

Adenosine-Containing Neurons in the Brain Localized by Immunocytochemistry

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Specific sensitive rabbit antisera directed against the adenosine derivative laevulinic acid ($O^{2,3}$ -adenosine acetal), which are capable of detecting as little as 1 pmol of adenosine by radioimmunoassay and which require more than 1000- to 40,000-fold greater concentrations of adenine nucleotides to displace adenosine binding to antisera, have been developed. These antisera were employed to localize adenosine immunoreactivity throughout the rat CNS using the peroxidase-antiperoxidase (PAP) complex and avidin-biotin-peroxidase complex (ABC) immunocytochemical techniques. Intense staining for adenosine immunoreactivity was localized to the cytoplasm of perikarya and fibers in neuronal cell groups of discrete rat brain regions. Areas containing highest levels of immunoreactivity included the pyramidal cells of the hippocampus, the granule cells of the dentate gyrus, subnuclei of the thalamus, amygdala, and hypothalamus, the primary olfactory cortex, and many motor and sensory nuclei of the brain stem and spinal cord. High levels also occurred in certain layers of the cerebral cortex, the caudate-putamen, the septal nuclei, and the Purkinje cell layer of the cerebellum. Varying the extent of tissue hypoxia altered only the levels of endogenous immunoreactive adenosine without changing the pattern of distribution of the immunoreactivity. Staining was abolished by immunoabsorption and by pretreatment of tissue sections with adenosine deaminase. The localization of adenosine to discrete neuronal groups in the brain supports the possibility of a neurotransmitter or neuromodulatory role for adenosine.

The numerous influences of the purine nucleoside adenosine on interneuronal communication within the CNS suggest a role as a neurotransmitter or neuromodulator. Adenosine exerts its effects via 2 distinct receptor subtypes coupled to adenylate cyclase: a high-affinity inhibitory A_1 receptor and a low-affinity stimulatory A_2 receptor (Cooper et al., 1980; Van Calker et al., 1979). Binding sites associated with adenosine A_1 receptors have been localized in the brain using *in vitro* autoradiographic techniques with ^3H -cyclohexyladenosine (Goodman and Snyder, 1981, 1982; Lewis et al., 1981). Lesion studies suggest that these sites are associated with axon terminals of excitatory neu-

ronal pathways (Goodman et al., 1983), which is consistent with inhibitory neurophysiological actions of adenosine that appear to involve blockade of excitatory neurotransmitter release (Fredholm and Hedqvist, 1980; Harms et al., 1979; Kocsis et al., 1984; Kostopoulos and Phillis, 1977; Kostopoulos et al., 1975; Phillis and Wu, 1981; Phillis et al., 1979).

The correlation of behavioral potencies of xanthines with an affinity for adenosine receptors, and the potent sedating effects of lipophilic adenosine derivatives suggest that the behavioral effects of xanthines arise from blockade of adenosine receptors (Barraco et al., 1983; Katims et al., 1983; Snyder et al., 1981).

Similar to classical neurotransmitters, adenosine is released from nerve terminals following depolarization, although the calcium dependence of this process is unclear (Bender et al., 1981; Dunwiddie and Fredholm, 1984; Pull and McIlwain, 1977; Stone, 1981). In addition, there exists an energy-dependent, high-affinity uptake system for adenosine, which could provide a mechanism for removal of synaptically released adenosine (Bender et al., 1980; Kuroda and McIlwain, 1974; Shimizu et al., 1972). Autoradiographic studies utilizing the potent adenosine uptake inhibitor ^3H -nitrobenzylthioinosine (Jarvis and Young, 1980; Marangos et al., 1982) have demonstrated discrete regional localization of adenosine uptake sites (Bisserbe et al., 1985; Nagy et al., 1985). Whether adenosine uptake occurs specifically into adenosine-containing neurons remains to be established. Adenosine deaminase, which is involved in the conversion of adenosine to inosine, has also been localized immunocytochemically to discrete brain regions (Nagy et al., 1984a, 1985).

Despite all the evidence suggesting a role for adenosine in the brain, it is not clear whether adenosine in the brain occurs specifically in neurons or other cell types. Previously, histochemical methods have not been available to identify adenosine in the CNS. We have utilized sensitive and specific antisera to localize adenosine to specific neuronal cell groups throughout the rat CNS using immunocytochemical techniques. Preliminary results have been described earlier (Braas et al., 1985).

Materials and Methods

Tissue preparation

Adult male Sprague-Dawley rats (150-250 gm) given ad libitum access to commercial rat chow and tapwater were maintained on a 12 hr light/12 hr dark cycle. For routine immunocytochemical studies, animals under sodium pentobarbital anesthesia were sequentially perfused intracardially with balanced salt solution, buffered fixative, and 7% sucrose in 0.15 M sodium phosphate buffer, pH 7.4. Fixatives included 4% paraformaldehyde, 2% paraformaldehyde, 4 or 2% paraformaldehyde containing 0.1, 0.2, or 0.5% glutaraldehyde, picric acid formaldehyde, 1% glutaraldehyde, 2.5% glutaraldehyde, periodate lysine paraformaldehyde (McLean and Nakane, 1974), and periodate lysine paraformaldehyde glutaraldehyde (Gendelman et al., 1983). Fixatives were pre-

Received Aug. 5, 1985; revised Dec. 16, 1985; accepted Dec. 30, 1985.

This work was supported by United States Public Health Service Grants MH-18501, DA-00266, NS-16375, Research Scientist Award DA-00074 to S.H.S., and a grant from the International Life Sciences Institute. K.M.B. is a recipient of United States Public Health Service Fellowship NS-07073. We are grateful to Dawn C. Dodson and Nancy Bruce for their efforts in the preparation of this manuscript. We also thank Victor May for his scientific support and his input into the writing of this manuscript.

