Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 4165–4169, July 1980
Developmental Biology

Presumptive common precursor for neuronal and glial cell lineages in mouse hypothalamus

(immunocytochemistry/ventricular cell/S-100 protein/differentiation)

F. DE VITRY*, R. PICART*, C. JACQUE†, L. LEGAULT‡, P. DUPOUEY§, AND A. TIXIER-VIDAL*

*Groupe de Neuroendocrinologie Cellulaire, Collège de France, 11 Place Marcelin-Berthelot, 75231 Paris Cedex 05, France; †Laboratoire de Neurochimie, Institut National de la Santé et de la Recherche Médicale Unité 134, Salpétrière, 75013 Paris; ‡Laboratoire de Biochimie Cellulaire, Collège de France, 11 Place Marcelin-Berthelot, 75231 Paris Cedex 05, France; and §Laboratoire de Biochimie des Antigènes, Institut Pasteur, 75015 Paris, France

Communicated by Alex B. Novikoff, March 21, 1980

ABSTRACT **The cellular localization of a neuronal and a glial cell specific protein (14-3-2 and S-100, respectively) has been explored in mouse hypothalamus in order to trace cell lineages. This study was performed on fixed slices, at the light microscope level, by using either the indirect peroxidase-labeled immunoglobulin technique or immunofluorescence. In the adult, only S-100 immunoreactivity was found in the ependymal layer. In contrast, the magnocellular neurons of the preoptic area displayed strong 14-3-2 immunoreactivity. At neonatal stages (fetal day 17–postnatal day 3), both 14-3-2 and S-100 immunoreactivities developed simultaneously in the *same* cells lining the ventral part of the third ventricle. Transient detachment of some of these ventricular cells could be visualized before migration in the hypothalamus where they remained as bipotential cells up to postnatal day 10. Later in the development, they differentiated into separate cells, one type containing 14-3-2 and the other S-100, like neurons and glial cells. These results argue for a developmental stage during which cells lining the ventricle are bipotential and may thus be candidates for the role of stem cells for both neuronal and glial lineages.**

The study of embryonic brain development has long been hampered by lack of cell markers for early developmental stages. The chief purpose of the present study was to explore the localization of neuronal and glial cell-specific proteins in mouse hypothalamus during development, in order to trace nerve cell lineages from adult cells to early stem cells.

In regard to the histogenesis of the nervous system, differences in opinion have been expressed concerning the nature and behavior of cells lining the neural tube which appeared identical in structure and behavior. By general consensus, the cells of the ventricular zone are considered to be the ultimate progenitors of all neurons and glia in the adult central nervous system (1). Nevertheless, it remained to be determined whether or not neuronal and glial cell lineages derive from the same ventricular stem cells, and whether neurons and glial cells become irreversibly committed to a given phenotype while within the germinal epithelium.

The hypothalamus consists of the lateral and ventral walls of the third ventricle. In the mouse, it begins to be distinguishable at day 10 of gestation. Cells of the ventricular zone divide rapidly and then migrate to their final position. Before embryonic day 16, most neurons in the mouse are already grouped into several hypothalamic nuclei (2, 3). Ultimately, the ventricular cells stop dividing and differentiate, mainly postnatally, into multiciliated ependymal cells and tanicytes, both attributed to the glial lineage. Thus, it appears that development of the mouse hypothalamus begins early in fetal life and terminates slowly postnatally.

The present study concerned cell identification, primarily in the ependymal zone of the third ventricule of the mouse brain, by using both immunoperoxidase-labeled globulins and the immunofluorescence technique.

The 14-3-2 protein (neuron-specific enolase) is found in the brain of most animal species with a high degree of immunological crossreactivity, and it has been located specifically in neurons (4, 5). The highly soluble S-100 protein is present in the central nervous system of all vertebrates. Its exclusively glial localization is still controversial. Although some authors reported a neuronal localization (6–8), most authors agree to glial localization in both the central and peripheral nervous systems (see review in ref. 9). It recently has also been detected in the ependymal lining of the neonate rat (10).

Our experiments demonstrate the presence of both 14-3-2 and S-100 proteins in the same ventricular cells, at the neonatal stage. Their differentiation into separate neuronal and glial cell lineages has been followed during development.

