tential in the prejunctional dendrite or a local active response in the dendrite of the impaled neuron. Too little is known about the electrophysiological characteristics of neocortical dendrites to resolve this issue.

Our data provide both morphological and electrophysiological evidence for electrotonic coupling of superficial neurons in the neocortical slice. This form of intercellular communication may also be a feature of the neocortex in vivo (*18*), where it could be of great functional significance. It is intriguing that five of the dye-coupled aggregates were organized in vertically oriented columns, a structural arrangement of importance in current concepts of the mechanisms of neocortical information processing (*19*). Under normal conditions, the activities of electrotonically coupled neurons might not necessarily be highly synchronized, since complex interplay between electrotonic and chemical forms of transmission can result in a flexible repertoire of intercellular interactions (*13, 20*) On the other hand, in pathological conditions such as epilepsy, the presence of electrotonic connections might be an important factor in achieving the characteristic high degree of neuronal synchronization.

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References and Notes

1. K. J. Roney, A. B. Scheibel, G. L. Shaw, *Br. Res. Rev.* **1**, 225 (1979).
2. C. F. L. Hinrichsen and L. M. H. Larramendi, *Brain Res.* **7**, 296 (1968); C. Sotelo and S. L. Palay, *ibid.* **18**, 93 (1970); R. Baker and R. Llinas, *J. Physiol. (London)* **212**, 45 (1971); C. Sotelo, R. Llinas, R. Baker, *J. Neurophysiol.* **37**, 560 (1971); R. M. Wylie, *Brain Res.* **50**, 179 (1973); H. Korn, C. Sotelo, F. Crepel, *Exp. Brain Res.* **16**, 225 (1973); Y. Yarom and R. Llinas, *Neurosci. Abstr.* **5**, 109 (1979).
3. J. J. Sloper, *Brain Res.* **44**, 641 (1972); ——— and T. P. S. Powell, *Proc. R. Soc. London* **203**, 39 (1978).
4. W. W. Stewart, *Cell* **14**, 741 (1978).
5. M. V. L. Bennett, M. E. Spira, D. C. Spray, *Dev. Biol.* **65**, 114 (1978).
6. C. Yamamoto, *Exp. Brain Res.* **14**, 423 (1972); P. A. Schwartzkroin, *Brain Res.* **85**, 423 (1975). The standard perfusion solution contained NaCl, 124 m*M*; KCl, 5 m*M*; NaH_2PO_4, 1.25 m*M*; $MgSO_4$, 2 m*M*; $CaCl_2$, 2 m*M*; $NaHCO_3$, 26 m*M*; and dextrose, 10 m*M*.
7. Lucifer Yellow CH was provided by W. W. Stewart.
8. Lucifer injection was attempted in neurons that had membrane potentials greater than 50 mV. With use of a bridge circuit, dye was subjected to electrophoresis by injection of 1- to 2-nA, 750-msec negative current pulses at 1 Hz for 5 to 10 minutes. Because of the high resistance of the Lucifer electrodes, they could not be used for reliable electrophysiological measurements. After the injection period, the slice was left in the chamber for 5 minutes to 2 hours and was then removed and fixed in 4 percent buffered Formalin. The tissue was dehydrated in alcohols, cleared in xylene, whole-mounted, and examined and photographed with an epifluorescence microscope and appropriate filters. The tissue was then counterstained with cresyl violet to verify the laminar distribution of dye-filled neurons. Neurons whose resting membrane potentials had fallen below 35 mV by the end of the injection period did not stain.
