Reconstruction of neural tube-like structures *in vitro* from primary neural precursor cells

(collagen gel matrix/morphogenesis/nestin)

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ABSTRACT   Vertebrate central nervous system develops from a neural tube derived from the embryonic ectoderm. In mouse, the neural tube around embryonic day 10 primarily consists of neural precursor cells (NPCs). During the development of embryonic central nervous system, NPCs proliferate and migrate outward; thus later stages show NPCs toward the lumen of the neural tube and neurofilament-positive differentiated cells toward the periphery. In conventional liquid culture, NPCs isolated from mouse on embryonic day 10 proliferate and differentiate into neurofilament-positive neurons. In the present communication, we show that fragments of neural tubes and aggregates of NPCs, when placed into collagen gel matrix, form three-dimensional structures which resemble the neural tube formed *in vivo* in the developing embryos. Even dissociated NPCs form the three-dimensional structures in the collagen gel matrix. Our results indicate that individual NPCs or fragments of neural tubes carry morphogenetic information which allows them to reconstruct neural tube-like structures *in vitro*.

Vertebrate central nervous system (CNS) derives from a neural tube, in which pluripotent neural precursor cells (NPCs) proliferate and differentiate into various types of neuronal and glial cells (1–4). The neural tube is initially consisted of pseudostratified NPCs, then increases in thickness as NPCs proliferate, and finally becomes multilayered. In mouse, neurons become detectable in the hindbrain region around embryonic day 10 (E10) and are localized only in the marginal zone of the tube (5). The spatial as well as temporal control of CNS development requires the precise proliferation, migration, and differentiation of NPCs. Although the dynamic morphogenesis in the early CNS development has attracted investigators for near a century (6–9), the mechanisms controlling the programmed development of NPCs remain poorly understood, primarily due to technical difficulties associated with the study of mammalian CNS development.

As the first step to circumvent these difficulties we have established a simple method to isolate NPCs from embryonic mouse head at E10 (10). In primary cultures, NPCs differentiate into neurons and glia by following a time schedule similar to that observed in embryos. These findings prompted us to develop models of the CNS development *in vitro*. Collagen gel has been used as a matrix for studies of three-dimensional growth, differentiation, and morphogenesis of epithelial cells (11–16). In this study, we have utilized collagen gel culture of NPCs to observe their morphogenetic behavior three-dimensionally. NPCs were found to proliferate, extend neurites, and reconstruct neural tube-like structures in collagen gel matrix.

MATERIALS AND METHODS

NPC Preparation and Culture. An enzymatic method for preparation of NPCs from E10 embryos has been described (10). Trypsinization of mouse embryo heads, followed by gentle trituration, resulted in fragments of neural tubes. These fragments formed spherical structures with a diameter around 100–200 μm during overnight incubation in medium containing 10% fetal bovine serum (FBS). Fragments were further trypsinized into single cells. Fragments of neural tubes and dissociated NPCs were embedded in collagen gel prepared from mouse tails as described (11). In brief, collagen tendons from tails were sterilized in 70% ethanol and dissolved in sterile 0.017 M glacial acetic acid solution (4 mg/ml). The solubilized collagen was neutralized by a mixture of sterile 0.34 N NaOH and sterile 10× Waymouth's medium without bicarbonate (1:2). All solutions were kept on ice prior to use. Fragments or dissociated NPCs were suspended in neutralized collagen solution, pipetted into culture dishes, and incubated at room temperature until a gel had formed before addition of culture medium. Aggregates were made by incubation of dissociated NPC in 10% FBS-containing medium for 20 hr at 37°C. These NPC aggregates were then embedded in collagen gel as described above. The culture medium was a 1:1 mixture of Ham's nutrient mixture F12 and Dulbecco's modified Eagle's medium, supplemented with FBS (10%), insulin (10 μg/ml; Sigma), transferrin (10 μg/ml; Sigma), cholera toxin (10 ng/ml; Sigma), and sodium selenite (10 nM; Sigma). Cells were embedded in 1 ml of collagen and cultured in 12-well dishes (Falcon) coated with collagen (0.5 ml/well). Medium was changed every second day.

