References and Notes

1. M. Nei, *Genetics* **68**, s47 (1971).
2. ———, in *Genetic Distance*, J. F. Crow, Ed. (Plenum, New York, in press); *Amer. Natur.* **106**, 283 (1972).
3. At the nucleotide level, there must be at least one codon difference between any two alleles (nucleotide sequences) at a locus. Thus, $d_X = 1 - J_X$ is a minimum estimate of the expected number of codon differences between two randomly chosen genes from population X, while D_X is the average value of d_X over all loci (genomes). Similarly, D_Y and D_{XY} are minimum estimates of the codon differences per locus. Therefore, $D = D_{XY} - (D_X + D_Y)/2$ may be regarded as a minimum estimate of net codon differences between X and Y when intrapopulational codon differences are subtracted. Note that in a randomly mating population D_X is equal to average heterozygosity.
4. If the individual codon changes are independent and follow a Poisson distribution, the mean number of net codon differences (D) is given by using $D_{XY} = -\log_e J_{XY}$, $D_X = -\log_e J_X$, and $D_Y = -\log_e J_Y$, where J_{XY}, J_X, and J_Y are the arithmetic means of j_{XY}, j_X, and j_Y, respectively. If the rate of codon changes varies from locus to locus, the geometric means of j_{XY}, j_X, and j_Y are better than the arithmetic means because the quantities being considered are the logarithms of these values. The geometric means always give a larger estimate of D than the value obtained from the arithmetic means. However, under certain circumstances the geometric means give an overestimate of D [see (*2*)].
5. The protein loci used are as follows: acid phosphatase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucomutase (PGM1, PGM2, PGM3), adenylate kinase, peptidase (A, B, C, and D)* [see (*6*)], NADH diaphorase, adenosine deaminase, glutamic-pyruvic transaminase, mitochondrial glutamic oxaloacetic transaminase*, soluble glutamic oxaloacetic transaminase, lactate dehydrogenase*, phosphoglycerate kinase, catalase, carbonic anhydrase*, malic dehydrogenase, hexokinase, citrate dehydrogenase, nucleoside phosphorylase, red-cell oxidase, red-cell pyrophosphatase*, pyruvate kinase*, triosephosphate isomerase*, phosphohexose isomerase, placental acid phosphatase*, placental alkaline phosphatase, urinary amylase*, hemoglobin (α and β), haptoglobin (α and β), transferrin, Gc-globin, α_1-acid glycoprotein, α_1-antitrypsin, ceruloplasmin*, cholinesterase (locus 2)*, albumin Naskapi*, and the third component of complement (C'3)*. For the gene frequency data and the sources, see (*11*).
6. Gene frequency data for the protein loci with asterisks in (*5*) were not available for the Japanese population.
7. A locus is defined as polymorphic if the frequency of the second most common allele is equal to or larger than 0.01.
8. H. Harris, *Brit. Med. Bull.* **25**, 5 (1969).
9. M. Kimura and T. Ohta, *Nature* **229**, 467 (1971).
10. T. E. Reed, *Science* **165**, 762 (1969).
11. A. K. Roychoudhury and M. Nei, in preparation.
12. L. L. Cavalli-Sforza, *Proc. Int. Congr. Genet. 12th* **3**, 405 (1969); in *Disadvantaged Child*, J. Hellmuth, Ed. (Brunner/Mazel, New York, 1970), vol. 3, p. 111.
13. M. Nei, *Amer. Natur.* **105**, 385 (1971).

14. We thank Drs. C. Stern, J. V. Neel, and P. L. Workman for their comments on an earlier draft of this report. Supported by PHS grant GM-17719 and NSF grant GB-21224.

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Adenosine 3′,5′-Monophosphate Is Localized in Cerebellar Neurons: Immunofluorescence Evidence

Abstract. *Adenosine 3′,5′-monophosphate is localized in specific cerebellar neurons, as shown by fluorescence immunocytochemistry with a specific rabbit immunoglobulin. Positive staining is exhibited by Purkinje neurons and granule cells. The increase in concentration of cyclic adenosine monophosphate in the cerebellum, which is known to follow decapitation, is represented by greatly increased fluorescence of Purkinje neurons only. These immunofluorescence data provide the first evidence for localization of cyclic adenosine monophosphate in specific neurons and may permit further exploration into the role of this cyclic nucleotide in neuronal function.*