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parc'd in 0.15 M sodium phosphate buffer, pH 7.4. In initial experiments, 2.5% glutaraldehyde provided the best preservation of antigenicity and tissue structure; accordingly, this fixative was used in subsequent studies. Following perfusion, the brains were rapidly removed and cut into 2–3 mm coronal slices using a Jacobowitz rat brain slicer (Jacobowitz, 1974), and placed in fresh buffered sucrose. Tissue slices were embedded either in brain paste and rapidly frozen for cryostat sectioning, or in 2% agar for vibratome sectioning.

Since cellular levels of ATP and adenosine vary greatly depending upon the physiological state of the animal, brain tissue was prepared by 3 methods to determine their effects on the immunocytochemical stain. In one group of animals, the duration of the initial intracardial perfusion wash was varied (0.0, 0.5, 1.0, and 5.0 min) prior to fixation with 2.5% glutaraldehyde. The second group of rats was killed by decapitation. Brains were cut into 1–2 mm coronal sections, fixed with buffered 2.5% glutaraldehyde, washed in buffered sucrose, and processed for cryostat sectioning. The third group of rats was killed by focused microwave irradiation (2.5–4.5 sec, 3.5 kW, 2.45 GHz; Litton Menu-master 70/50) and the brains processed for immunocytochemical staining.

Immunocytochemical stain

Vibratome (30 μ m) or cryostat (8 μ m) tissue sections were stained using a modification of the peroxidase–antiperoxidase (PAP) complex technique (Braas et al., 1983; Moriarty and Halmi, 1972; Sternberger et al., 1970) or the avidin–biotin–peroxidase complex (ABC) technique (Childs and Unabia, 1982; Hsu et al., 1981). The procedures for adenosine haptenization and the production and characterization of specific antisera have been described previously (Newby and Sala, 1982). Rabbits were immunized with laevulinic acid (*O*^{2,3}-adenosine-acetal) synthesized by the method of Seela and Cramer (1975), which was coupled to human serum albumin. The antisera from 3 animals (rabbits 1, 3, and 4), which had demonstrated binding of more than 100 pmol of adenosine/ml of serum, were screened for their immunoreactivity under staining conditions. The staining procedure was as follows: (1) pretreatment of tissue—incubation with 0.2% Triton-X 100 in 0.1 M sodium phosphate buffer, pH 7.4, for 20 min, followed by a 0.1 M sodium phosphate buffer wash; (2) blocking reaction—incubation with 1:200 normal goat serum (NGS) for 15 min, then blotting off excess; (3) primary antibody reaction—incubation in 1:100–1:50,000 primary antiserum for 48 hr at 4°C, then washing with 0.1 M phosphate buffer containing 0.05% Triton-X 100; (4) blocking reaction; (5) second antibody reaction—incubation in either 1:200 goat anti-rabbit IgG or 1:400 biotinylated goat anti-rabbit IgG in 1:200 NGS for 60 min, followed by a phosphate buffer wash containing 0.05% Triton-X 100; (6) blocking reaction; (7) label complex—incubation with either 1:100 PAP complex or 1:200 ABC in 1:200 NGS for 60 min, followed by a phosphate buffer wash; (8) peroxidase reaction—incubation with diaminobenzidine and H₂O₂ as substrates (0.3 mg/ml diaminobenzidine and 0.03% H₂O₂ in 0.05 M Tris buffer, pH 7.6) for 10 to 30 min, followed by a wash with phosphate buffer; (9) osmium tetroxide reaction—exposure to osmium tetroxide vapors for 5–10 min to enhance the reaction product. All antisera were diluted in sterile 0.05 M sodium phosphate buffer, pH 7.4, containing 2.5 mg/ml human serum albumin.

Staining controls

The specificity of the immunocytochemical stain was determined using immunoabsorption, enzymatic degradation of antigen, and method controls. Displacement of adenosine binding to the antisera by other compounds has been previously described (Newby and Sala, 1982). All control studies were performed on tissue from rats that were perfused for 0.5 min with prewash followed by buffered 2.5% glutaraldehyde.

For immunoabsorption tests, primary antiserum (1,3) was absorbed with adenosine or heterologous antigen (1 ng to 100 μ g/ml of serum) at 4°C for 24–48 hr prior to immunocytochemical staining of serial rat brain vibratome sections or cryosections.

Solid phase immunoabsorption was also performed. Antiserum (1,3), diluted in 0.15 M sodium phosphate buffer, pH 7.0, was applied to a laevulinoyladenylsepharose (10.3 μ mol adenosine/gm wet resin; 5 ml bed volume) column (Seela and Waldek, 1975), and eluted with 0.15 M sodium phosphate buffer, pH 7.0. Fractions of 1.0 ml (0.1 ml/min flow rate) were collected and assayed for specific binding according to the method of Newby and Sala (1982). Samples of representative frac-

tions were lyophilized, dissolved in staining buffer containing 1:200 NGS, and used to immunocytochemically stain serial vibratome sections.

To destroy endogenous adenosine, serial cryosections were incubated with adenosine deaminase (0.2 U/ml to 2 U/ml) for 5, 15, 30, or 60 min at 37°C prior to immunocytochemical staining. Serial control sections received enzyme vehicle only.

Brain sections were also stained using normal rabbit (preimmune) serum to replace the primary antibodies. Additional sections were stained omitting primary antibody, goat or biotinylated goat anti-rabbit IgG, or ABC or PAP complex.

Materials

Reagents were obtained from the following sources: calf intestine adenosine deaminase, Boehringer-Mannheim (Indianapolis, IN); nucleotides, nucleosides, and their bases, Sigma (St. Louis, MO); human serum albumin, Calbiochem-Behring (La Jolla, CA). All other reagents were analytical grade. Immunochemicals were obtained from the following sources: goat anti-rabbit IgG and normal rabbit serum, Kallestad (Chaska, MN); PAP complex, N. L. Cappel Laboratories (Cochranville, PA); normal goat serum, biotinylated goat anti-rabbit IgG, and ABC, Vector Laboratories (Burlingame, CA).