NPCs were also prepared at E9–9.5 by the same method described above and dissociated into single cells by trypsinization and trituration. To prevent dilution of culture medium, 48-well dishes were coated with a minimum amount of collagen (0.2 ml per well), and NPCs (2–5 × 10^5 per well) were embedded in 0.2–0.5 ml of collagen. They were fed with the medium described above. Higher concentrations (20% and 30%) of FBS were tested without any supplement. Medium was changed every second day.

Histology and Immunocytochemistry. Collagen gels containing cells were washed with phosphate-buffered saline (pH 7.4) and fixed with 4% paraformaldehyde in phosphate-buffered saline for 4 hr at 4°C. Gels were cut into halves, one dehydrated and embedded in paraffin, the other embedded in polyester wax (BDH). Sixty to 80 serial sections were cut at 6 μm, mounted on slides, and stained with hematoxylin and eosin or processed for immunocytochemistry as described (10). Mouse monoclonal anti-160-kDa neurofilament protein (NF) (1:50; no. RPN1104; Amersham) rabbit anti-glial fibril-

Abbreviations: CNS, central nervous system; NPC, neural precursor cell; NF, neurofilament protein; GFAP, glial fibrillary acidic protein; E*n*, embryonic day *n*; FBS, fetal bovine serum.

lary acidic protein (GFAP) (undiluted; no. 1400; Lipshaw Manufacturing, Detroit) and a rabbit anti-nestin serum (1:100) were used as primary antibodies for immunocyto-chemistry. A 16-amino acid peptide corresponding to residues 1247–1262 of rat nestin (4) was synthesized and used to raise antibodies in rabbits (unpublished work). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Tago; no. 6250) diluted 1:100 for anti-NF, FITC-conjugated goat anti-rabbit IgG (Tago; no. 6200) diluted 1:200 for anti-GFAP and anti-nestin, and rhodamine-conjugated goat anti-mouse IgG (Tago; no. 6350) diluted 1:50 for 5-bromodeoxy-yuridine (BrdUrd) were used as secondary antibodies. Samples were counterstained with 0.01% Evans blue (Merck; no. 3169). For analysis of NPC proliferation, aggregates in collagen gels were incubated with the BrdUrd–FrdUrd labeling reagent (Amersham; no. RPN20) for 2 hr at 37°C prior to fixation. Photomicrography was performed with an Olympus BH2-RFK fluorescence microscope.

RESULTS AND DISCUSSION

Around E10, the mouse neural tube consists of mostly nestin-positive cells, a few NF-positive cells, and virtually no GFAP-positive cells (Fig. 1). Fragments of neural tube curl up during the overnight preparation period. Histological and immunocytochemical observations of fragments revealed that undifferentiated cells are almost always located toward the inside and differentiated ones at the outer surface, indicating that they maintain the initial positional memory as in the neural tube (Fig. 1). However, a lumen or ventricle

formation was not observed in fragments at this stage. Fragments were cultured in collagen gel and fed with the medium described in *Materials and Methods*. Fragments began to extend neurites within 24 hr in culture (Fig. 2). By 3–5 days in culture, fragments increased severalfold in diameter, as did the neurites increase in length (Fig. 2). Histological and immunocytochemical observations revealed that (*i*) the majority of fragments developed a ventricle or lumen structure lined by layers of nestin-positive NPCs, (*ii*) each fragment had a single lumen, (*iii*) the lumen was in general located in the center of fragments but occasionally was in the marginal zone, and (*iv*) differentiated NF-positive neurons were toward the outer layers of fragments (Fig. 3). Because of structural similarities with the neural tube, we call these organoids "neural balls." Random growth of neurites outward, as observed with the *in vitro* neural balls, is not seen in embryos, because the neural tube is surrounded by the basement membrane (17, 18). Little information is available on the role of basement membrane in morphogenesis of the CNS at these early stages. It may be of interest to examine whether these neural balls cultured in collagen gel matrix express extracellular matrix protein, including basement membrane components. Collagen matrix has been shown to support growth, differentiation, and three-dimensional organization of epithelial cells (11–16). It seems to provide structural support unavailable in conventional liquid culture for growing fragments.