An involvement of adenosine 3′,5′-monophosphate (cyclic AMP) in brain function is inferred from biochemical studies on brain slices (*1*) or homogenates (*2*); these studies show that central neurotransmitters, particularly norepinephrine (*1, 3*), can regulate the activity of adenylate cyclase (*3*). However, biochemical analysis of brain has not yet demonstrated intercellular or intracellular differences in cyclic AMP content among various types of neurons and glia. We report that localization of cyclic AMP within specific central neurons can be detected by a technique of fluorescence immunocytochemistry (*4*). Furthermore, the pattern of histochemical reactivity varies with experimental conditions that are known to produce marked alterations in the cyclic AMP content of brain extracts.

The indirect immunofluorescence technique has been described (*4*). In this technique, rabbit antiserums were prepared against the 2-*O*-succinyl derivative of cyclic AMP, which was conjugated to either human serum albumin or to keyhole limpet hemocyanin (*5*), and the immunoglobulin (Ig) G fractions of these antiserums were used. In the final step, a fluorescein isothiocyanate–conjugated goat immunoglobulin G (IgG) that was prepared against rabbit IgG (*6*) was used to stain the complex of cyclic AMP and rabbit Ig. Portions of the rabbit Ig used for the immunocytochemical staining were also used for a specific and sensitive assay for cyclic AMP in tissue extracts (*5*). Cryostat sections (10 to 14 μm thick) of unfixed rat cerebellum and brainstem, frozen in liquid nitrogen, were stained by the immunofluorescence method and examined by dark-field fluorescence microscopy. We selected rat cerebellum for this analysis because of its uniquely high endogenous concentrations of cyclic AMP (*7*).

Concentrations of cyclic AMP in the cerebellum climb rapidly after the animal is decapitated, plateau after 90 to 100 seconds, and remain elevated for at least 5 minutes; concentrations of adenosine triphosphate (ATP) fall during this period (*8, 9*). When cerebellums were frozen in liquid nitrogen 90 to 150 seconds after decapitation and cryostat sections were prepared for immunofluorescence staining of cyclic AMP, discrete cell staining of high intensity was observed only in specific cerebellar cortical neurons (Fig. 1, A and C). Approximately 80 to 90 percent of Purkinje (P) cells show positive staining under these conditions: Most reactive P cells are stained exclusively in the cytoplasm, but a few P cells are stained mainly in the nucleus or in both nucleus and cytoplasm. Positive staining is also observed in the granule cell layer (Fig. 1A); the source of reactivity in the granule cell layer is the granule cell, because the fluorescence is undiminished when mossy fibers degenerate after total unilateral cerebellar pedunculectomy.

Moderate fluorescence that is seen occasionally in the molecular layer is presumably from P cell dendrites; strongly reactive primary and secondary dendritic branches could occasionally be traced back to the perikaryon. Virtually no immunoreactivity could be seen within the cerebellar white matter (Fig. 1, A and C). The perikaryon and proximal dendritic branches of some large multipolar neurons of the pontine reticular formation and deep cerebellar nuclei also exhibited strong cytoplasmic reactivity (Fig. 1B).

Several immunological reactions establish the specificity of the indirect immunocytochemical staining and confirm that the staining observed in these neurons is attributable to their cyclic AMP content. No staining of cerebellar sections was observed when the Ig fraction of nonimmunized rabbits was used as the primary immunoreagent or when

the fluorescein-conjugated goat IgG was reacted with tissue sections that had not been exposed to rabbit Ig against cyclic AMP. The positive discrete neuronal staining patterns observed only when both immunoreagents were used could be blocked if portions of the rabbit Ig to be used for staining were first incubated with 0.1 or 1.0 m*M* cyclic AMP for 4 hours at 4°C (*10*).

When the rabbit Ig against cyclic AMP was first incubated (4 hours, 4°C) alone or with 5.0 m*M* concentrations of ATP, succinate, adenosine 5′-monophosphate, or the cyclic monophosphates of guanosine, inosine, or uridine, there was no observable effect on the cell staining (*10*). Furthermore, identical cytological patterns of positive staining were seen with antibodies prepared to cyclic AMP that was conjugated to either human serum albumin or keyhole limpet hemocyanin. This result indicates that positive staining could not be due to cross-reactivity between an unknown brain protein of rodents and antibodies to the carrier proteins.