MATERIALS AND METHODS

Preparation of Purified Antigens. *14-3-2 protein.* The protein was prepared according to Grasso (11) and its purity was verified by $NaDodSO_4$/polyacrylamide gel electrophoresis.

S-100 protein. The S-100 protein was purified from beef brain by the method of Moore (12), slightly modified. A linear gradient of NaCl was used for elution from the DEAE-cellulose column instead of the three-chambered device which produces a parabolic gradient. The purity of the preparation was checked on acidic polyacrylamide gels and also by means of crossed immunoelectrophoresis compared with a standard batch of purified S-100 provided by P. Calissano.

Preparation and Specificity Control of Antisera. *14-3-2 protein.* Rabbit antiserum directed against pure bovine 14-3-2 protein was obtained by using Freund's adjuvant and subcutaneous injections. It gave a unique common precipitin line when purified 14-3-2 and soluble brain extracts were used as antigens. Furthermore, an immunocytolocalization was performed on the hippocampal area of rat brain (13) and in mouse cerebellum: 14-3-2-positive cells were exclusively neuronal cells; glial cells were always negative.

S-100 protein. Rabbit antisera against S-100 were obtained and tested as described (14). Controls were performed in order to ensure the glial specificity of our antiserum. First, by means of crossed immunoelectrophoresis with an intermediate gel, it was compared with a reference immune serum provided by E. Bock. Second, its behavior was glial specific in a series of studies on neural tumors (14). Third, when tested with immunocytochemical procedures on sections of adult mouse cerebellum, only glial cells were visualized; neurons were never stained.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: P_i/NaCl, phosphate-buffered saline.

Absorption of Antisera. *14-3-2 antiserum.* The equivalence point was previously defined by a quantitative precipitin determination. Antiserum was absorbed by immunoprecipitation in liquid medium with the optimal quantity of antigen.

S-100 antiserum. For immunohistochemical controls, the anti-S-100 antiserum was treated on an immunoadsorbant: 1 ml of undiluted serum was fixed with 600 mg of CNBr-activated Sepharose 4B previously loaded with 9 mg of S-100 protein. The mixture was stirred for 1 hr at room temperature and the absorbed serum was obtained by filtration.

Tissue Preparation for Immunocytochemistry. Mouse brains (C57 BL/6J) of different ages were used. At least six animals in each of the following groups were taken: embryonic days 17, 18, 19, and 20; postnatal days 1, 2, 4, 10, 15, 21, and 30; and ages 2 and 4 months. Presence of a vaginal plug determined the first day of gestation.

Brains were rapidly dissected out and cut into 2- to 4-mm-thick tissue blocks containing the hypothalamus. The blocks were then immersed for 10–24 hr in cold 4% paraformaldehyde/0.1 M phosphate, pH 7.4, and rinsed in phosphate-buffered saline (P_i/NaCl) containing 10% sucrose. Frontal slices of hypothalami 25–30 μm thick were cut on a TC2 tissue sectioner (Sorvall). They were treated with 0.1 M $NaIO_4$ in P_i/NaCl for 15 min, rinsed with P_i/NaCl for 15 min, and incubated with $NaBH_4$ (10 mg/ml of P_i/NaCl) for 10 min according to Schachner (15). This improved the contrast by decreasing the background staining. After four changes of P_i/NaCl, sections were incubated for 30 min in normal sheep serum to prepare them for the immunoperoxidase or immunofluorescence reaction.

Immunocytolocalization of 14-3-2 and S-100 Antigens. *Immunoperoxidase single staining.* When not mentioned, dilutions and washing were performed in P_i/NaCl. The slices were incubated for 3 hr at room temperature with 14-3-2 antiserum (dilution, 1:50) or with S-100 antiserum (dilution, 1:30), washed four times for 30 min, and incubated with sheep anti-rabbit globulin coupled with horseradish peroxidase (Institut Pasteur) for 1 hr (dilution, 1:40). After washing, slices were incubated with 3,3′-diaminobenzidine, HCl (Sigma), 0.25 mg/ml in Tris buffer (pH 7.6), for 20 min at room temperature. Slices were then treated for 10 min with the same solution to which 0.001% H_2O_2 had been added, washed with Tris buffer, and mounted in glycerol/gelatin (Sigma).