To test whether dissociated NPCs from E10 embryos can form neural balls in collagen matrix, cells were embedded in the collagen gel (1–2 × 10^5 cells per well of 24-well dishes) and
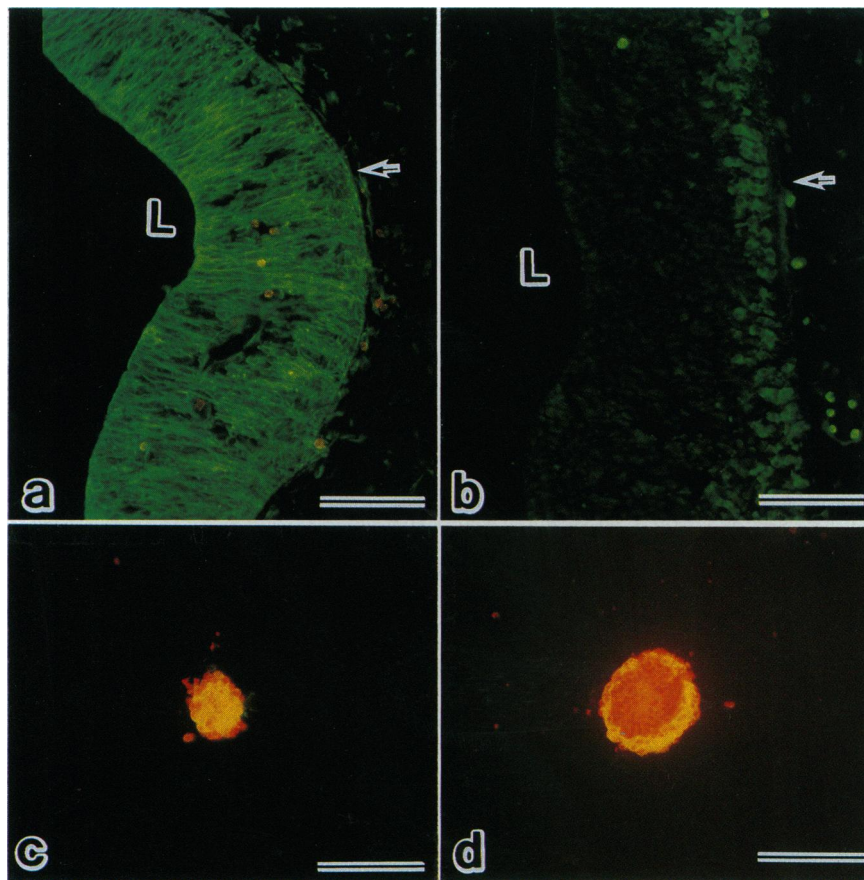