9. Under the microscope, the three-dimensional character of the columnar-shaped dye-coupled aggregates was evident when the plane of focus was shifted back and forth. Also, examples of this dye-coupling pattern were obtained in slices cut in the sagittal as well as the coronal plane.
10. B. A. MacVicar and F. E. Dudek, *Brain Res.* **196**, 494 (1980).
11. U. Kuhnt, M. J. Kelly, R. Schaumberg, *Exp. Brain Res.* **35**, 371 (1979).
12. W. R. Lowenstein and T. Kanno, *J. Cell Biol.* **22**, 565 (1964); G. D. Pappas and M. V. L. Bennett, *Ann. N.Y. Acad. Sci.* **137**, 495 (1966); A. Kaneko, *J. Physiol. (London)* **213**, 95 (1971); M. V. L. Bennett, in *Intracellular Staining Techniques in Neurobiology*, S. D. Kater and C. Nicholson, Eds. (Elsevier, New York, 1973); I. Imanga, *J. Membr. Biol.* **16**, 381 (1974); W. R. Lowenstein, *Cold Spring Harbor Symp. Quant. Biol.* **40**, 49 (1976).
13. For references, see: M. V. L. Bennett, in *Handbook of Physiology*, E. R. Kandel, Ed. (Williams & Wilkins, Baltimore, 1977), vol. 1, section 1, pp. 357-416; E. Korn and D. S. Faber, in *The Neurosciences: Fourth Study Program*, F. O. Schmitt and F. G. Worden, Eds. (MIT Press, Cambridge, Mass. 1979).
14. Extracellular stimulation was applied with bipolar electrodes consisting of side-by-side electrolytically sharpened and insulated tungsten wires with an intertip distance of about 100 μm. Constant current stimuli were 25 to 250 μA and 50 to 250 μsec.
15. U. Meiri and R. Rahamimoff, *Science* **176**, 308 (1972).
16. Antidromic latencies corresponded to axonal conduction velocities as slow as 0.5 m/sec, which is well within the range reported for axons in the mammalian central nervous system [S. G. Waxman and H. A. Swadlow, *Prog. Neurobiol. (Oxford)* **8**, 297 (1977)].
17. R. Werman and P. Carlen, *Brain Res.* **112**, 395 (1976).
18. The presence of gap junctions in primate somatosensory cortex has been described (*3*). However, dye coupling after intraneuronal injection of Lucifer Yellow was not reported in a recent study on cat cerebral cortex [M. Takato and S. Goldring, *J. Comp. Neurol.* **186**, 173 (1979)]. This might reflect differences between the in vivo and the in vitro preparations, or a true species difference. Alternatively, it may reflect the tendency to select for deeper neurons in the in vivo intracellular studies.
19. For references, see V. Mountcastle, in *The Mindful Brain*, G. M. Edelman and V. Mountcastle Eds. (MIT Press, Cambridge, Mass. 1978), pp. 7-50.
20. M. S. Berry and V. W. Penroth, *Comp. Biochem. Physiol.* **57A**, 289 (1977).
21. Supported by grants NS 06477 and NS 12151 from the National Institute of Neurological and Communicative Disorders and Stroke, and by the Morris Research Fund. M.J.G. is on leave from the Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel. We thank U. J. McMahan and B. Wallace for critically reviewing the manuscript.