Results

Immunocytochemical stain for adenosine: general properties

Endogenous levels of the adenine nucleotides in the brain are 100–1000-fold higher than those of adenosine (Winn et al., 1980). It was therefore necessary to develop antisera with considerable selectivity for adenosine. The antisera employed in the present study were obtained by immunization of rabbits with the adenosine derivative laevulinic acid (*O*^{2,3}-adenosine acetal) (Seela and Cramer, 1975) coupled to human serum albumin. This haptenization of adenosine preserved both the purine and ribose rings of the native adenosine structure. The resultant antisera are both sensitive and selective, and are capable of detecting 1 pmol of adenosine by radioimmunoassay (Newby and Sala, 1982). Greater than 1000–40,000-fold higher concentrations of ATP, ADP, AMP, cAMP, inosine, hypoxanthine, and adenine are required to displace adenosine binding from the antisera, while 2'-deoxyadenosine has a similar affinity to adenosine (Newby and Sala, 1982).

In initial experiments, we detected adenosine-like immunoreactivity in discrete neuronal cell groups throughout the brain. Adenosine immunoreactivity was most prominent throughout the cytoplasm of select perikarya, while staining was absent over nuclei. Staining often extended into dendritic and axonal processes of neurons. Numerous densities of fibers and axon terminals were also observed in regions of the hindbrain. Antisera (1,3), (3,5), and (4,5) produced similar immunocytochemical staining patterns, and serum (1,3), a later bleeding of the antiserum (1,2) previously described by Newby and Sala (1982), was selected for routine use. The optimal staining dilutions for antiserum (1,3) were 1:7000 to 1:12,000.

To validate that the immunoreactivity observed in our studies was specific for adenosine itself, we conducted a number of control experiments (Table 1). In liquid phase immunoabsorption studies, staining was reduced to background levels when tissue sections were stained with anti-adenosine preabsorbed with adenosine. Staining remained intense, however, when the antiserum was preabsorbed with other adenine nucleotides and related compounds (Table 1). 2'-Deoxyadenosine also eliminated staining, while higher amounts of cAMP and adenine (100 μ g/ml serum) reduced staining intensity. These results paralleled those observed for the specificity of the antisera by radioimmunoassay.

Solid phase immunoabsorption of anti-adenosine was also performed. Antiserum was applied to a column containing laevulinic acid (*O*^{2,3}-adenosine acetal) linked to Sepharose. Bind-

Table 1. Immunocytochemical staining controls

Controls	Immunoreactivity ^a
Immunoabsorption ^b	
Adenosine	—
Laevulinic acid (<i>O</i> ^{2,3'} -adenosine acetal) ^c	—
ATP	+
ADP	+
AMP	+
cAMP	±
Guanosine	+
Inosine	+
Uridine	+
Thymidine	+
Hypoxanthine	+
2'-Deoxyadenosine	—
Adenine	±
Adenosine deaminase pretreatment of tissue section	—
Normal rabbit (preimmune) serum	—
Omission of stain component	
Anti-Adenosine	—
Goat anti-rabbit IgG	—
ABC or PAP complex	—

Primary antiserum was absorbed with the indicated antigens for 24–48 hr prior to immunocytochemical staining. An aliquot of anti-adenosine was also applied onto a laevulinoyladenylspherose column as described and the eluted fractions screened for immunoreactivity. Adult male rats were perfused with saline for 0.5 min, followed by 2.5% glutaraldehyde in 0.15 M sodium phosphate buffer, pH 7.4. Serial coronal vibratome or cryostat tissue sections were immunocytochemically stained, as described in Materials and Methods. Additional sections were treated with adenosine deaminase prior to immunocytochemical staining or were stained with normal rabbit serum. Method controls included omission of individual components of the stain. Each control experiment was performed 2–5 times, with identical results.

^a Immunoreactivity: +, unchanged; —, abolished; ±, reduced.

^b 1 ng–100 µg Antigen/ml anti-adenosine.

^c Linked to Sepharose.

ing was diminished from 4.3 pmol/ml to an undetectable level (<0.05 pmol/ml) after elution through the column. Solid phase immunoabsorption, which removes the antibodies from the serum, also eliminated the immunocytochemical stain for adenosine (Table 1). Staining was abolished when serial vibratome sections were incubated with solid phase absorbed antiserum (data not shown).

Further evidence for the authenticity of the adenosine staining was obtained by enzymatic degradation of endogenous tissue adenosine prior to the immunocytochemical stain. Increasing pretreatment times of serial cryosections with adenosine deaminase, which catalyzes the conversion of adenosine to inosine, gradually reduced the stain intensity to background levels (Table 1). Staining was also absent when normal rabbit (preimmune) serum replaced the primary antibody. Additionally, staining was abolished when the primary antibody, secondary antibody, or either the ABC or PAP complex was omitted from the staining procedure (Table 1).

Endogenous adenosine levels vary depending upon the metabolic state of the tissue, increasing dramatically in hypoxia (Berne et al., 1974; Kleihues et al., 1974; Wojcik and Neff, 1982). Conceivably, adenosine may be homogeneously distributed in the brain, but increase to detectable levels only in metabolically active neurons. One would then expect the pattern of adenosine-like immunoreactivity in various brain regions to vary depend-

Table 2. Effect of fixation protocol on immunoreactive adenosine

Protocol	Relative stain intensity ^a	
	Hippocampus	Caudate-putamen
Prefixation saline perfusion time (min)		
0.0	3.5	1–1.5
0.5	3.5–4	2–2.5
1.0	4	2.5
5.0	4+	2.5–3
Decapitation	4+	3
Microwave irradiation	3.5	1–1.5

Adult male rats were intracardially perfused with saline for the indicated times prior to fixation with buffered 2.5% glutaraldehyde. Additional animals were either decapitated or microwave-irradiated and the tissues processed for cryostat sectioning. Tissue cryosections were stained using 1:7000 anti-adenosine, as described in Materials and Methods. Each experimental protocol was repeated 2–6 times, with similar results.

^a Relative stain intensity: 4 = most intense; 0 = no stain.

ing upon the degree of tissue hypoxia. Accordingly, we conducted immunocytochemical stains for adenosine in brain tissue prepared by several methods. One group of animals was killed by microwave irradiation, which rapidly destroys enzymatic activities. The duration of the perfusion prewash prior to fixation with glutaraldehyde was varied in the second group of rats. A third group was killed by decapitation (Table 2).

