Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1326–1330, February 1982
Neurobiology

Neurotypy: Regional individuality in rat brain detected by immunocytochemistry with monoclonal antibodies

(neuronal diversity/gene recombination/neuropeptide/hybridoma/lymphocyte–neuron homology)

LUDWIG A. STERNBERGER, LEE W. HARWELL, AND NANCY H. STERNBERGER

Center for Brain Research, University of Rochester School of Medicine, Rochester, New York 14642

Communicated by Berta Scharrer, November 9, 1981

ABSTRACT **Hybridomas from spleen cell fusions of six BALB/c mice immunized with hypothalamus were analyzed by immunocytochemistry for antibodies reactive with paraffin sections of fixed rat brain. In a total of 135 antibody producers, 60% were brain specific. Among these, 54% reacted with glial elements, pituitary cells, or basal lamina of intracerebral capillaries, with little variation among individual hybridomas in each of these groups. Forty-six percent of brain-specific antibodies reacted with neuronal structures, localizing on nerve fibers, neurofibrils, or perikarya. Neuron-specific hybridomas could be classified into groups that localized in anatomically defineable overall patterns. Within these patterns individual hybridomas exhibited extensive qualitative localization diversity ("neurotypy"). Conceivably, the genetic message for a common "proantigen" within an overall pattern may be slightly modified during differentiation of a neuron, thus leading to minor variability in antigenic expression. During antibody formation, similar minor changes occur in the differentiation of the genetic message for the antibody variable region. Apparently, minor changes in the antibody combining site among groups of hybridomas is reflected in the detectability of minor neurotypic changes among differentiated neuronal proantigens. If neurotypy proves to be the result of single-base substitutions or of variability in alignment of peptide-coding exons, the Scharrer concept of the fundamental significance of neurosecretion could also become applicable to neuronal specialization.**

The Scharrer concept of the fundamental biologic role of neurosecretion (1, 2) ushered in the discovery of an increasing number of neuropeptides in diverse regions of the nervous system (3). The existence of more than one peptide sequence in individual prohormones (4–7) and the coexistence in a single cell of given peptides with a variety of other peptides, unsuspected from the structure of known prohormones (8–11), suggest a great variability in the expression of prohormones among different neurons. On the supposition that such differences may be an expression of the functional diversity of "experienced" neurons, we explored its existence with the use of hybridoma antibodies to whole brain homogenate. Because each monoclonal antibody is reactive with a single antigenic determinant, it defines an antigen even if it has not been chemically isolated. The only requirement for such application of monoclonal antibodies was the use of a technique for their detection that does not require availability of isolated antigen. Immunocytochemical analysis fulfills this requirement, because it defines an antibody clone by the anatomical distribution of its localization rather than the nature of the antigen with which it reacts. With the use of immunocytochemistry for intracellular antigens, we have found that a large proportion of antibodies to whole brain are brain specific, and that among these a sizeable fraction is neuron specific.

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.

MATERIALS AND METHODS

Six BALB/c mice were immunized with Freund's complete adjuvant containing homogenized hypothalami recovered from Fischer 344 rats perfused with saline while they were under Nembutal anesthesia. Four days after an intraperitoneal booster injection of homogenate, 10^8 spleen cells were fused with 6 $\times$ 10^7 mouse myeloma cells, P3-X63-Ag8-653, using 35% (wt/vol) polyethylene glycol 1500 with 5% dimethyl sulfoxide (wt/vol) (12–14). The total fusion mixture from each mouse was distributed into a sufficient number of microcultures to ensure that, with a high probability, each culture would be monoclonal. In addition to cells and medium, each microculture well contained 4 $\times$ 10^3 normal mouse peritoneal cells and 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT) (15). Supernates from vigorously growing cultures were assayed immunocytochemically and the cells were stored frozen. Most of the data in the present communication were obtained from these supernates. Cell suspensions from immunocytochemically interesting supernates were recloned twice by limiting dilution to ensure stability and monoclonality, and selected clones were propagated in bulk culture or ascites.

