAP-2의 합성도 증가하였다. 따라서, 신경아세포의 형태적 분화와 생화학적 분화는 서로 밀접하게 연관되어 일어나는 것으로 추측되며, insulin은 신경특이단백질의 합성을 촉진시킴으로써 신경아세포가 신경돌기를 형성하며 분화되어가는 데 결정적인 역할을 하는 것으로 보인다.