Immunofluorescence single staining. Indirect immunofluorescence staining was performed under the time and temperature conditions described above. Sheep anti-rabbit globulin coupled with fluorescein isothiocyanate (dilution, 1:20) or rhodamin (dilution, 1:50) were used (a gift from C. Tougard). Slices were mounted in phosphate-buffered glycerol (pH 7.5) and examined with a Zeiss reflection fluorescence microscope equipped with a 200-W Hg lamp.

Immunoperoxidase double staining. After the 14-3-2-containing cells were stained brown by the diaminobenzidine reaction, the specific antibody and the peroxidase-labeled antibody were selectively eluted from the tissue sections by incubation for 1 hr in 0.05 M glycine/HCl buffer, pH 2.2. After rinsing for 30 min with constant agitation, the S-100 antigen was localized in a similar way as 14-3-2 by using as the peroxidase substrate 4-chloronaphthol (0.25 mg/ml) which stains blue. Double-labeled cells appeared black. Identical results were obtained regardless of the antigen used first.

Immunofluorescence double staining. 14-3-2 protein was localized by using fluorescein isothiocyanate-conjugated sheep antirabbit globulin. Sections were then saturated for 2 hr with unlabeled sheep anti-rabbit IgG. The S-100 protein was then detected by using a rhodamine-coupled anti-rabbit globulin.

Tests for Specificity of the Cytochemical Staining. *Single staining.* Treatment with normal rabbit serum as well as with specific antisera previously absorbed by the homologous protein (see above) produced no staining.

Immunoperoxidase double staining. Completeness of pH 2.2 elution was controlled by omitting diaminobenzidine in the first staining step and, after elution, treating slices again with sheep anti-rabbit globulin and diaminobenzidine. Specificity of the reaction was tested by replacing one of the specific antisera by the same absorbed antiserum.

Immunofluorescence double staining. To rule out a possible artifactual double staining of the same antigen by mutual displacement of labeled antibodies, the following test was used. In a first step, the 14-3-2 or S-100 protein was revealed by using the appropriate specific antiserum and fluorescein-labeled sheep anti-rabbit globulin. In a second step, before the rhodamine-labeled globulin was used, the anti-S-100 or anti-14-3-2 antiserum was replaced by the corresponding absorbed antiserum. Because no staining could be observed with the rhodamine-labeled antibodies, we can assume that no mutual displacement of the labeled antibodies occurred. This control was achieved in both conditions: fluorescein- or rhodamine-labeled antibodies used first.

RESULTS

14-3-2 Protein. In the adult, cells with a neuronal appearance were seen as reactive throughout most of the hypothalamus. The magnocellular neurons in the preoptic area were particularly striking (Fig. 1). The 14-3-2 protein was absent from the ependymal zone of the ventricle (Fig. 2*a*). However, a few positive cells lying on the ependyma were seen and might correspond to the "liquor-contacting neurons" already observed by other methods (see review in ref. 16).

At the earliest prenatal stage examined (17–18th days of gestation), 14-3-2 protein was detected in 10–20% of the ependymal cells lining the ventral face of the ventricle. At embryonic day 19, it was found in about 40% of these cells (Fig. 2*b*); at embryonic day 20 and at birth, it was in the majority of the ventricular cells lining the ventral part of the third ventricle. In the dorsal part of the ventricle no positive cells could be seen in the ependymal layer. Small rounded or ovoid positive cells were also found in the lateral wall of the third ventricle at a certain distance from the ependymal layer at all stages.

Perinatally, in several cases 14-3-2-positive cells were seen detaching from the ventricle and beginning their migration (Fig. 2*b*). At days 3 and 4, positive cells were still present in the ependymal layer lining the ventral part of the third ventricle whereas, in the lateral part, ciliated cuboidal ependymal cells were never seen as positive. Then, frequency of positive cells in the ventral lining of the ventricle progressively decreased,

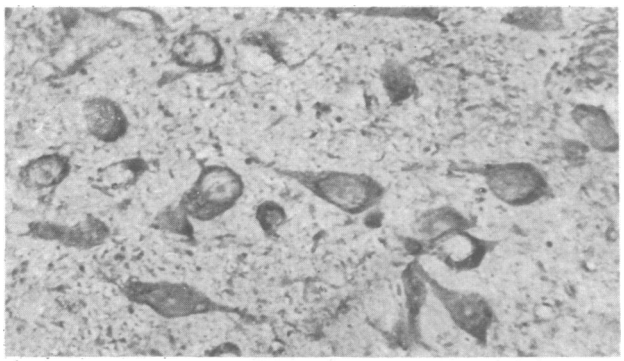

FIG. 1. Adult hypothalamus. Magnocellular neurons of the preoptic area stained with 14-3-2 antiserum. (× 340.)