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Identification of Tissue-Specific Nuclear Antigens Transferred to Nitrocellulose from Polyacrylamide Gels

Abstract. *Nonhistone protein antigens resolved by electrophoresis in sodium dodecyl sulfate were identified immunochemically after being transferred to nitrocellulose. Use of antiserum to dehistonized chromatin from Novikoff hepatoma revealed numerous protein antigens specific to the chromatin of Novikoff hepatoma in comparison to that of normal rat liver.*

Nonhistone protein antigens specific for cell types, both normal and transformed, have been repeatedly detected with the use of antiserums to dehistonized chromatins from their respective sources (*1-3*). The presence of such specific antigens is consistent with a role for some of the chromosomal proteins in cellular differentiation. Procedures for the identification, isolation, and characterization of the proteins responsible for the immunological specificity are facilitated by combining the sensitivity and specificity of immunochemistry with the resolution capability of protein electrophoresis in polyacrylamide gels. Recently Towbin *et al.* (*4*) reported methods for transfer of proteins by electrophoresis from polyacrylamide gels to nitrocellulose sheets. By combining this approach with a peroxidase antiperoxidase (PAP) antigen localization technique (*5*), we can now rapidly visualize those nonhistone proteins exhibiting antigenic activity.

The methods for preparation of chromatins (*6*) and antiserum to dehistonized chromatin (*1*) have been described. The materials include Novikoff ascites hepatoma (transplanted in 150- to 200-g male Sprague-Dawley rats) and normal rat liver (excised from healthy 150- to 200-g male Sprague-Dawley rats). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method (*7*) was used to separate chromatin proteins without extraction or enzymatic digestion of the DNA. Chromatin samples in deionized water and 0.1 m*M* phenylmethyl sulfonyl fluoride, at a concentration not exceeding 1 mg/ml in DNA as determined by absorbance at 260 nm, were treated with a Sonifier cell disruptor (model W185) at 70 W eight times for 10 seconds each time, with the samples being cooled on ice for 20 seconds between sonications. The samples were mixed with 0.9 volume of a solution containing 0.139*M* tris-HCl (*p*H 6.8), 4.44 percent SDS (Bio-Rad), 22.2 percent glycerol, and Pyronine Y at 25 μg/ml and with 0.1 volume of 2-mercaptoethanol; they were then subjected to sonication without cooling for 90 seconds and heating in boiling water for 2 minutes. The samples were applied to

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7.5 percent polyacrylamide slab gels (0.75 mm thick) and subjected to electrophoresis (Bio-Rad model 220) at a constant current of 5 mA per gel until the samples were through the stacking gel (3 percent polyacrylamide, 1 cm long) and then at 10 mA per gel.

After their separation (Fig. 1A), the proteins were transferred to nitrocellulose sheets as described for SDS gels (*4*). As can be seen in the amido black-stained (*8*) nitrocellulose sheet in Fig. 1B, the transferred proteins were representative of those in the polyacrylamide gel seen in Fig. 1A. Although Towbin *et al.* (*4*) had used peroxidase-, fluorescein-, or isotopically labeled second antibodies to detect antigens, we adapted the PAP method (*5*) to this purpose. The nitrocellulose sheets onto which the proteins had been transferred were saturated with protein by incubating for 1 hour at 40°C in 10 m*M* phosphate-buffered saline (PBS), *p*H 7.2, containing 3 percent bovine serum albumin and 10 percent calf serum that had been heat-inactivated. The same buffer was used to make all antiserum dilutions. The protein-saturated sheets were incubated for 1 hour at room temperature with dilutions of rabbit antiserum to dehistonized chromatin from Novikoff hepatoma and then washed for a total of 30 minutes with five successive 100-ml portions of PBS. The nitrocellulose sheets were next incubated for 30 minutes with antiserum to rabbit immunoglobulin G (Janus Laboratories) diluted 1:40 and then washed as above. The final incubations were for 20 minutes in rabbit PAP (Miles Biochemicals) diluted 1:200. After the sheets were again washed as above, antigenic bands were stained in 50 m*M* tris-HCl (*p*H 7.5), 3,3′-diaminobenzidine (0.3 mg/ml), and 0.005 percent hydrogen peroxide.

The first two lanes of Fig. 1C show the localization of the antigenic protein bands in Novikoff hepatoma and in normal rat liver chromatins with antiserum to dehistonized chromatin from Novikoff hepatoma; because the antiserum was to Novikoff hepatoma chromatin, all antigenic activity in liver is necessarily a subset of that in Novikoff hepatoma. Even though many antigens appear to be common to both Novikoff and liver chromatins, a few bands, primarily those migrating with molecular weights of less than 94,000, are specific to Novikoff chromatin.

The second two lanes of Fig. 1C show the localization of antigenic proteins when the above antiserum is adsorbed with chromatin from normal liver. Numerous antigens show determinants that are specific to the proteins of Novikoff chromatin in comparison with the proteins of liver chromatin. However, an intense band in the Novikoff chromatin, migrating with an apparent molecular weight of about 80,000, does not localize after adsorption of the antiserum. This suggests either that the protein is common to the two tissues but in greatly differing quantities or that the antigenic determinant is present on another protein in the liver chromatin. Many bands (particularly those migrating with apparent molecular weights between 100,000 and 200,000) appeared to be antigenically common to both Novikoff and liver chromatins before adsorption (Fig. 1C, lanes 1 and 2), but after being adsorbed with liver chromatin (Fig. 1C, lanes 3 and 4), they showed activities specific to Novikoff. This suggests that many common nonhistone proteins (thus possessing potential determinants common to several tissues) may be differentially modified between tissues to produce antigenic determinants that are tissue-specific.