For immunocytochemistry, Fischer 344 or male Sprague–Dawley rats were perfused with Bouin's fixative and their brains, with attached pituitaries, pineals, trigeminal nerves, and ganglia, were embedded in paraffin. Seven micrometer-thick parasagittal sections were stained by the sequence of 3% normal goat serum for 30 min; hybridoma supernate for 24 hr; goat anti-mouse IgG, diluted 1:20, for 30 min; mouse peroxidase-antiperoxidase complex (PAP) diluted to contain 0.017 mg of peroxidase and 0.006 mg of antiperoxidase per ml, for 30 min; and 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide for 8 min (16, 17).

RESULTS

Among a total of 135 antibody-producing cultures, 5 were probably biclonal, because 4 of them stained pituitaries and 1 of them brain endothelium in addition to neuronal elements. Final verification of monoclonality by repeated limiting-dilution subcloning has been established for some of the 135 hybridomas but is not yet complete for others. Except for elimination of the occasional biclonality, there was no qualitative immunocytochemical difference between original cultures and limiting-dilution subclones.

Surprisingly, only 54 out of 135 antibody-containing supernates were not specific to brain structures within the limited range of test tissues used. The majority of these yielded uniform staining of brain tissue and attached non-brain structures, and thus were discarded. Also discarded were seven hybridomas with anti-nuclear antibodies and three hybridomas with anti-

Abbreviation: PAP, peroxidase-antiperoxidase complex.

Table 1. Staining patterns of brain-specific hybridoma antibodies

General structures stained	Hybridoma number*
Neurofibrillar patterns	
Widespread fibrils and perikarya	02-40†
Selected fibrils and perikarya	1B-2† 02-124
Selected fibrils, no perikarya	03-4† 03-33 03-44† 03-48‡ 04-07, 06-17
Neuronal patterns (not neurofibrils)	
Perikarya in brain stem and midbrain	03-57†
Widespread neurons and projections	02-161
Purkinje cells and hippocampus	02-135†
Purkinje and pyramidal cells	06-32† 06-53†
Cerebellar patterns	
Cerebellar cortex, stellate fibers only	02-172
Cerebellar cortex, glomeruli	02-24†
Various proportions of cerebellar cells and fibers	02-19 02-28 02-29† 02-88 02-99 02-158 02-164 02-187 02-169 02-170 02-184 02-185 06-05
Cytoplasm of neurons, nuclei of other cells	04-5
Unmyelinated fibers	02-54 02-61 02-78 06-50†
Myelinated fibers	02-189 03-26 06-39†
Oligodendrocytes	1A-43 02-80 03-31† 06-45† 06-51† 06-59†
Basal lamina of brain capillaries	02-04 02-30 02-33 02-36 02-41† 02-47 02-48 02-55 02-63 02-77 02-92 02-106 02-132 02-133 02-134 02-143 02-163 02-168 02-180
Pituitary, pars intermedia	02-174
Pituitary, selected pars anterior cells	02-21 02-38 02-39 02-43 02-61 02-77 02-82 02-87 02-98 02-127 02-138 02-149 02-157 02-166 02-170 02-175 02-178 02-190

* Fusion number followed by culture number.
† Stability and monoclonality established by subcloning.
‡ Antibody-producing genome lost during subcloning.

endothelial basement lamina antibodies reactive within the brain as well as attached peripheral structures.

Eighty-one hybridomas were specific to identifiable brain structures, 37 to neuronal and 44 to non-neuronal elements (Table 1). Not all major non-neuronal components were represented. Thus, none of the hybridomas reacted with astrocytes. Many antibodies reacted with discrete cells in the pars anterior of the pituitary and others with the basal lamina of brain capillaries, but not with capillaries of pituitary, pineal, ependyma, and trigeminal nerve. Only six antibodies were specific for glial elements; they stained oligodendrocytes. Each individual hybridoma staining any of these structures reacted identically without variation in regional distribution of immunocytochemical stain.