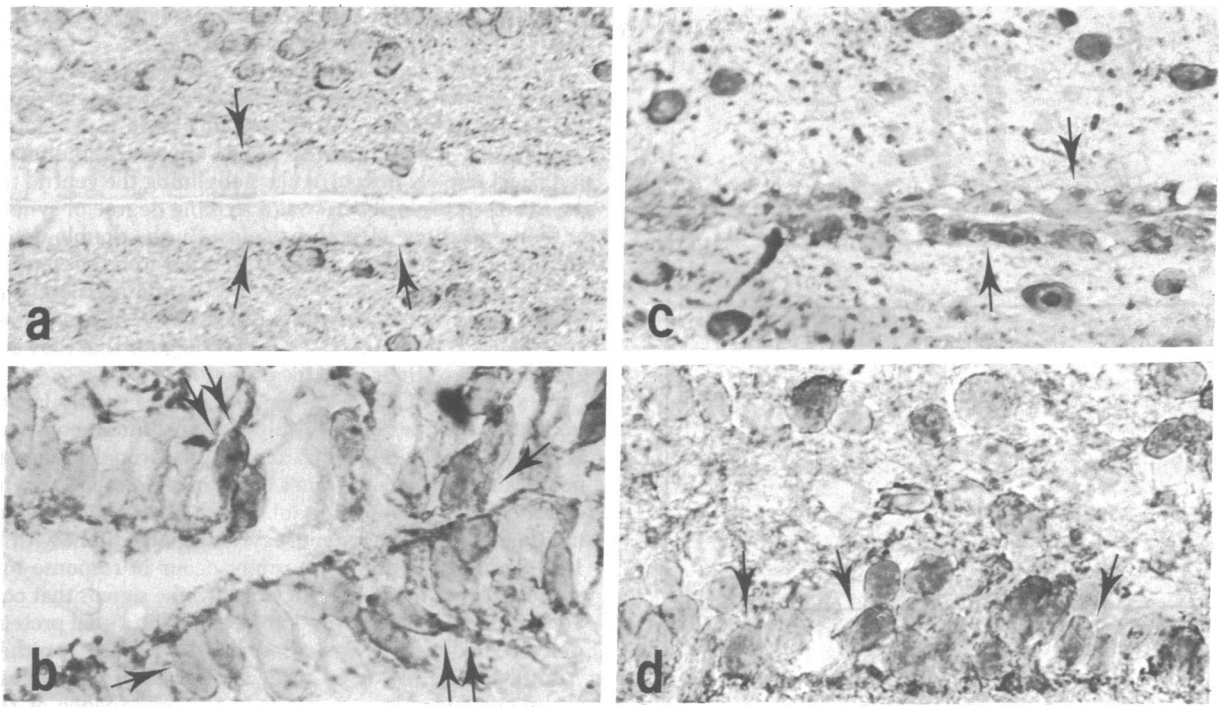

FIG. 2. Immunoperoxidase single staining. (×340.) (*a* and *b*) 14-3-2 antiserum. (*a*) In adult, ependymal cells are unstained (→); (*b*) in 19-day embryo, numerous ependymal cells are stained (→), some of them in migration (⇉). (*c* and *d*) S-100 antiserum. (*c*) In adult, ependymal cells are strongly stained (→); (*d*) in 19-day embryo, numerous ependymal cells are stained (→).

and at day 10 only a few positive cells persisted in this zone. From day 15 on, the adult pattern stabilized.

S-100 Protein. In the adult, ependymal cells, ciliated or not, were highly positive for S-100 protein (Fig. 2*c*). In addition positive rounded cells were also found in the lateral wall of the third ventricle (Fig. 2*c*). Although the accuracy of classifying glial cell types at the light microscope level is limited, in the adult these cells might represent oligodendrocytes. No typical filamentous astrocytes were visualized with the S-100 antiserum at any stage.