To determine whether the pattern or the intensity of the intraneuronal staining for cyclic AMP would reveal changes associated with the state of functional activity, we examined cerebellums frozen within 30 seconds after decapitation (that is, before the onset of the major rise in the cerebellar concentration of cyclic AMP after decapitation) as well as frozen biopsies of the cerebellar cortex (*11*) taken from animals anesthetized for 30 minutes or longer with halothane (1 percent), chloral hydrate (350 mg/kg, intraperitoneally), or pentobarbital (35 mg/kg, intraperitoneally).

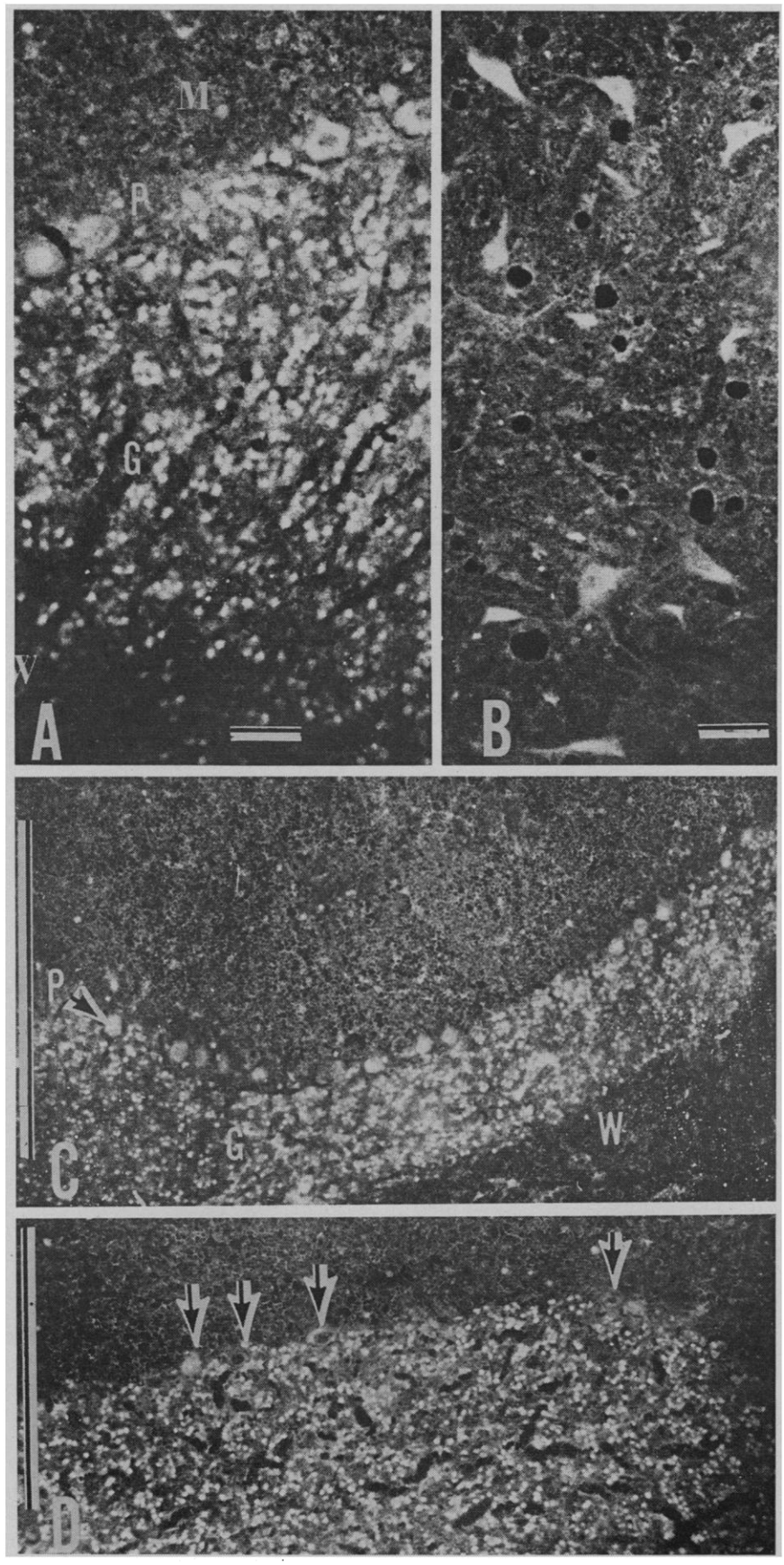

Fig. 1. Dark-field fluorescence micrographs showing the immunocytochemical staining for cyclic AMP. (A) Positive staining is seen in the nuclei of granule cells (*G*) and in the Purkinje cells (*P*) of the cerebellar cortex 150 to 180 seconds after decapitation. Low reactivity is seen in the molecular layer (*M*) and in the white matter (*W*) (calibration = 25 μm). (B) Positive immunocytochemical staining of neurons of the lateral reticular formation is seen in a specimen frozen 150 to 180 seconds after decapitation. Staining of these neurons appears to be almost entirely cytoplasmic (calibration = 25 μm). (C) A preparation similar to (A) but at low magnification shows the frequency of positive staining in Purkinje cells 150 to 180 seconds after decapitation (calibration = 250 μm). (D) This section is comparable to (C), except that the specimen was frozen within 30 seconds of decapitation. Fluorescence within the granule cell layer can still be seen, but only a small proportion of the Purkinje cells (arrows) now exhibit reactivity (calibration = 250 μm).

In cerebellar samples taken within 30 seconds after decapitation, only a few (10 to 15 percent) of the P cells exhibited positive immunofluorescence (Fig. 1D). In contrast, fluorescence in the granule cell layer was only slightly reduced in intensity and showed essentially no differences in the distribution of positively stained cellular elements. These samples showed minimal positive staining in the molecular layer or in the white matter. If the histochemical staining pattern is correlated with biochemical estimations of cyclic AMP content 30 and 120 seconds after decapitation (*8*), the previously reported rise in cyclic AMP content is manifested by increased immunocytochemical staining detectable mainly in P neurons (Fig. 1, C and D).