In contrast, diversity was high with antibodies to neuronal elements. All these antibodies revealed a high predilection for structures in the brain stem, cerebellum, and limited cerebral cortex regions and absence or paucity of structures in hypothalamus and thalamus. Among the antibodies that gave the appearance of neurofibrillar staining, hybridoma 02-40 stained most profusely and seemed to detect all neurofibrils and many perikarya indiscriminately, at least in brain stem and cerebellum (Fig. 1*a*). Hybridoma 1B-2 still stained many neurofibrils and, in the cerebellum, revealed white matter fibers strongly, but basket cell fibers only weakly (Fig. 2*a*). In contrast, 03–4, 03–33, 03–44, and 03–48 stained neurofibrils in white cerebellar matter only weakly, but basket cell fibers strongly (Fig. 2*b*). 1B–2 stained most motor neurons, many neurons in the mesencephalic reticular formation, most, but not all, cells of the mesencephalic nucleus of the trigeminal nerve (Fig. 3*a*), and selected pyramidal cells in the cerebral cortex (Fig. 4*a*). In contrast, 03–4, 03–33, 03–44, and 03–48 stained no perikarya at all. Although these four antibodies exhibited only minor variations in their staining patterns, they did not stain identical antigens as revealed by differences in stainability of normal and pathologic human brain (unpublished). Hybridoma 04–07 also stained neurofibrils and no perikarya. However, the distribution was much more selective, delineating the various layers of

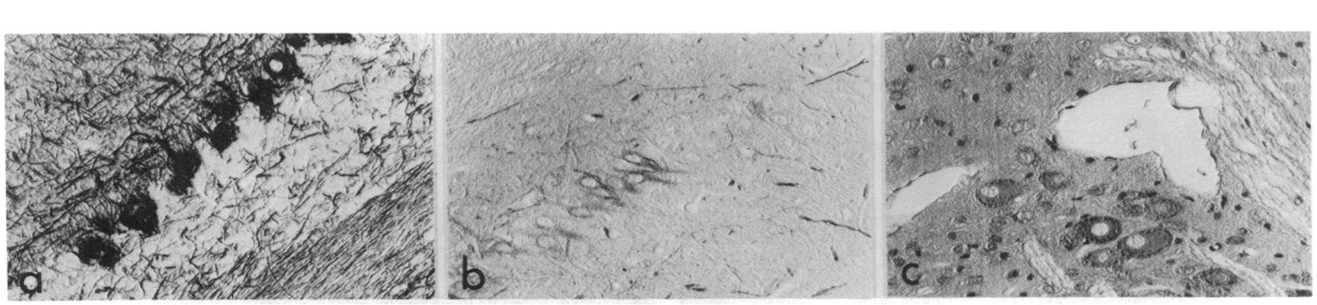

FIG. 1. (*a*) Cerebellum stained by hybridoma 02–40. Indiscriminate neurofibrillar staining includes Purkinje somata. Compare with Fig. 2. (*b*) Hippocampus. Hybridoma 02–135 seems to stain pyramidal cells in area CA2, but not in area CA1c. (*c*) Hybridoma 04–05 stains perikarya of neurons of the mesencephalic nucleus of the trigeminal nerve, but stains nuclei of nearby oligodendrocytes and endothelial and ependymal cells. (×130.)