The lowest intensities of adenosine-like immunoreactivity occurred in tissues from rats killed by microwave irradiation, while the highest immunoreactivities were found in tissue from animals killed by decapitation. Prolonging the duration of the perfusion prewash also increased staining intensities. However, regardless of the amount of endogenous immunoreactivities adenosine, the pattern of distribution of the immunoreactive throughout the cytoplasm of specific neuronal subpopulations in different regions of the brain showed no variation. The observed staining patterns, therefore, most likely reflected the localizations of endogenous adenosine in the intact animal.

Immunocytochemical stain for adenosine: localization

Adenosine-like immunoreactivity observed throughout rat brain was confined to neurons. Staining was most pronounced over neuronal cell bodies where immunoreactivity occurred throughout the cytoplasm, while staining was absent over nuclei (Fig. 1). Staining frequently extended into the dendritic and axonal processes of neurons. The most extensive staining of neuronal fibers and axon terminals was observed in brain stem and spinal cord.

Considerable regional variations in adenosine staining were detected (Figs. 2 and 3; Table 3). Strikingly discrete localization of adenosine-like immunoreactivity occurred in the hippocampal formation (Fig. 1, A–D). Intense stain was observed throughout the cell somas and fibers of the pyramidal cell layer (CA1–CA4) of the hippocampus and the granule cell layer of the dentate gyrus.

A high density of immunoreactivity occurred in the primary olfactory cortex, where adenosine was localized in the discrete band of large neuronal cell somas. Other regions of the cerebral cortex also contained moderate to high immunoreactivity. Staining was most intense in deeper layers, especially in the perikarya and fibers of the pyramidal cells in layer V, while no staining was observed in more superficial layers.

Moderately high levels of staining were observed in the thalamus, with variations among the thalamic subnuclei. Moderate to high adenosine immunoreactivity occurred throughout the

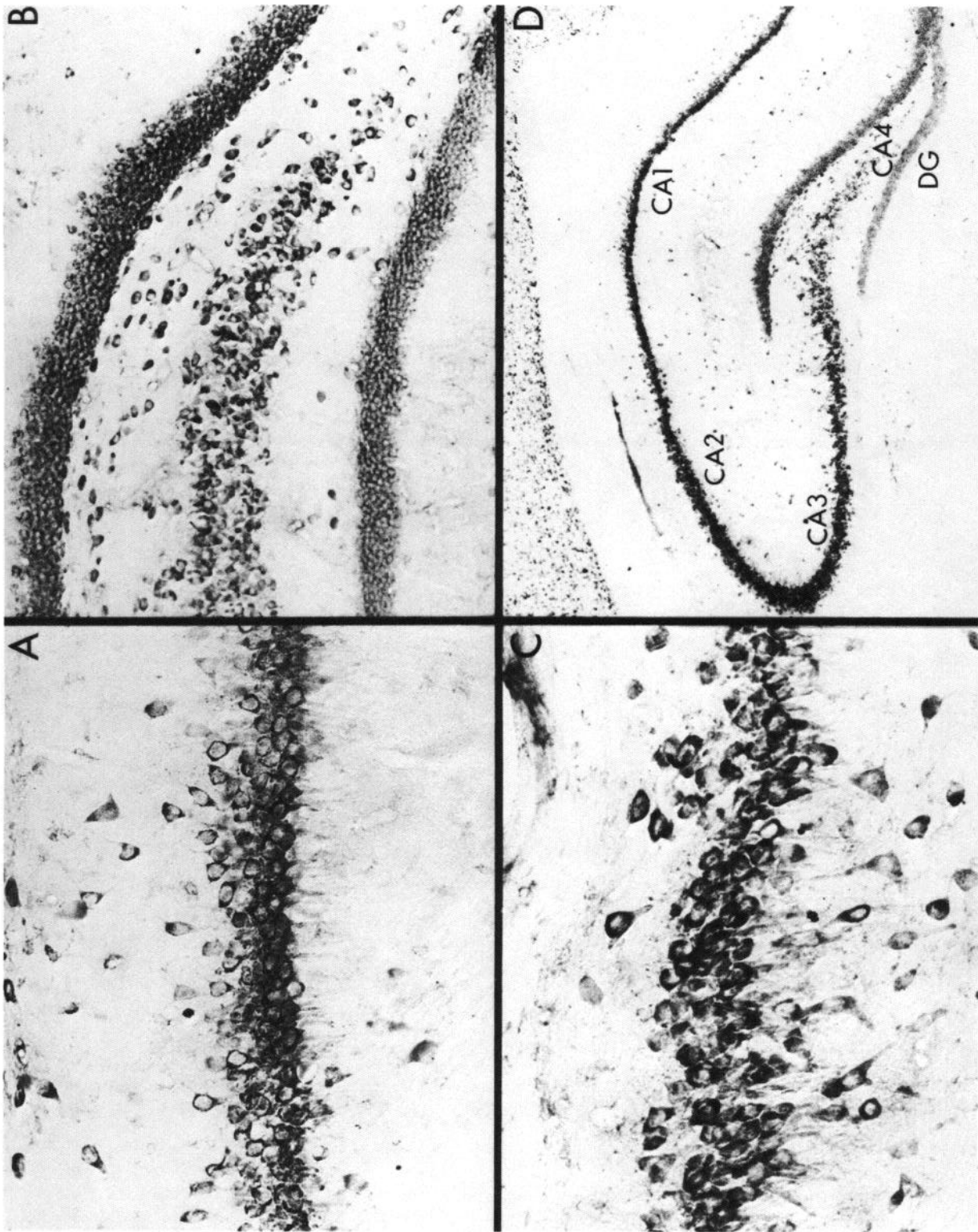


Figure 1. Immunocytochemical staining of hippocampus for adenosine. Rat brain tissue was perfusion-fixed, vibratome-sectioned, and immunocytochemically stained with 1:10,000 anti-adenosine, as described in Materials and Methods. Adenosine immunoreactivity was specifically localized to the cytoplasm of perikarya and fibers in the pyramidal cell layers of the hippocampus, including CA1 (*A, D*), CA3 (*C, D*), CA4 (*B, D*), CA1–CA4, Field CA1–CA4 of Ammon's horn; *DG*, dentate gyrus.