We have been able to identify nonhistone protein antigens separated by SDS-PAGE and to determine those that are specific for cell type by electrophoretically transferring them to, and immunochemically localizing them on, nitrocellulose sheets. Although immunoelectrophoresis can be used to separate and identify many protein antigens, it cannot be used to identify antigens that form nonprecipitating complexes with antibodies. Since such complexes have often been observed with antiserums to nonhistone proteins (*3*), our methods for identifying antigens, including those that are nonprecipitating, are of great value.

Because these methods allow direct correlation of antigenic activities to apparent protein molecular weights, information useful in the purification of antigens is immediately obtained. They also provide a rapid and sensitive means of monitoring the fractionation of numerous antigens simultaneously. Other electrophoretic techniques (such as isoelectric focusing and two-dimensional separation), when used with the transfer and localization methods, should contribute additional information on the properties and specificities of nonhistone protein antigens.

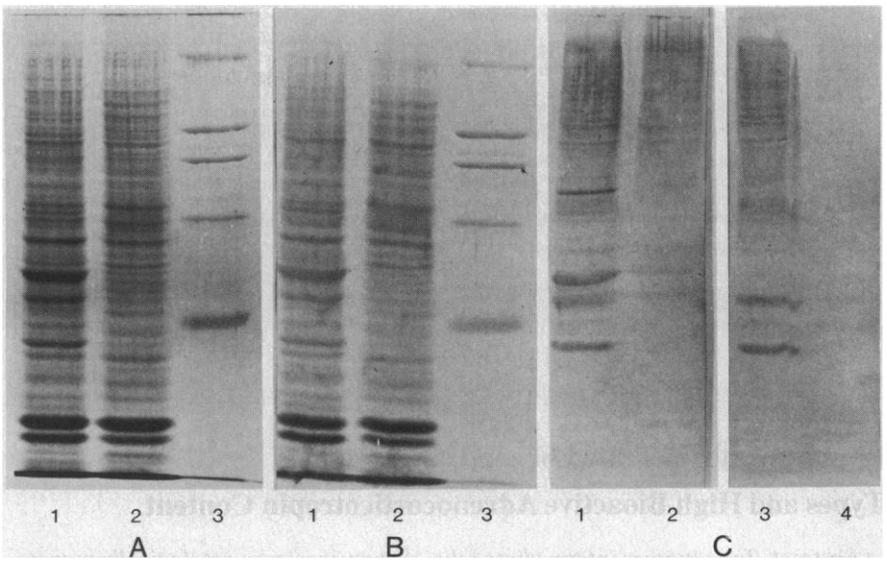

Fig. 1. (A) Electrophoretic separation in SDS of (lane 1) proteins from Novikoff ascites hepatoma chromatin (25 μg as DNA), (lane 2) proteins from normal rat liver chromatin (25 μg as DNA), and (lane 3) molecular weight standards (Bio-Rad) myosin (200,000), β-galactosidase (116,500), phosphorylase B (94,000), bovine serum albumin (68,000), and ovalbumin (43,000). (B) Samples duplicating those in (A), after electrophoretic transfer from a polyacrylamide gel to a nitrocellulose sheet. The two heavily stained bands migrating near the dye front are H1 histones. (C) Immunochemical localization reaction of electrophoretically separated and transferred proteins from (lane 1) Novikoff ascites hepatoma chromatin (25 μg as DNA) with antiserum to Novikoff dehistonized chromatin diluted 1:200, (lane 2) normal rat liver chromatin (25 μg as DNA) with antiserum as in lane 1, (lane 3) Novikoff ascites hepatoma chromatin (25 μg as DNA) with antiserum as in lane 1 adsorbed with normal rat liver chromatin (200 μg/ml as DNA), and (lane 4) normal rat liver chromatin (25 μg as DNA) with antiserum as in lane 3.