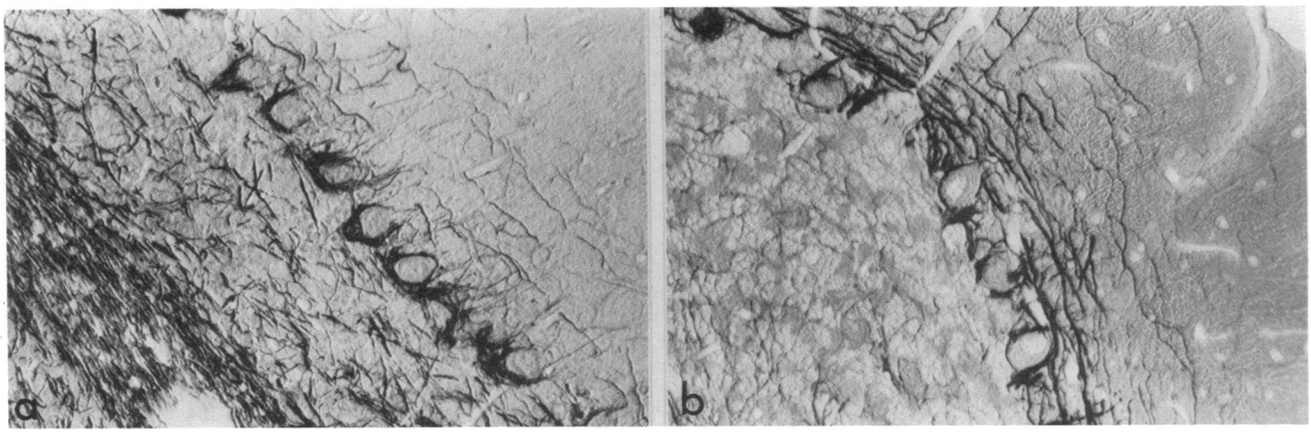

FIG. 2. (*a*) Cerebellum stained with hybridoma 1B–2. Prominent deep cerebellar fibers seem to contact fiber "baskets" around unstained Purkinje somata. (*b*) Cerebellum stained with hybridoma 03–44. Prominence of basket cell fibers and paucity of deep cerebellar fibers. (×200.)

the cerebral cortex, staining the fornix strongly and the corpus callosum, anterior commissure, and optic nerve only weakly. Neurofibrillar material and perikarya were also stained by 02–124, but the distribution was again different. The red nucleus was prominent, as was the medial lemniscus and the decussation of the trapezoid body. However, there were only few cell bodies and dispersed fibers in the reticular formation and only sparse fibers in the cerebellum. Also, in contrast to the other neurofibrillar-staining hybridomas, there was no staining anterior to the level of the red nucleus.

Five hybridomas stained selective neurons and their immediately associated fibers but revealed few intervening fibers. Each one of them had a different staining pattern. In the cerebellar cortex, 02–135 stained only Purkinje cells with their dendrites and axons (Fig. 3*b*). In other areas it revealed strong and selective staining of the red nucleus (Fig. 5*a*), and it stained stellate cells in the cerebral cortex (Fig. 4*b*) and seemed to delineate, in the hippocampus, stained area CA2 from unstained area CA1c (Fig. 1*b*) (18).

Several hybridomas, all from the same fusion, were distinguished by their cerebellar patterns. The molecular layer of the cerebellum was the only brain structure stained by 02–172 (Fig. 5*b*). Thus, it appears to stain outer stellate cell fibers, because these are the only cerebellar fibers that do not leave the molecular layer (19). However, among 12 other hybridomas, some stained parallel fibers strongly and glomeruli weakly (Fig. 4*c*), some stained both structures along with Purkinje cells (Fig. 4*d*), and others stained glomeruli strongly, molecular layer weakly, and Purkinje cells not at all. All these 12 hybridomas also stained to variable degrees fibers in the pons and medulla and, with some of them, cell bodies, such as those in the fastigial nucleus or in the trapezoid body, exhibited granular membrane staining. Thus, there seemed to occur, despite an overall similar pattern, considerable individual variation among these antibodies in the relative degrees of staining of parallel fibers, Purkinje cells, glomeruli, and neuronal cell membranes in deeper nuclei.

Supernates from four hybridomas appeared to stain fibers in unmyelinated areas without staining myelinated areas. They outlined, in negative image, cell bodies and projections of some of the larger neurons and also stained cerebellar glomeruli (Fig. 4*e*). Three of the hybridomas stained fibers in myelinated areas but not in unmyelinated areas. Another hybridoma stained the nuclei in oligodendrocytes and ependymal, endothelial, pituitary, and cerebellar granule cells, but the cytoplasm in neurons (Fig. 1*c*). Perhaps this antibody reacts with an antigen found in the cytoplasm of highly differentiated cells but in the nucleus of less differentiated cells.