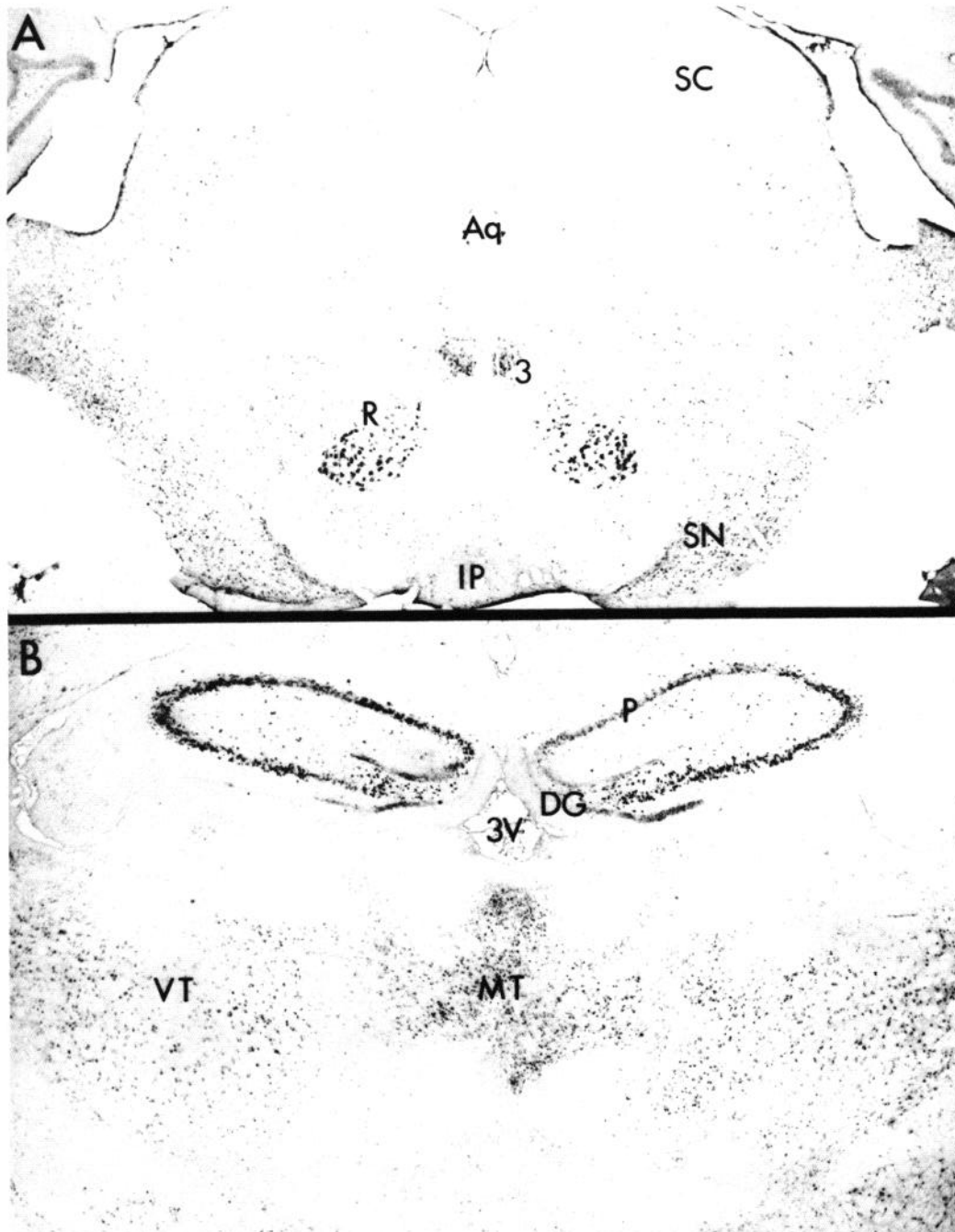


Figure 2. Staining of midbrain and forebrain regions for adenosine immunoreactivity. Vibratome tissue sections from the midbrain (*A*) and forebrain (*B*) were immunocytochemically stained using 1:7000 or 1:10,000 anti-adenosine. The red nucleus and oculomotor nucleus were prominently stained in the midbrain, with lesser staining in the substantia nigra, superior colliculus, and interpeduncular nuclei. In the forebrain, staining in the ventral and medial thalamus, as well as in the hippocampal formation, was prominent. Staining of the hippocampal formation is shown in greater detail in Figure 1. SC, Superior colliculus; 3, principal oculomotor nucleus; R, red nucleus (magnocellular); SN, substantia nigra; IP, interpeduncular nuclei; Aq, cerebral aqueduct; P, pyramidal cell layer, hippocampus; DG, dentate gyrus; MT, medial thalamus; VT, ventral thalamus; 3V, third ventricle.

cytoplasm of cell bodies and extended into the fibers of neurons in the ventral and medial nuclei, while weaker or no stain was observed in the lateral and dorsal nuclei (Fig. 2*B*). The closely adjacent hypothalamus possessed substantially less adenosine-like immunoreactivity than the thalamus. Variations in staining were observed within the hypothalamus, with highest staining intensities in the lateral and mammillary areas.

Moderate staining was found in the corpus striatum and the

amygdala. Staining in the caudate-putamen occurred in cell bodies along the superior and inferior borders, as well as in discrete patches within the body of the caudate-putamen. In the corpus striatum, the method of sacrifice strongly influenced the intensity of the staining (Table 2). Weak to no staining was observed in the globus pallidus. The amygdala possessed moderate immunoreactivity in cell bodies, with higher intensities in the basolateral nuclei and lesser intensities in the central nuclei.