Before birth, evolution of S-100 protein in the ependymal zone was virtually synchronous with that of 14-3-2. S-100 antigenicity was first detectable in a few cells lining the ventral part of the ventricle at embryo day 17. Then, the number of positive cells increased with embryonic age. S-100-positive cells detaching from the ventricular zone before their migration were observed from embryonic day 19 to postnatal day 4 (Fig. 2*d*).

From postnatal day 3 on, all cells lining the ventricle contained the S-100 protein.

Double Detection of 14-3-2 and S-100 Proteins. Single staining has shown a remarkable coincidence in the appearance and evolution of 14-3-2 and S-100 antigenicity from embryo day 17 to postnatal day 3. To determine whether, at the neonatal development stage, the two specific neuronal and glial antigens were present in the same cells of the same section, we used the double staining method with 14-3-2 antiserum followed by S-100 antiserum and vice versa. In all cases, most cells of the ependymal layer in the ventral part of the ventricle were clearly positive for both antigens at the following stages: day 20 of gestation, neonate, day 5 (Fig. 3 *a* and *b*), and postnatal day 7. The staining due to 14-3-2 progressively disappeared around postnatal day 10, in contrast to the S-100 staining which persisted in the adult.

In the lateral wall of the third ventricle at the same developmental stages (embryo day 20 to postnatal days 7–10), most of the rounded small cells were doubly stained (Fig. 3 *a* and *b*). Then, a progressive segregation of the two cell lineages could be observed at postnatal day 10 and afterward. Two kinds of immunoreactive cells were simultaneously present in same section. In the periventricular zone double-stained cells contained both 14-3-2 and S-100 antigens (Fig. 4, postnatal days

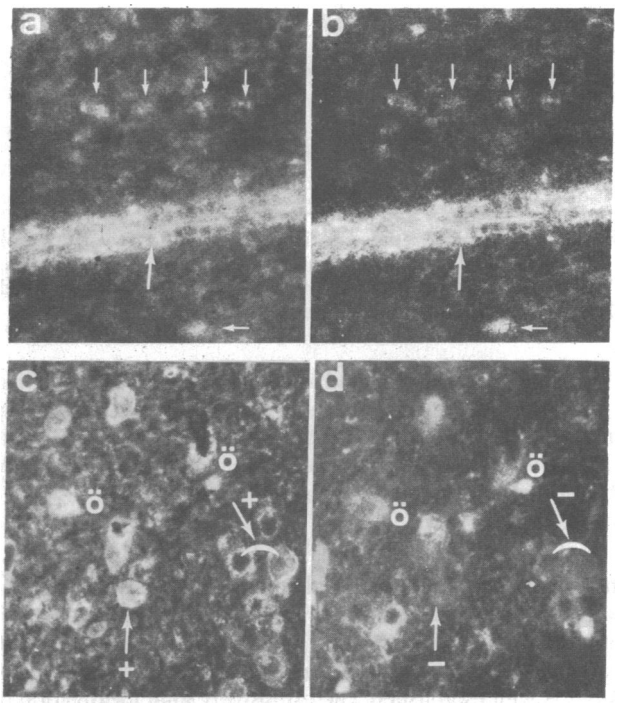

FIG. 3. Immunofluorescence double detection in the same section at two developmental stages: 14-3-2 antiserum (*a* and *c*) and S-100 antiserum (*b* and *d*). At postnatal day 5, antigens are located in the same cells (→) (*a* and *b*). In the adult (*c* and *d*), antigens are located in separate cells (+ →, − →); a few cells display both antigenicities (ö). (× 212.)