DISCUSSION

Previous work on neuronal individuality by immunocytochemistry with monoclonal antibodies was mainly concerned with the cell surface and the discovery of "recognition antigens." Thus, on immunization with retinal membrane pellets, Barnstable (20)

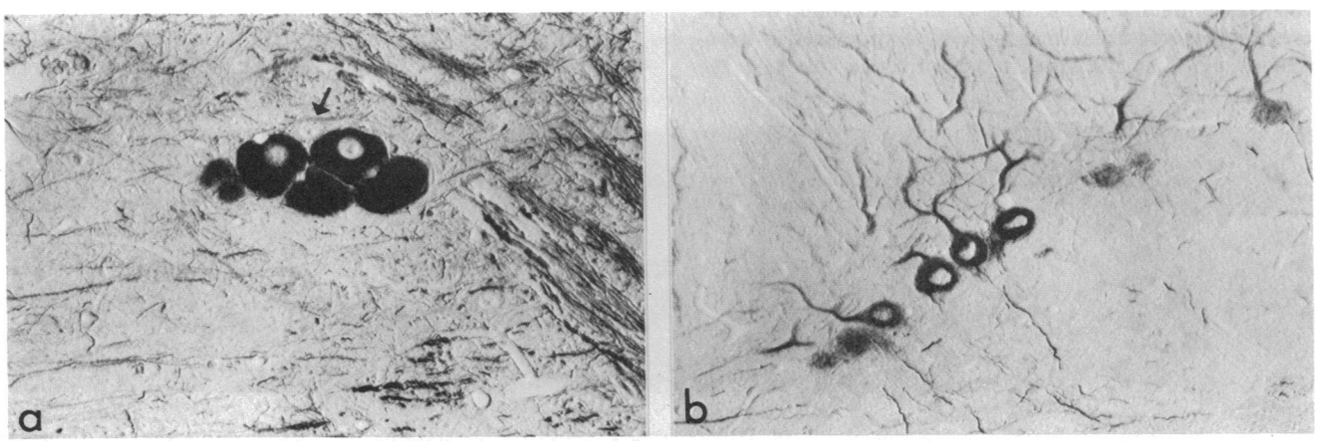

FIG. 3. (*a*) Mesencephalic nucleus of the trigeminal nerve stained with hybridoma 1B–2. Arrow points to an unstained neuron. (*b*) Purkinje somata, axons, and dendrites stained with hybridoma 02–135. Compare with Figs. 2 and 5*b*. (×200.)