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References and Notes

1. F. Chytil and T. C. Spelsberg, *Nature (London) New Biol.* **233**, 215 (1971).
2. L. S. Hnilica, J. F. Chiu, K. Hardy, H. Fujitani, R. Briggs, *The Cell Nucleus*, H. Busch, Ed. (Academic Press, New York, 1978), vol. 5, p. 307; L. S. Hnilica and R. C. Briggs, in *Cancer Markers*, S. Sell, Ed. (Humana, Clifton, N.J., 1980), p. 463.
3. R. C. Briggs, W. M. Krajewska, L. S. Hnilica, G. Lincoln, J. Stein, G. Stein, in *Nuclear-Cytoplasmic Interactions in the Cell Cycle*, G. L. Whitson, Ed. (Academic Press, New York, in press).
4. H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979).
5. L. A. Sternberger, *Immunochemistry* (Prentice-Hall, New York, 1974), pp. 129-171.
6. T. C. Spelsberg, L. S. Hnilica, A. T. Ansevin, *Biochim. Biophys. Acta* **228**, 550 (1971). Chromatins were prepared from purified nuclei [J. S. Wilhelm, A. T. Ansevin, A. W. Johnson, L. S. Hnilica, *ibid.* **272**, 220 (1972); G. Blobel and V. R. Potter, *Science* **154**, 1662 (1966)] as described, except that after the last extraction with 0.08*M* NaCl and 0.02*M* EDTA, the chromatins were washed once with 1.4 m*M* NaCl and 0.14 m*M* sodium citrate, *p*H 7.0, and centrifuged at 31,000*g* for 20 minutes. The chromatins were hydrated as described in the text.
7. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
8. W. Schaffner and C. Weissmann, *Anal. Biochem.* **56**, 502 (1973).
9. Supported by PHS training grant HD-07043-04 (W.F.G.) and grants CA-26412 and CA-26948 awarded by the National Cancer Institute.

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Pituitary Intermediate Lobe in Dog: Two Cell Types and High Bioactive Adrenocorticotropin Content

Abstract. *The pituitary intermediate lobe of most species is cytologically monotonous, but that of the dog is composed of two immunocytochemically distinct cell types. The predominant* A *cells are typical pars intermedia cells: they stain immunocytochemically for α-melanotropin and, more weakly, for adrenocorticotropin and β-lipotropin. The* B *cells are like the corticotrophs of the anterior lobe: they stain intensely for adrenocorticotropin and β-lipotropin but not for α-melanotropin. The* B *cells may account for the high concentration of bioactive adrenocorticotropin measured in the canine pars intermedia, and may explain why in dogs adenomas causing Cushing's disease through hypersecretion of adrenocorticotropin can arise from the intermediate as well as the anterior pituitary lobe.*

Recent studies have greatly clarified the biogenesis of adrenocorticotropin (ACTH). It is now evident that ACTH and β-lipotropin (β-LPH) are cleaved from a common precursor glycoprotein molecule (variously termed pro-ACTH/endorphin, pro-opiocortin, pro-corticomelanotropin, and pro-corticolipotropin) (*1*). In the corticotrophs of the hypophysial pars distalis (PD) the prohormone is predominantly processed to authentic ACTH [ACTH(1-39)], β-LPH, and variable amounts of γ-LPH and β-endorphin (*2*). In the pars intermedia (PI) of species thus far studied (rat, cow, and pig), ACTH(1-39) is *N*-acetylated and then cleaved into an NH_2-terminal tridecapeptide that, upon subsequent *C*-amidation, yields α-melanotropin (α-MSH); concomitantly, ACTH(18-39) (corticotropin-like intermediate lobe peptide, CLIP) is formed from the COOH-terminal portion of ACTH(1-39). β-LPH is also further processed in the PI to β-endorphin (the 31 COOH-terminal amino acids of β-LPH) and its metabolites (*3*). In the PI, therefore, ACTH and β-LPH appear to serve as intermediates in the biosynthetic pathway to α-MSH and β-endorphin, respectively. Since biological activity of ACTH, in a potency comparable to that of ACTH(1-39), minimally requires the sequence ACTH(1-18), it is not surprising that little bioactive ACTH has been found in the PI of rats (*4*, *5*). Inability of the rat PI to maintain the structure and function of the adrenal cortex after removal of the PD also indicates that the PI does not secrete physiologically significant amounts of bioactive ACTH (*6*).