Cerebellar biopsies from rats anesthetized with halothane showed staining patterns essentially identical to those obtained within 30 seconds after decapitation of unanesthetized rats; moderate staining was widely distributed in the granule cell layer and within a few (10 to 20 percent) of the P cells. On the other hand, cerebellar biopsies from animals anesthetized with chloral hydrate or pentobarbital showed even less neuronal immunoreactivity than did biopsies from halothane-anesthetized rats (*12*). The reduced staining in the rats treated with chloral hydrate or pentobarbital was exhibited by both the P cells and the granule cells. These results suggest that anesthetics alter factors that regulate cyclic AMP concentrations in P cells and granule cells.

The localization of cyclic AMP within the specific neurons of the rat cerebellar cortex implies that the cyclic nucleotide is not freely diffusible within the positively stained neurons under the conditions of the staining reaction (*13*). However, no precise quantitative relation between immunocytochemical staining and direct chemical estimation of cyclic AMP content has been established, and it is not known how much cyclic AMP was lost from tissue sections during processing and whether cyclic AMP was bound extracellularly or less firmly to other cells, such as glia (*14*). Nevertheless, with the use of the immunofluorescence technique for detecting localization of cyclic AMP, the cellular distribution of this important nucleotide can now be assayed. The rise in cerebellar content of cyclic AMP after decapitation thus can be correlated with elevated concentration within the P neurons. It remains to be determined whether the alterations in immunocytochemical staining of cyclic AMP shown by P cells after decapitation can be triggered in vivo by activation of specific cerebellar afferent synaptic pathways or by specific neurotransmitters.