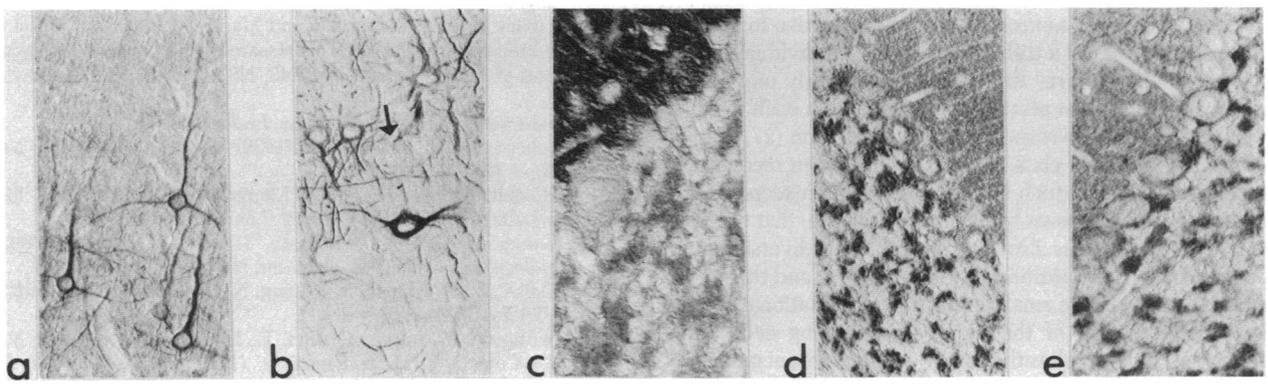

FIG. 4. (*a*) Cerebral cortex. Pyramidal cells stained by hybridoma 1B–2. (*b*) Cerebral cortex. Stellate cells stained by hybridoma 02–135. Arrow points to unstained pyramidal cell. (*c*) Cerebellum. Parallel fibers extend as fine projections from granule cells and expand in the molecular layer. Staining by hybridoma 02–186. (*d*) Cerebellum. Granular layer, Purkinje cells, and molecular layer stained by hybridoma 02–164. (*e*) Cerebellum stained by hybridoma 02–78. Glomeruli are the most prominent structures recorded. (*a*, *b*, *d*, and *e*, ×170; *c*, ×340.)

isolated hybridomas reactive with different retinal cell types. Trisler *et al.* (21) described a retinal antigen distributed in a dorsoposterior–ventroanterior gradient, Vulliamy *et al.* (22) and Cohen *et al.* (23) distinguished peripheral from central neurons, and Zipser and McKay (24) produced an antibody specific to the penile evertor neurons in the leech nerve cord. Because of the unavailability of mouse PAP, these studies either employed relatively insensitive immunofluorescence or, when the PAP technique was used (24), detected mouse hybridoma antibodies by staphylococcal protein A and rabbit PAP. However, protein A possesses only low affinity for mouse IgG1 (25). The relatively low sensitivity of immunocytochemical detection of antibodies required the use of cultured cells, frozen sections, or fixed whole mounts. Penetration of antibodies is poor in these preparations and, therefore, the scope of investigation had to be restricted to cell surface antigens or to antigens in relatively simple nervous systems, such as that of the leech. The present investigation was aimed at detecting antigens in the interior of the cell because it was assumed that a greater variety of antigens is possessed by the whole cell than by its surface alone. To facilitate their detection in mammalian brain, we utilized extensively fixed rat brain and sensitive immunocytochemical technique involving the homologous sequence of anti-mouse immunoglobulin and mouse PAP.

With this technique, we found an unexpectedly large proportion of hybridomas that were brain specific, either to neuronal or non-neuronal elements. However, certain abundant structures, such as astrocytes or their fibrillar projections, remained undetected. Even though hypothalamus was used for immunization, there was a conspicuous absence of staining in thalamus or hypothalamus, with the exception of dispersed fibers, which may have entered these areas from other structures. Also surprising was the high frequency of neurofibrillar and neuronal staining and the predilection for the cerebellum. The peculiar distribution of these overall patterns and their appearance in several of the fusions, to the exclusion of other neuronal patterns, is probably due to the use for immunization of inbred mice, who share a similar complement of *Ir* genes (26). This uniformity of response is extended to each of the hybridomas from individual fusions that reacted with non-neuronal elements. Thus, each hybridoma within the same fusion specific for brain endothelial basement membranes reacted in an anatomically identical manner. This, of course, is the expected result if there exists little antigenic difference in basement lamina antigens within different regions of the brain.

Among the total of all fusions, only few overall neuronal patterns could be distinguished, and those termed neurofibrillar, cerebellar, and neuronal were most prevalent (Table 1). The similarity of these overall patterns extended from one fusion to another. Thus, in general, but not in detail, the neurofibrillar staining pattern was similar in fusions 1B, 02, 03, and 04. Therefore, the permissiveness of response to antigens in these patterns must also be inscribed in the *Ir* genes of the BALB/c mouse.