It is of interest that Cushing's disease, that is, hypercortisolism due to excessive secretion of ACTH, can occur in dogs in association with tumors of the PI as well as the PD (*7*). Among 11 dogs with Cushing's disease associated with pituitary adenomas that we have thus far examined, eight had tumors of the PD and three of the PI. The fact that tumors secreting bioactive ACTH can arise from the canine PI suggested to us that the PI of this species may fundamentally differ from that of others such as the rat. We have therefore studied the canine PI (i) by immunocytochemistry (ICC) for ACTH and other derivatives of the prohormone molecule, (ii) by bioassay for ACTH, and (iii) by radioimmunoassay (RIA) for ACTH combined with physicochemical characterization of the reactive molecular species.

The dog hypophyses used were from 18 adult male and female German shepherds and Labrador retrievers, five of which were studied by ICC. For ICC comparison pituitaries were obtained from five rats, five mice, four cats, six rhesus monkeys, and nine human beings who had died from nonendocrine diseases. The antibodies used were the following. (i) Antibody to midportion ACTH (West) (supplied by the NIAMDD Hormone Distribution Program), which reacts with ACTH(1-39) and ACTH(11-24) on an equimolar basis, but not with α-MSH, β-MSH, ACTH(1-10), or ACTH(17-39). (ii) Antibody to human β-LPH whose antigenic determinant resides in the NH_2-terminal(1-36) sequence and which reacts with β-LPH and γ-LPH on an equimolar basis (*8*); although this antibody shows only partial cross-reaction with dog β-LPH, its cross-reactivity is sufficient for ICC. (iii) Antibody to α-MSH (supplied by H. Vaudry) that cross-reacts, when tested by RIA, less than 0.2 percent with human and porcine (p) ACTH(1-39), ACTH(1-10), and ACTH(1-16)-NH_2, and not at all with β-LPH or β-, γ-, or α-endorphin (*5*).

By ICC we found that in dogs, as in other species, the PD corticotrophs stained with the antibody to ACTH, and a very few of them stained with the antibody to α-MSH. In the canine PI we found two distinct cell types (Table 1): *A* cells, which comprised more than 90 percent of the cell population, and *B* cells,

Table 1. Immunostaining of the dog hypophysis for ACTH and its biosynthetic congeners. Glands were fixed in Bouin's fluid, embedded in paraffin, and sectioned at 5 μm. Pituitary peptides were demonstrated with the unlabeled antibody-peroxidase-antiperoxidase technique (*9*). The antibody dilutions used were 1:9000 for antibody to ACTH, 1:3000 for antibody to β-LPH, and 1:1000 for antibody to α-MSH. The antibodies were applied to the sections for 24 hours at 4°C, and the sites of antigen-antibody interaction demonstrated with 3,3'-diaminobenzidine (30 mg/ml) in the presence of 0.05 percent hydrogen peroxide as the chromogen. In control experiments we substituted normal rabbit serum for the immune serums, and absorbed the antibodies with the appropriate antigens (1 μg/ml) for 48 hours before applying them to the slides. Only staining abolished by such incubation of the antibody with the antigen was considered specific. The number of plus signs indicates the intensity of staining.

Cell type	ACTH	β-LPH*	α-MSH
Pars distalis corticotrophs	+++	+++	−−
Pars intermedia			
A cells	+	+	++
B cells	+++	+++	−−

*We have confirmed, in all species studied, that cells staining for ACTH also stain for β-LPH (*10*). No component of the dog hypophysis stained, at dilutions of 1:9000 and 1:3000, with an antibody against human β-endorphin that stained the PI of rats and the PD corticotrophs of cats reliably, PD corticotrophs of rats and mice erratically, and those of human beings not at all.

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