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References and Notes

1. S. Kakiuchi and T. W. Rall, *Mol. Pharmacol.* **4**, 367 (1968); H. Shimuzu, J. W. Daly, C. R. Creveling, *J. Neurochem.* **16**, 1609 (1969); E. C. Palmer, F. Sulser, G. A. Robison, *Pharmacologist* **11**, 157 (1969).
2. L. M. Klainer, Y.-M. Chi, S. L. Freidberg, T. W. Rall, E. W. Sutherland, *J. Biol. Chem.* **237**, 1239 (196→ B. Weiss and E. Costa, *Biochem. Pharmacol.* **17**, 2107 (1968); B. Weiss and A. D. Kidman, *Advan. Biochem. Psychopharmacol.* **1**, 131 (1969).
3. T. W. Rall, *Ann. N.Y. Acad. Sci.* **185**, 520 (1971).
4. H. J. Wedner, B. J. Hoffer, E. Battenberg, A. L. Steiner, C. W. Parker, F. E. Bloom, *J. Histochem. Cytochem.* **20**, 293 (1972).
→ A. L. Steiner, D. M. Kipnis, R. Utiger, C. W. Parker, *Proc. Nat. Acad. Sci. U.S.A.* **64**, 367 (1969); A. L. Steiner, C. W. Parker, D. M. Kipnis, *Advan. Biochem. Psychopharmacol.* **3**, 89 (1970).
6. Obtained from Miles Laboratories, item 64-173, lots 12 and 13; only this partially purified goat immunoglobulin prepared against purified rabbit IgG was found to be useful in the immunocytochemical detection of cyclic AMP.
7. B. Weiss and E. Costa, *Biochem. Pharmacol.* **17**, 2107 (1968); M. J. Schmidt, D. E. Schmidt, G. A. Robison, *Science* **173**, 1142 (1971).
8. B. Breckenridge, *Proc. Nat. Acad. Sci. U.S.A.* **52**, 1580 (1964); P. Uzunov and B. Weiss, *J. Neuropharmacol.* **10**, 697 (1971).
9. O. H. Lowry, J. V. Passonneau, F. X. Hasselberger, D. W. Schulz, *J. Biol. Chem.* **239**, 18 (1964).
10. The relatively high concentrations of cyclic AMP required to block immunocytochemical staining reflect the large binding capacity of the undiluted rabbit Ig against cyclic AMP used in the staining and blocking comparisons, and has no meaning in terms of a binding constant.
11. Cerebellar biopsies were obtained by mounting the anesthetized rats in a stereotactic apparatus. The cerebellar surface was exposed and then covered with thin slabs of warm agar (3 percent, in saline); small wedges of the outer folia were removed with a Hartman alligator forceps that had been chilled in liquid nitrogen before use. The excised sample was reimmersed into the liquid nitrogen within 5 seconds.
12. M. I. Paul, G. L. Pauk, B. R. Ditzion, *Pharmacology* **3**, 148 (1970); M. J. Schmidt, J. T. Hopkins, D. E. Schmidt, G. A. Robison, *Brain Res.*, in press. When comparisons were made between the effects of the anesthetics on the postdecapitation rise in immunocytochemical staining for cyclic AMP, six to eight stained sections from each biopsy sample were coded and examined by a double-blind protocol.
13. M. Ebadi, B. Weiss, E. Costa, *Arch. Neurol.* **24**, 353 (1971); G. N. Gill and L. D. Garren, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 786 (1971).
→ A. G. Gilman and M. Nirenberg, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 2165 (1971). Although we were unable to see neuroglia distinctly stained by the immunocytochemical procedure applied to cerebellum, we have seen such cells in unstimulated sympathetic ganglia from frog and calf (F. E. Bloom, F. F. Weight, J. Kebabian, unpublished results).
15. A.L.S. is an Established Investigator of the American Heart Association. Supported by NIH grants 1R01AM15676-01 to A.L.S. and A100219-10 to H.J.W. and C.W.P. We thank F. F. Weight, M. J. Schmidt, and A. G. Gilman for critical discussions.

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Photoreception in a Barnacle: Electrophysiology of the Shadow Reflex Pathway in Balanus cariosus

Abstract. *The photoreceptors in the median ocellus of the rock barnacle depolarize when illuminated. This depolarization spreads passively to the axon terminals in the supraesophageal ganglion. A small number of cells in the supraesophageal ganglion hyperpolarize when the median ocellus is illuminated and depolarize when it is shadowed. Nerve impulses are superimposed on the slow depolarization of the ganglion cells. Impulse activity in response to shadowing the median ocellus is recorded in a few fibers of the circumesophageal connectives. Picrotoxin blocks this shadow-induced activity. A model of the shadow reflex pathway is presented.*

The sensory pathway that mediates the shadow withdrawal reflex in the rock barnacle *Balanus cariosus* has been studied (*1*). When a shadow passes across an animal, the normal rhythmical behavior quickly stops, the cirri are withdrawn into the shell, and the opercular plates close tightly. We have never observed a response to an increase in the light intensity in these animals.

One group of the primary photoreceptors that initiate the shadow response in *B. cariosus* is in the median ocellus (*1*). The ocellus contains six to nine photoreceptors; each photoreceptor has multiple distal dendritic branches capped by microvilli and a large axon that runs in the median ocellar nerve to the supraesophageal ganglion (*2*).

A portion of the central nervous system containing the median ocellus,