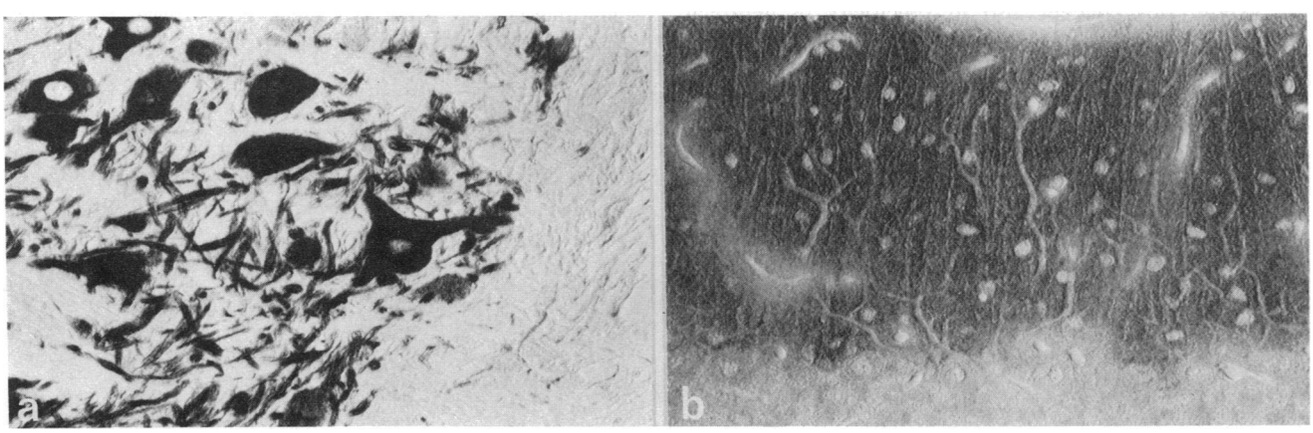

FIG. 5. (*a*) Red nucleus stained with hybridoma 02–135. (*b*) Cerebellum stained by hybridoma 02–172. Outer stellate cell fibers in the molecular layer surround unstained Purkinje dendrites. (×200.)

However, each anti-neuronal hybridoma reactive in a given overall pattern yielded a different, detailed, individual distribution within its pattern, distinguishing not only one fusion from the other, but also most hybridomas within each fusion.

During the immune response, allelic exclusion (27) results in a shift from the IgM class, expressed in antigen recognition units, to other classes, such as IgG, expressed in secreted antibodies. It has been shown by Gaehardt *et al.* (28) that the shift from IgM to IgG class is also accompanied by a diversification of the sequence of the antibody variable region, and thus of its specificity, ascribable to small changes from mutational events during differentiation of the antibody-producing cell. Such small changes in antibodies that occur during immunization are not easily detectable by immunocytochemistry if the antigen involved is invariant. Thus, even if changes occur in the specificities of individual hybridomas reactive, for example, with endothelial basement lamina of rat brain, these changes are not detectable if all capillaries possess the same antigen. However, if a tissue antigen appears in a variety of forms, each expressing a minor change in structure, variations in antibody may reveal differences in distribution of immunocytochemical stain. Such differences were, indeed, observed for each hybridoma antibody reactive within a given overall neuronal pattern. This phenomenon may be interpreted by ascribing to each overall staining pattern a general "proantigen," whose genome undergoes, during differentiation of the brain, and perhaps concomitant with neuronal experience, a number of minor somatic mutations, which are expressed in a variety of "neurotypes," each differing in minor respects from the overall structure of the proantigen.

It is interesting that a small variability in the antibody combining site results in reaction with a different neurotype within a given overall neuroanatomic reaction pattern, rather than yielding nonreaction with brain or a reaction with constituents outside the prescribed overall pattern. Conceivably, *Ir* genes might determine only whether or not a host will respond to the overall structure of the proantigen. They may not discriminate between neurotypes. Perhaps, small changes from proantigen to neurotype may be detected immunologically only with antibodies differing from each other by small changes, such as occur somatically during differentiation of the antibody-producing cells. There may be a parallelism in permissible variability of neuronal proantigens with that of *Ir* gene-determined antigen recognition capacity, reminiscent perhaps of the distribution of shared antigens among brain and T cells (29, 30).