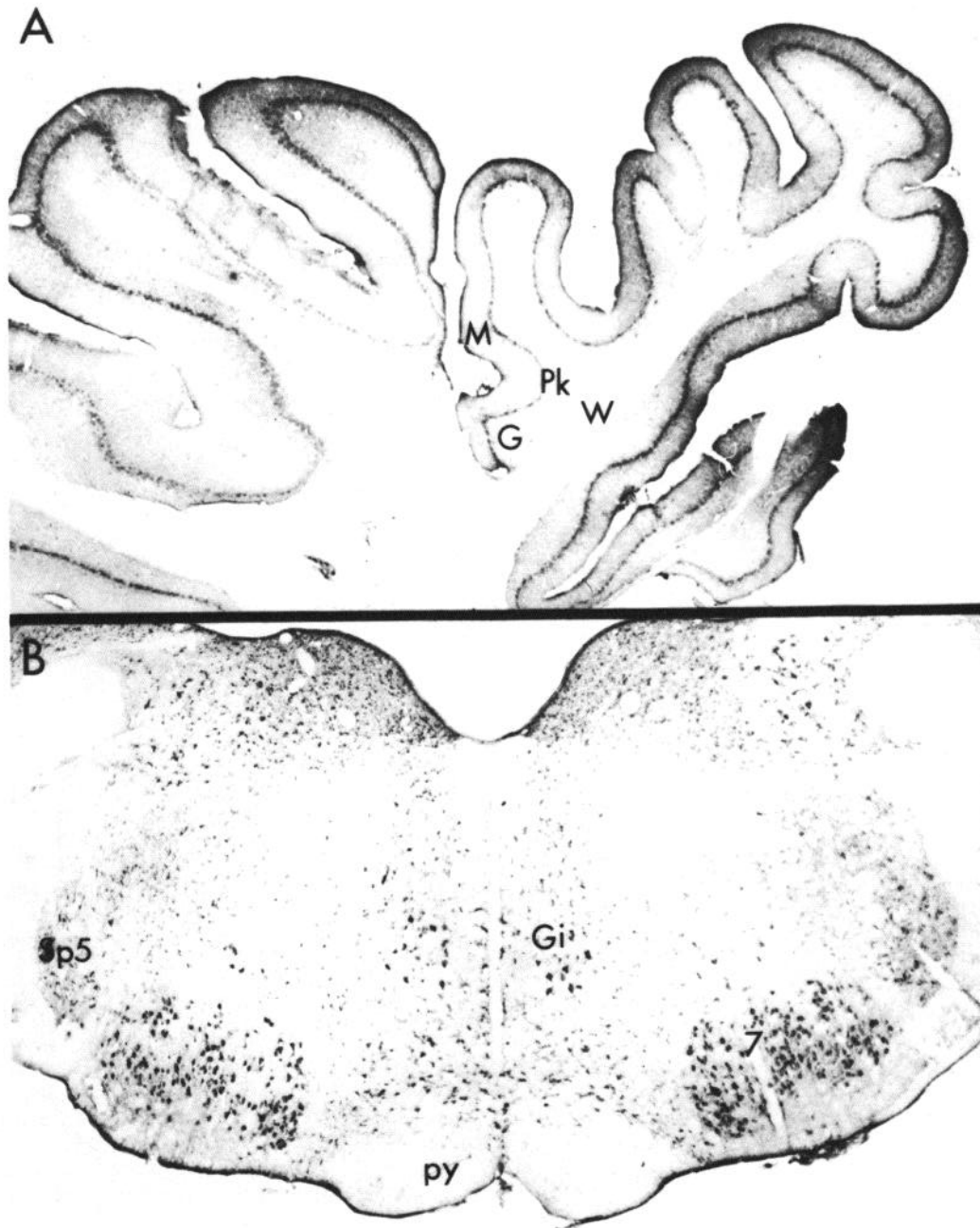


Figure 3. Immunocytochemical staining for adenosine in cerebellum and hindbrain. Vibratome sections through the cerebellum (*A*) and hindbrain (*B*) were processed for immunocytochemistry using 1:7000 or 1:10,000 anti-adenosine. The perikarya of the Purkinje cell layer of the cerebellum, as well as their dendrites extending into the molecular layer, were intensely stained. This is shown in greater detail in Figure 4*B*. No staining was observed in white matter. Adenosine immunoreactivity was found in neuronal cell bodies in discrete nuclei of the hindbrain, including the facial nucleus and gigantocellular reticular nucleus. *M*, Molecular layer; *Pk*, Purkinje layer; *G*, granular layer; *W*, white matter; *7*, facial nucleus; *Gi*, gigantocellular reticular nucleus; *Sp5*, nucleus spinal tract trigeminal nerve; *py*, pyramidal tract; *4V*, fourth ventricle.

In the brain stem, several patterns of localization occurred. Adenosine-like immunoreactivity was intensely concentrated in motor nuclei, including the red nucleus, the facial nucleus, the oculomotor nucleus, the motor nucleus of the trigeminal, and the hypoglossal nucleus (Figs. 2*A*, 3*B*, 4*C*). Moderate staining was apparent in sensory nuclei, such as the lateral vestibular nucleus (Fig. 4*D*) and the sensory nucleus of the trigeminal. The most prominent grouping of adenosine-containing fibers and terminals occurred in the spinal nucleus of the trigeminal nerve (Fig. 4*A*).

In the spinal cord, adenosine staining was similar to the patterns observed in the brain stem. The most intense immunoreactivity occurred in the motor nuclei in the ventral horn, while a band of staining was also apparent in fibers and terminals in the substantia gelatinosa.

Discrete localizations of adenosine immunoreactivity were also found in the cerebellum. Staining was highest in the Purkinje cells and in their dendrites extending into the molecular layer (Figs. 3*A*, 4*B*). Weaker staining was observed in the granular layer, while staining was absent in the white matter.