FIG. 1. Immunofluorescence micrographs of E10 neural tube (*a* and *b*) and fragments (*c* and *d*). Sections of E10 heads and fragments were stained with anti-nestin (*a* and *c*) and anti-160-kDa NF (*b* and *d*) antibodies. E10 neural tube is occupied mostly with nestin-positive NPCs and some NF-positive neurons toward the periphery. L, lumen of neural tube; arrow, outer limiting membrane. Nestin-positive NPCs are located in the center (*c*), while NF-positive neurons are at the periphery of fragments (*d*). *c* and *d* were counterstained with Evans blue. (Bars = 100 μm.)
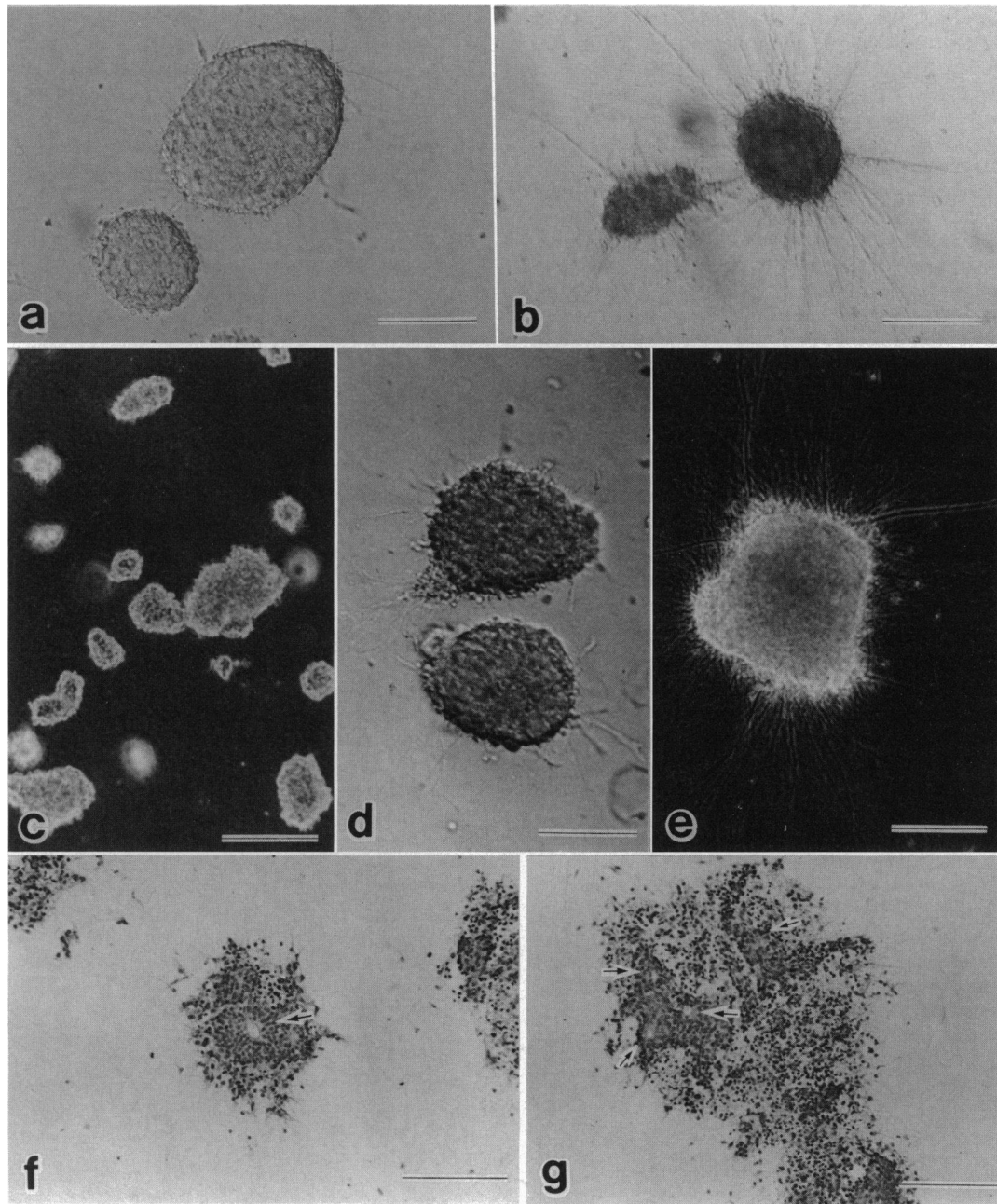
FIG. 2.   Phase-contrast micrographs show fragments (*a* and *b*) and aggregates (*c–e*) cultured in collagen gel. Fragments began to extend neurites within 24 hr (*a*) and increased in number and length of neurites during 3 days in culture (*b*). Aggregates of dissociated NPCs were embedded in collagen gel and photographed at 0 (*c*), 2 (*d*) and 5 (*e–g*) days in culture. Aggregates begin to extend neurites within 2 days (*d*) and the number and length of neurites had increased around day 5 (*e*). Small neural balls had a single neural tube unit (*f*), while in the larger ones multiple units were present (arrows) (*g*). [Bars = 50 μm (*a* and *d*) or 100 μm (*b*, *c*, and *e–g*).]

cultured. Under these conditions, cell aggregation was not observed and the majority of the cells died, but a few surviving ones formed neurites (data not shown). Aggregate suspension culture of neural cells is known to cause cyto-differentiation (19–21), and aggregates of dissociated fetal brain cells project neurites when they are placed in collagen gel (22). Therefore we incubated dissociated cells in 10% FBS-containing medium for 20 hr to form small aggregates (Fig. 2), prior to embedding in the collagen matrix. When cultured in this way, NPCs gradually extended neurites within 2 days and increased in diameter (Fig. 2). These early aggregates, when examined immunocytochemically, showed random distribution of NPCs and neurons, and many cells in aggregates were mitotic (Fig. 3). However, by day 5 in culture they became morphologically indistinguishable from the neu-

ral balls developed from fragments (Fig. 2). Relatively small neural balls had a neural tube unit: a single lumen covered with an inner NPC layer and an outer layer of neurons. Larger neural balls had multiple neural tube units (Fig. 2). However, immunocytochemical studies revealed that some small neural balls had only clusters of NPCs and neurons (pseudo neural balls) (data not shown).