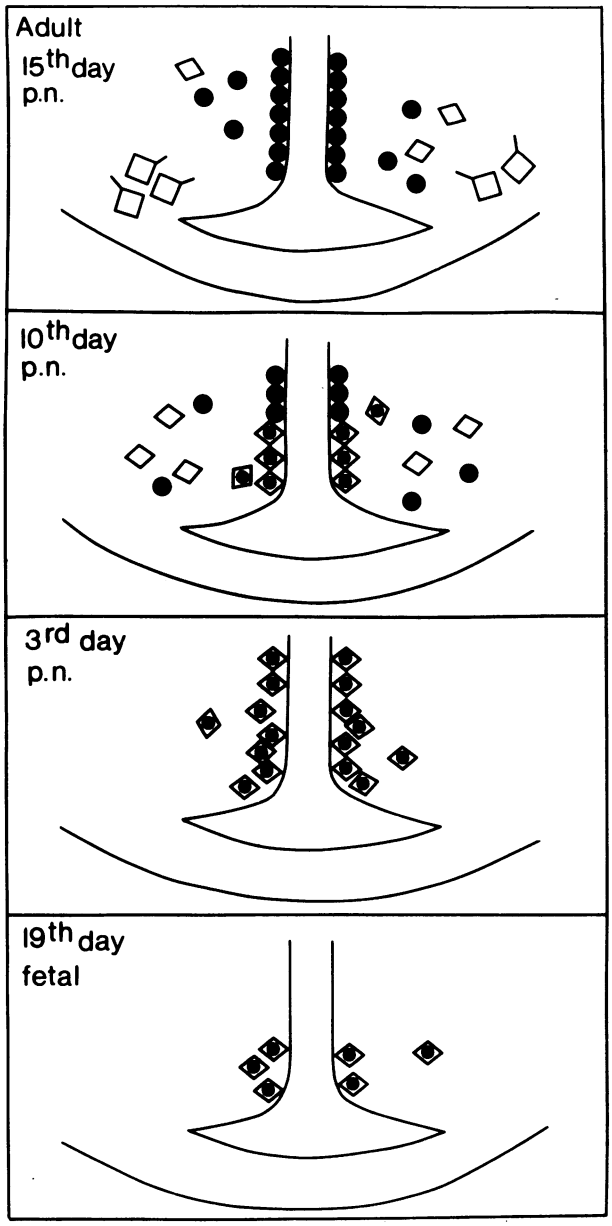

FIG. 4. Schematic representation of the cellular localization of 14-3-2 and S-100 during development of the mouse hypothalamus. ◇, 14-3-2-positive cell; ●, S-100-positive cell; ●, double-stained cell; ⊐—, magnocellular neurons; p.n., postnatal.

3–10). They might correspond to bipotential cells in the course of migration. Far away from the ventricle, single-stained cells contained either the 14-3-2 or the S-100 antigen (Fig. 3 *c* and *d*; Fig. 4, day 10–adult). However, even in the adult, a few cells retained both antigenicities (Fig. 3 *c* and *d*).

DISCUSSION

The present study was undertaken to identify a possible common precursor to neuronal and glial lineages in the mouse hypothalamus by using antisera specific to 14-3-2 and S-100 proteins, respectively. Whereas the neuronal specificity of 14-3-2 is well established, the exclusively glial localization of S-100 protein is still controversial (see Introduction). Nevertheless, our S-100 antiserum did not reveal either magnocellular neurons in the hypothalamus or Purkinje cells in the cerebellum. It never stained typical filamentous astrocytes; these were stained with a glial fibrillary acidic protein (GFA) antiserum in the adult only which did not permit tracing of their lineage during development (unpublished data).

Our observations show that, in the adult, magnocellular neurons contain 14-3-2 protein whereas ciliated ependymal cells and tanicytes contain S-100 protein. During development, 14-3-2 and S-100 immunoreactivities appeared simultaneously and progressively in ventricular cells lining the ventral part of the third ventricle. There was a striking degree of synchrony in the appearance of both proteins. By the double-detection method, both antigens have been found to be present in the same cells, between embryonic day 19 and postnatal day 10. At the same time, cells in the process of detaching from the ventricular zone could be seen. This suggests that they are migrating toward their final location (Fig. 4).

By showing that two antigens which are mostly located in separate cell types in the adult appear simultaneously in the same cells in the late fetus, our results strongly suggest that neurons and glia derive from a common stem cell. The neuronal differentiation is concomitant with a loss of the S-100 antigenicity, that of the oligodendrocytes is concomitant with loss of 14-3-2. Such a segregation might occur in response to local stimuli during migration. In contrast, the signals that commit ventricular cells to express both neuronal and glial proteins are delivered when cells are still within the germinal epithelium. Additional studies are required to determine whether cell detachment and migration occur as a consequence of the expression of both antigens.