Table 3. Distribution of immunoreactive adenosine in rat brain

Brain region	Relative stain intensity ^a
Primary olfactory cortex	3.5-4
Frontoparietal cortex	
Lamina V, VI	3-3.5
Lamina IV	1.5
Lamina II, III	2.5
Lamina I	0
Cingulate cortex	3.5
Corpus callosum	0
Fornix	0
Anterior commissure	0
Caudate-putamen	2-3
Globus pallidus	0.5
Septal nuclei (medial and lateral)	3
Accumbens nucleus	2.5
Hippocampus	
Pyramidal cell layer	4
Granule cell layer of dentate gyrus	3
Amygdala	
Basolateral nucleus	3
Central nucleus	2
Medial nucleus	1.5
Lateral nucleus	2
Thalamus	
Lateral	1-2
Ventral	3.5
Medial	2.5-3.5
Anterior	0.5-2.5
Hypothalamus	
Lateral	3
Mammillary	3
Superior colliculus	2.5-3
Central grey	1.5
Principal oculomotor nucleus	3
Red nucleus	4
Interpeduncular nuclei	2.5-3
Substantia nigra	2.5-3
Motor trigeminal nucleus	4
Pontine reticular nucleus	3.5-4
Parvocellular reticular nucleus	3.5-4
Gigantocellular reticular nucleus	3.5
Nucleus mesencephalic tract trigeminal nerve	3

Discussion

The present study demonstrates the localization of adenosine-like immunoreactivity in select neurons within the rat brain. Extensive biochemical and immunocytochemical control studies have established that the staining likely involves chemically authentic adenosine. Since the immunocytochemical staining patterns were unaltered by the duration of hypoxia prior to tissue-processing, the observed localizations most probably reflect the endogenous disposition of adenosine in intact animals.

Examination of adenosine-like immunoreactivity throughout the CNS of the rat has failed to demonstrate staining in any structures other than neurons. The heterogeneous distribution of these neurons throughout the CNS resembles the disposition of neuronal pathways for most neurotransmitters (Bjorklund and Hokfelt, 1983, 1984; Bjorklund et al., 1984; Krieger et al., 1983). Interestingly, staining of neurotransmitters, especially

Table 3. Continued

Brain region	Relative stain intensity ^a
Trapezoid body, medial and lateral	3-3.5
Facial nucleus	4
Principal sensory trigeminal nucleus	3
Nucleus spinal tract trigeminal nerve	3
Vestibular nuclei	2.5-3.5
Hypoglossal nucleus	3.5
Inferior olive	2.5-3
Cerebellum	
Molecular layer	2.5
Purkinje layer	3.5
Granule layer	1.5-2
White matter	0
Spinal cord	
Substantia gelatinosa	2.5-3
Motor nuclei	4

Adult male rats were intracardially perfused for 0.5 min with prewash prior to fixation with buffered 2.5% glutaraldehyde. Vibratome or cryostat tissue sections were immunocytochemically stained using 1:7000-1:12,000 anti-adenosine, as described in Materials and Methods. Relative stain intensity in brain sections taken at 500 μ m intervals was evaluated according to coronal levels in the atlas of Paxinos and Watson (1982). Experiments were repeated 10 times, with similar results.

^a Relative stain intensity: 4 = most intense; 0 = no stain.

neuropeptides, often detects fiber systems but reveals cell bodies only upon blockade of axoplasmic transport. In contrast, adenosine is primarily localized to perikarya, while dense plexi of fibers are most apparent in the brain stem and spinal cord.

A variety of biochemical markers that may be related to adenosine has been localized at a microscopic level in specific regions of the brain. These studies have included localizations of adenosine receptors, adenosine uptake sites, and the enzymes involved in adenosine metabolism. Adenosine deaminase, which catalyzes the conversion of adenosine to inosine, has been localized by immunocytochemistry (Nagy et al., 1984a, b, 1985). Adenosine uptake sites have been localized by autoradiography with the uptake inhibitor ³H-nitrobenzylthioinosine, while adenosine A₂ receptors have been localized with ³H-cyclohexyladenosine (Bisserbe et al., 1985; Goodman and Snyder, 1981, 1982; Lewis et al., 1981; Nagy et al., 1985). 5'-Nucleotidase, an enzyme involved in the dephosphorylation of adenosine monophosphate to adenosine, has also been localized (Marani, 1977; Scott, 1964, 1967).

Adenosine deaminase has a very restricted localization. High levels of adenosine deaminase immunoreactivity appear predominantly in the tuberal, caudal, and postmammillarycaudal magnocellular neuronal cell bodies and fibers of the basal hypothalamus (Nagy et al., 1984a). Although this distribution differs from the much more widespread occurrence of adenosine-like immunoreactivity, the most intense staining of endogenous adenosine within the hypothalamus also occurs in perikarya and fibers of this region. Adenosine deaminase immunoreactivity has also been detected in cell bodies of small type B neurons in the dorsal root ganglion and in neurons in the superior colliculus (Nagy et al., 1984b, 1985). Although the dorsal root ganglion was not examined for endogenous adenosine immunoreactivity, moderate levels of staining were observed in axons and terminals in the substantia gelatinosa, where sensory neurons terminate.

While there are similarities among the localizations of the various biochemical markers, some marked differences also exist. For instance, although immunoreactive adenosine, adeno-