Aggregates of dissociated cells can develop neural balls which are superficially indistinguishable from those developed from fragments. However, the internal architecture of the former is more sophisticated. NPCs and neurons are actively motile on the surface of a plastic culture dish and gradually organize into aggregates. The molecular mechanisms which cause the aggregation of neural cells *in vitro* have been extensively studied (23, 24). Neural cells (NPCs
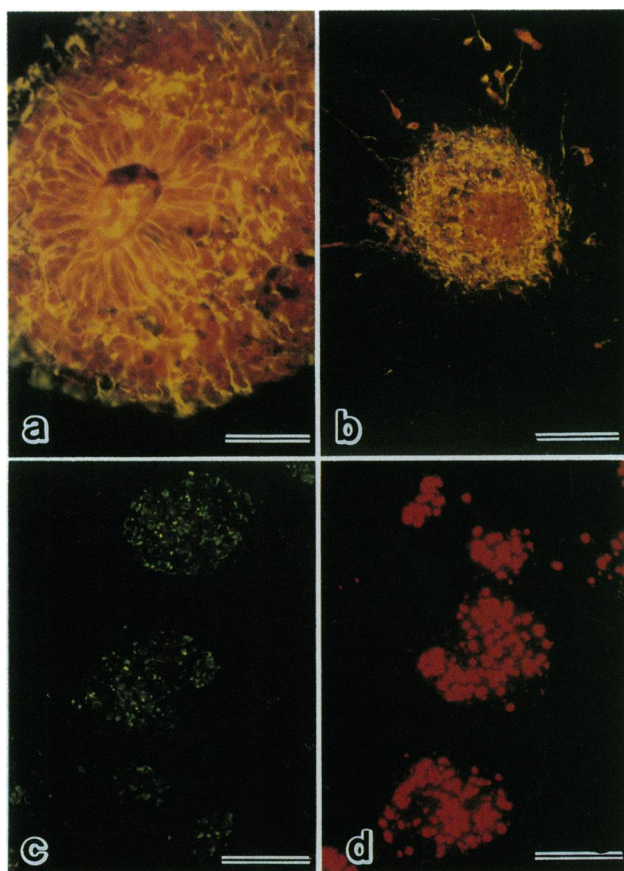
FIG. 3. Immunofluorescence micrographs of neural balls and aggregates. Neural balls developed from fragments formed a lumen lined inside with nestin-positive NPCs which extended long cytoplasmic processes by 3 days in culture (*a*). The marginal zone of neural balls is occupied by NF-positive neurons on day 5 of culture (*b*). Aggregates cultured for 2 days have randomly distributed nestin-positive (*c*) and BrdUrd-incorporating (*d*) cells. *a* and *b* were counterstained with Evans blue. [Bars = 50 μm (*a*) or 100 μm (*b–d*).]

and differentiated neurons) must share common surface marker(s) to recognize and aggregate, but nonneural cells such as contaminating fibroblasts lack the marker and therefore fail to aggregate (results not shown). Many NPCs are proliferative in aggregates (Fig. 3); therefore enlargement of aggregates is due to an increase in cell number. However, some small balls fail to reorganize neural tube units. They might lack a sufficient number of proliferative NPCs and therefore be unable to grow and form neural tube units. It is of interest to know the mechanisms by which NPCs and neurons distributed randomly in aggregates reorganize into neural tube units.

The observations described above indicate that proliferative activity of NPCs is very important in reconstructing neural tube-like structures. The most-proliferative NPCs are located near the ventricle of neural tube and they are dominant in neural epithelium of early embryo. The primitive NPC population might be diluted with their descendants during the active proliferative period. A recent study demonstrated that differentiated neurons are detectable even at 9 days of pregnancy (25). The failure in our attempt to construct neural balls from dissociated cells might have been caused by the limited number of highly proliferative NPCs and/or poor culture conditions. To test this hypothesis we prepared dissociated NPCs from embryos at an earlier stage (E9–9.5) in which neural tubes are occupied by mainly primitive NPCs. Dissociated NPCs were directly embedded in collagen gel and cultured in various media with modified