Our observations of neonatal ventricular cell detachment and migration are consistent with a late development of the ventral part of the hypothalamus. Indeed, although most of the hypothalamic neurons are in place on day 16 of gestation, those occupying the anteromedial and posteromedial positions settle during the latter stage of embryonic life (2, 3). The median eminence becomes progressively differentiated within 2 weeks after birth in the rat (see review in ref. 17).

The evidence presented here that, perinatally, most of the hypothalamic neural cells are bipotential and contain 14-3-2 and S-100 proteins throws a new light on contradictory findings concerning the localization of S-100 (6–9). Our data might explain why many established nerve cell lines of tumor origin or obtained after carcinogen treatment of embryos have been found unexpectedly to contain both neuronal and glial characteristics (18, 19).

We tentatively conclude that neuronal and glial cell lineages, in the ventral part of hypothalamus, are derived from a common stem cell. This precursor cell located, in the neonatal stage in the ventricular zone, contains both 14-3-2 and S-100 proteins. It then migrates and differentiates into either neurons or glia. Further studies are needed to establish whether the same conclusion might be extended to other areas of the brain.

We thank C. Pennarun and F. Lachapelle for their skillful technical assistance. This work was supported by grants from the Centre National de la Recherche Scientifique (ER 89 and ATP 3925) and from the Fondation de la Recherche Médicale.

1. Boulder Committee (1970) *Anat. Rec.* **166**, 257–262.
2. Niimi, K. I., Harada, I., Kusara, Y. & Kishi, S. (1962) *Tokushima J. Exp. Med.* **8**, 203–238.
3. Shimada, M. & Nakamura, T. (1973) *Exp. Neurol.* **41**, 163–173.
4. Cicero, T. J., Cowan, W. M., Moore, B. W. & Suntzeff, V. (1970) *Brain Res.* **18**, 25–34.
5. Pickel, V. M., Reis, D. J., Marangos, P. J. & Zomzely-Neurath, C. (1976) *Brain Res.* **105**, 184–187.
6. Tabuchi, K. & Kirsch, W. M. (1975) *Brain Res.* **92**, 175–180.
7. Haglid, K. G., Hamberger, A., Hanson, H. A., Hyden, H., Persson, L. & Rönnback, L. (1975) *Nature (London)* **258**, 748–749.

8. Donato, R., Michetti, F. & Miani, N. (1975) *Brain Res.* **98,** 561–573.
9. Eng, L. F. & Bigbee, J. W. (1978) in *Advances in Neurochemistry,* eds. Agranoff, B. W. & Aprison, M. H. (Plenum, New York), Vol. 3, pp. 43–98.
10. Moore, B. W., Cimino, M. & Hartman, B. K. (1977) *Proc. Int. Soc. Neurochem.* **6,** 31 (abstr.).
11. Grasso, A., Roda, G., Angeletti-Hogue, R. A., Moore, W. B. & Perez, V. J. (1977) *Brain Res.* **124,** 497–507.
12. Moore, B. W. (1965) *Biochem. Biophys. Res. Commun.* **19,** 739–744.
13. Secchi, J., Lecaque, D., Cousin, M. A., Lando, D., Legault-Demare, L. & Raynaud, J. P. (1980) *Brain Res.,* **184,** 455–466.
14. Jacque, C. M., Kujas, M., Poreau, A., Raoul, M., Collier, P., Racadot, J. & Baumann, N. (1979) *J. Natl. Cancer Inst.* **62,** 479–483.
15. Schachner, M., Hedley-Whyte, E. T., Hsu, D. W., Schoonmaker, G. & Bignami, A. (1977) *J. Cell Biol.* **75,** 67–73.
16. Knowles, Sir, F. (1972) *Prog. Brain Res.* **38,** 255–270.
17. Tixier-Vidal, A. & de Vitry, F. (1979) *Int. Rev. Cytol.* **58,** 291–331.
18. Legault-Demare, L., Zeitoun, Y., Lando, D., Lamandé, N., Grasso, A. & Gros, F. (1980) *Exp. Cell Res.* **125,** 233–240.
19. Schubert, D., Heinemann, S., Carlisle, W., Tarikas, H., Kimes, B., Patrick, J., Steinbach, J. H., Culp, W. & Brandt, B. L. (1974) *Nature (London)* **249,** 224–227.