The Scharrers (1, 2) have originated the concept of the fundamental role of proteinaceous substances in cellular communication in brain and periphery. The diversity of neurotypes may extend this concept also to specificity of neuronal communication.

We thank Richard Bauserman and Margi Goldstein for skillful and devoted technical assistance. This research was supported by National Institutes of Health Grants NS 15843, NS 15809, and HD12932.

1. Scharrer, B. (1978) *Gen. Comp. Endocrinol.* **34**, 50–62.
2. Scharrer, E. & Scharrer, B. (1939) *Res. Publ. Assoc. Res. Nerv. Ment. Dis.* **20**, 170–194.
3. Hökfelt, T., Johannsson, O., Ljungdahl, A., Lundberg, J. M. & Schultzberg, M. (1980) *Nature (London)* **284**, 515–521.
4. Gainer, H., Loh, Y. P. & Sarne, Y. (1977) in *Peptides in Neurobiology*, ed. Gainer, H. (Plenum, New York) pp. 183–213.
5. Mains, R. E., Eipper, B. & Long, N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3014–3018.
6. Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. M. & Numa, S. (1979) *Nature (London)* **278**, 423–427.
7. Krieger, P. T. & Liotta, A. S. (1979) *Science* **205**, 366–372.
8. Sternberger, L. A., Greenwald, J. L., Hock, D., Elger, K. H. & Forssmann, W. G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5216–5220.
9. Joseph, S. A. & Sternberger, L. A. (1979) *J. Histochem. Cytochem.* **27**, 1430–1437.
10. Beauvillain, J. C., Tramu, G. & Dubois, M. P. (1981) *Cell Tissue Res.* **218**, 1–6.
11. Martin, R. & Voigt, K. H. (1981) *Nature (London)* **289**, 502–504.
12. Köhler, G. & Milstein, C. (1976) *Eur. J. Immunol.* **6**, 511–519.
13. De St. Groth, F. S. & Scheidegger, D. (1980) *J. Immunol. Methods* **35**, 1–21.
14. Kearney, J. F., Radbrach, A., Liesegang, B. & Rajewsky, K. (1979) *J. Immunol.* **123**, 1548–1550.
15. Littlefield, J. W. (1964) *Science* **145**, 709–710.
16. Sternberger, L. A., Hardy, P. H., Cuculis, J. J. & Meyer, H. G. (1970) *J. Histochem. Cytochem.* **18**, 315–333.
17. Sternberger, L. A. (1979) *Immunocytochemistry* (Wiley, New York), 2nd Ed.
18. Lorente de No, R. (1934) *J. Psychol. U. Neurol.* **46**, 113–177.
19. Carpenter, M. B. (1976) *Human Neuroanatomy* (Williams & Wilkins, Baltimore), 7th Ed.
20. Barnstable, L. J. (1980) *Nature (London)*, **286**, 231–235.
21. Trisler, G. D., Schneider, M. D. & Nirenberg, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2145–2149.
22. Vulliamy, T., Rattray, S. & Mirsky, R. (1981) *Nature (London)* **291**, 418–420.
23. Cohen, J. & Selvendran, S. Y. (1981) *Nature (London)* **291**, 421–423.
24. Zipser, B. & McKay, R. (1981) *Nature (London)* **289**, 549–554.
25. Chalon, M. P., Milne, R. W. & Vaerman, J. P. (1979) *Scand. J. Immunol.* **9**, 359–364.
26. Benacerraf, B. & Dorf, M. E. (1974) in *Progress in Immunology II*, eds. Brent, L. & Holborow, J. (North Holland, Amsterdam) pp. 181–190.
27. Honjo, T. & Kataoko, T. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2140–2144.
28. Gearhart, P., Johnson, H. D., Douglas, R. & Hood, L. (1978) *Nature (London)*, **291**, 29–34.
29. Selsing, J. W. (1981) *Cell* **25**, 47–58.
30. Hamann, A., Arndt, T., Klein, P. & Thiele, H. G. (1980) *Biochem. J.* **187**, 403–412.