culture conditions as described in *Materials and Methods*. Aggregation of these NPCs was not observed in collagen gel (Fig. 4*a*). Some NPCs extended neurites and remained as single neurons without aggregation (data not shown). Some NPCs enlarged to a diameter around 20 μm within 24 hr and gradually took the shape of a multicellular structure. They further developed into balls with a diameter of 30–60 μm by day 5 in culture (Fig. 4*c*). Neurites began to appear around day 3. Few balls were formed in medium with 20% or 30% FBS (data not shown). Histological observation revealed that balls had a primitive lumen at day 5 (Fig. 4*c*). The balls increased in size (50–100 μm; Fig. 4*d*), extended long neurites by day 9 in culture, and developed a radial organization of NPCs inside and neurons at the periphery (Fig. 3). Aggregation is often observed among neurons and NPCs when they are cultured on a plastic surface (10), but it is rarely observed in collagen gel culture. Presumably, collagen fibers inhibit active movement of neurons and NPCs. Our observations strongly suggest that single NPCs proliferate and form neural balls, although it should be confirmed in some experimental procedures. The observations from this experiment, using primitive NPCs, indicate that the stage of the precursor cells may be very important in determining their morphogenetic potential. NPCs from early embryonic stages might have true primitive potential, whereas those from later stages acquire a programmed fate and therefore lose some of
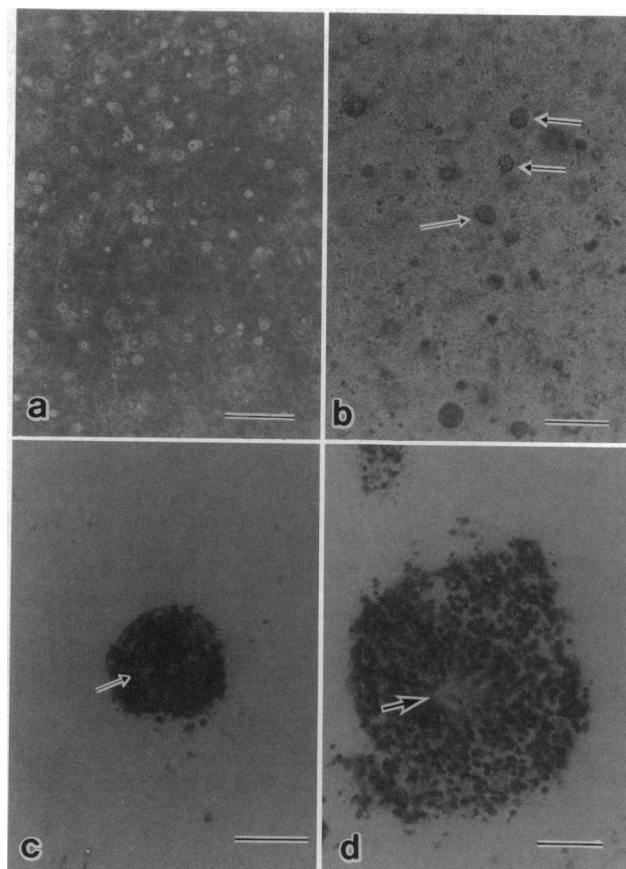

FIG. 4. (*a*) Dissociated NPCs from embryos at E9–9.5 were cultured in collagen gel for 24 hr. They were unaggregated cells. (*b*) Dissociated NPCs cultured for 3 days in 10% FBS-containing medium supplemented with insulin, transferrin, cholera toxin, and sodium selenite. These NPCs grew and formed neural balls (arrows). Phase-contrast micrographs are shown in *a* and *b*. (*c*) Neural ball cultured for 5 days, showing a primitive lumen (arrow). (*d*) Neural ball cultured for 9 days, showing a well-developed lumen (arrow). In *c* and *d*, cells were stained with hematoxylin and eosin. [Bar = 50 μm (*a* and *b*) or 30 μm (*c* and *d*).]

their proliferative ability. Identification of markers specific for the uncommitted primitive precursors capable of morphogenesis and proliferation might be helpful in answering these questions.