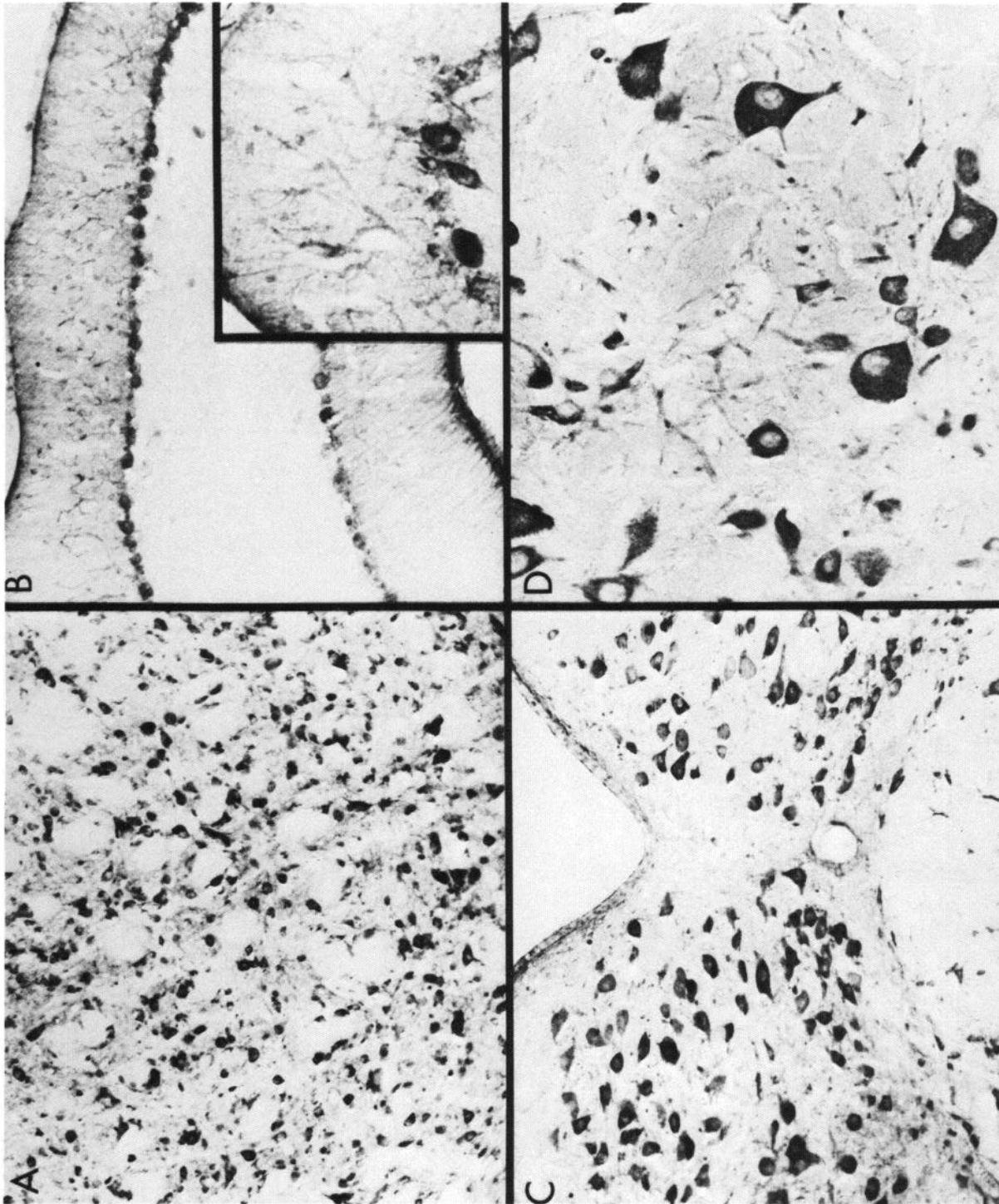


Figure 4. Immunocytochemical staining for adenosine in brain stem and cerebellum. Rat brains were processed, vibratome-sectioned, and stained with 1:10,000 anti-adenosine. Micrographs are representative of staining in neuronal cell bodies and fibers of the nucleus of the spinal tract of the trigeminal nerve (*A*), the hypoglossal nucleus (*C*), and the lateral vestibular nucleus (*B*). Staining in the cerebellum (*B*) was most pronounced in the cell soma and dendrites of Purkinje cells (*inset*).

sine uptake sites, A_1 receptors, and 5'-nucleotidase all occur in the thalamus, their relative levels differ in various thalamic subnuclei (Goodman and Snyder, 1982; Phillis and Wu, 1981; Scott, 1967). In the amygdala, immunoreactive adenosine is highest in the basolateral nucleus, which possesses the lowest levels of adenosine uptake sites and A_1 receptors. The central nucleus of the amygdala has the highest levels of A_1 receptor, but the lower levels of both adenosine immunoreactivity and uptake sites (Bisserbe et al., 1985; Goodman and Snyder, 1982). The hippocampus possesses high levels of 5'-nucleotidase, A_1 receptors, and adenosine immunoreactivity, but relatively low levels of adenosine uptake sites (Bisserbe et al., 1985; Goodman and Snyder, 1982; Scott, 1967). In the cerebellum, high levels of 5'-nucleotidase (Scott, 1964, 1967) and A_1 receptors (Goodman and Snyder, 1982; Goodman et al., 1983) are localized in the molecular layer; lower levels exist in the granule cell layer. Cerebellar 5'-nucleotidase is associated with Purkinje cell dendrites, parallel fibers, and synapses between parallel fibers and Purkinje cell spines (Marani, 1977). A_1 receptors appear to be located on axon terminals of excitatory granule cells (Goodman et al., 1983; Wojcik and Neff, 1983). In this instance, the results correspond fairly well to the localization of endogenous adenosine to Purkinje cells and their processes, which extend into the molecular layer of the cerebellum.

The marked differences in localization of various markers pose difficulties in formulating models of adenosine functions. There are many possible explanations for these discrepancies. Adenosine uptake sites and adenosine deaminase may be associated with metabolic stores of adenosine unrelated to neurotransmitter or neuromodulatory pools of adenosine. The observed staining patterns may reflect the distribution of neurons with high metabolic activity and hence relatively high levels of endogenous adenosine, rather than distribution of neuronal cells in which adenosine serves as a neurotransmitter. 5'-Nucleotidase may serve other metabolic functions aside from adenosine biosynthesis. Indeed, whether endogenous adenosine arises via this enzyme or through other pathways, such as *S*-adenosylhomocysteine hydrolase, has not been established. Most of the adenosine immunoreactivity described here is localized to neuronal cell somas. Receptor sites, on the other hand, would be expected to be in close proximity to adenosine nerve terminals that might be distant from the cell bodies. Similarly, adenosine uptake sites would be expected to be most concentrated in terminals. Finally, the localization of A_2 receptors has not been established. Whether most of the endogenous adenosine in the brain interacts with A_1 or A_2 receptors also remains unclear.

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