In the present study we have demonstrated that fragments of neural tubes and aggregates of NPCs can form neural tube-like structures. Even dissociated NPCs prepared from an earlier stage were shown to develop neural tube structures. This is intrinsic morphogenesis of NPCs without the help of the surrounding tissues and cell environment (26–29). The results strongly suggest that early primitive NPCs carry morphogenetic information which allows them to form neural balls from fragments of neural tubes or dissociated NPCs in collagen matrix. The three-dimensional collagen gel culture of NPCs is a useful model system to study CNS morphogenesis.

1. Chan, W. Y. & Tan, P. P. (1986) *J. Embryol. Exp. Morphol.* **96**, 183–193.
2. Moury, J. D. & Jacobson, A. G. (1989) *Dev. Biol.* **133**, 44–57.
3. Schoenwolf, G. C. & Smith, J. L. (1990) *Development* **109**, 234–270.
4. Lendahl, U., Zimmerman, L. B. & McKay, R. D. G. (1990) *Cell* **60**, 585–595.
5. Cochard, P. & Paulin, D. (1984) *J. Neurosci.* **4**, 2080–2094.
6. His, W. (1889) *Mark. Abhand. Kgl. Sach. Ges. Wiss. Math. Phys. Kl.* **15**, 313–372.
7. Schaper, A. (1894) *Morphol. Jahrb.* **21**, 625–708.
8. Penfield, W. (1932) in *Cytology and Cellular Pathology of the Nervous System*, ed. Penfield, W. (Hoeber, New York), Vol. 2, pp. 423–479.
9. Fujita, S. (1963) *J. Comp. Neurol.* **120**, 37–42.
10. Kitani, H., Shiurba, R., Sakakura, T. & Tomooka, Y. (1991) *In Vitro Cell. Dev. Biol.* **27A**, 615–624.
11. Imagawa, W., Tomooka, Y., Yang, J., Guzman, R., Richards, J. & Nandi, S. (1984) in *Methods for Serum-Free Culture of Cells of the Endocrine System*, eds. Barnes, D. W., Sirbasku, D. A. & Sato, G. H. (Liss, New York), Vol. 2, pp. 127–141.
12. Yang, J., Larson, L. & Nandi, S. (1982) *Exp. Cell Res.* **137**, 481–486.
13. Imagawa, W., Tomooka, Y. & Nandi, S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4074–4077.
14. Tomooka, Y., Harris, S. & McLachlan, J. A. (1985) *In Vitro Cell. Dev. Biol.* **21**, 237–244.
15. Iguchi, T., Uchima, F. D. A., Ostrander, L. & Bern, H. A. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3743–3747.
16. Montesano, R., Schaller, G. & Orci, L. (1991) *Cell* **66**, 697–711.
17. Sternberg, J. & Kimber, S. T. (1986) *J. Embryol. Exp. Morphol.* **91**, 267–282.
18. Martins-Green, M. & Erickson, C. A. (1986) *J. Embryol. Exp. Morphol.* **98**, 219–236.
19. DeLong, G. R. (1970) *Dev. Biol.* **22**, 563–583.
20. Shefield, J. B. & Moscona, A. A. (1970) *Dev. Biol.* **23**, 36–61.
21. Seeds, N. W. (1983) in *Advances in Cellular Neurobiology*, eds. Fedoroff, S. & Hertz, L. (Academic, New York), Vol. 4, pp. 57–79.
22. Mastuda, M. (1988) *In Vitro Cell. Dev. Biol.* **24**, 1031–1036.
23. Rutishauser, U. (1984) *Nature (London)* **310**, 549–554.
24. Takeichi, M. (1991) *Science* **251**, 1451–1455.
25. Easter, S. S., Jr., Ross, L. S. & Frankfurter, A. (1993) *J. Neurosci.* **13**, 285–299.
26. Brun, R. B. & Garson, J. A. (1983) *J. Embryol. Exp. Morphol.* **74**, 275–295.
27. Smedley, M. J. & Stanisstreet, M. (1985) *J. Embryol. Exp. Morphol.* **89**, 1–14.
28. Schoenwolf, G. C. (1988) *J. Comp Neurol.* **276**, 498–507.
29. Van Straaten, H. W. M., Hekking, J. W. M., Beursgens, J. P. W. M., Terwindt-Rouwenhorst, E. & Drukker, J. (1989) *Development* **107**, 793–803.