tively destroys Ia-bearing passenger lymphoid cells (*16*). Incubation or perfusion of graft tissue with perfluorocarbon may be a means of efficiently removing oxygen-sensitive lymphoid cells before transplantation.

RICHARD BUCALA*
MASANOBU KAWAKAMI†
ANTHONY CERAMI
Laboratory of Medical Biochemistry, Rockefeller University, New York 10021

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* To whom correspondence should be addressed.
† Present address: Department of Internal Medicine, University of Tokyo Hospital, Tokyo 113, Japan.

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Adenosine Receptors: Autoradiographic Evidence for Their Location on Axon Terminals of Excitatory Neurons

Abstract. *Adenosine receptors were made visible on light microscopy by autoradiography with tritiated cyclohexyladenosine. In the cerebellum, adenosine receptors were absent in Weaver mice, which lack granule cells, and were displaced in Reeler mice, which have displacements of granule cells. Thus, adenosine receptors appear to be located on the axon terminals of excitatory granule cells in the cerebellum. Removal of one eye of a rat depleted adenosine receptors in the contralateral superior colliculus, suggesting that the receptors occur on axon terminals of excitatory projections from retinal ganglion cells. The presence of adenosine receptors on excitatory axon terminals may explain synaptic inhibition by adenosine and the behavioral effects of xanthines.*

As a neuromodulator, adenosine inhibits the release of excitatory transmitter and also has postsynaptic effects (*1*). Behavioral stimulation by xanthines such as caffeine is linked to the blockade of adenosine receptors (*1, 2*). Several ^{3}H-labeled ligands have been used to identify these receptors (*3*). We located adenosine receptors in vitro by autoradiographic analysis (*4*) using ^{3}H-labeled cyclohexyladenosine ([^{3}H]CHA) (*5*); a similar finding was reported in a preliminary communication by others (*6*). In the present study using neurologic mutant mice as well as selective brain lesions in rats, we provide evidence for an association of adenosine receptors with cerebellar granule cells and retinal ganglion cells.

The cerebellum contains five types of neurons. Four of these (stellate, Golgi, basket, and Purkinje) are inhibitory and appear to use γ-aminobutyric acid as their neurotransmitter (*7*). The fifth type, the granule cell, is the only excitatory neuron in the cerebellum, but is the most frequently occurring cell type, accounting for about 80 percent of cerebellar neurons. The excitatory neurotransmitter of granule cells appears to be glutamic acid (*8*).

In normal rat cerebellum, the highest concentration of adenosine receptors was found in the molecular layer, with a lower concentration in the granule cell layer (*5*), observations we now confirm in mouse cerebellum. These receptors might be located on any of the distinct neuronal types.

To determine the location of cerebellar adenosine receptors, we used mutant mice with specific autosomally recessive neuronal defects. "Nervous" mice show a 90 percent loss of Purkinje cells, with other cerebellar cell types essentially normal (*9*). The overall pattern and density of [^{3}H]CHA grains in the cerebellum of nervous mice are the same as in littermate controls (data not shown). Thus, adenosine receptors in the cerebellum are not associated with Purkinje cells.

Weaver mice have an 80 percent deficiency of granule cells, with other cerebellar cell types being essentially normal (*9*). There were 70 to 80 percent fewer [^{3}H]CHA grains in the cerebellums of Weaver mice than in littermate controls (Fig. 1), as shown by grain counts.

The loss of adenosine receptors in Weaver mice indicates that the receptors are associated with granule cells. The extremely large number of adenosine receptors in the molecular layer of the normal cerebellum are thus confined to axons and terminals of parallel fibers of the granule cells in the molecular layer. We also examined Reeler mice in which the normal migration of granule cells does not occur so that granule cells and their axons remain in the external granular layer. In Reeler mice, [^{3}H]CHA grains were restricted to the external granular layer, the location of the granule cells (data not shown). This finding confirms an association of adenosine receptors with the axons and terminals of granule cells.

Ganglion cells of the retina are major excitatory neurons that project both to the lateral geniculate body and the superior colliculus. Their transmitter is not clearly established but appears to be one of the excitatory amino acids (*10*). Both the superior colliculus and the lateral geniculate have high densities of [^{3}H]CHA-labeled adenosine receptors (*5*).

To determine whether adenosine receptors might be associated with projections of retinal ganglion cells, we removed eyes unilaterally from Sprague-Dawley rats and conducted autoradiographic analysis of [^{3}H]CHA binding 21 days later (Fig. 2). Enucleation abolished [^{3}H]CHA labeling in the superior colliculus opposite the enucleation. The labeling in the lateral geniculate appeared to be normal on both sides after removal of the eye. Thus, adenosine receptors seem to be associated with excitatory projections from retinal ganglion cells to the superior colliculus. Although transynaptic effects cannot be ruled out, the similar loss of grains observed 4 and 21 days after enucleation fits a presynaptic location of the receptors.

Most ganglion cells are bifurcated, sending some processes to the superior colliculus and others to the lateral geniculate. If adenosine receptors are synthesized by these neurons, receptors would be lost from the lateral geniculate as well as from the superior colliculus. Adeno-

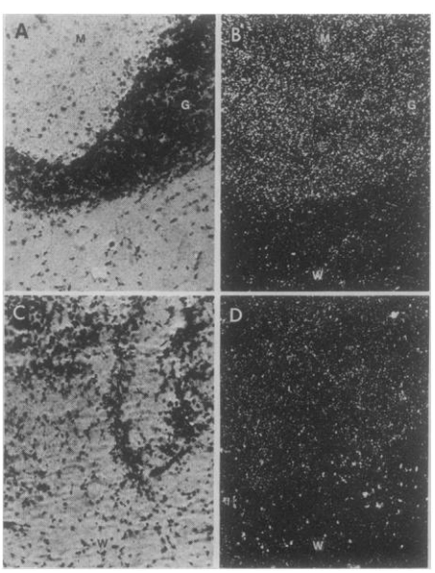
sine receptors may be transported by ganglion cell neurons down branches to the superior colliculus, but not to the lateral geniculate. Alternatively, adenosine receptors may be restricted to ganglion cells that project to the superior colliculus.

The cerebral cortex sends major excitatory efferent pathways to the thalamus and corpus striatum. We ablated one side of the cerebral cortex in some rats and conducted [^{3}H]CHA autoradiography 3 weeks after the lesions were produced. Careful examination of brain sections from these animals showed no decrease or altered pattern of [^{3}H]CHA grains in any nuclei of the thalamus or corpus striatum, in comparisons with the contralateral control side and with unlesioned rats. Thus, adenosine receptors do not appear to be located on terminals of the corticothalamic or corticostriate pathways.

The fornix contains axons of major pathways projecting from the hippocampus to the anterior thalamus and of pathways passing in the reverse direction. Three weeks after production of unilateral lesions of the fornix, we detected no abnormality in [^{3}H]CHA-labeled adenosine receptors in the hippocampus or thalamus. Lesioning both the fornix and mamillothalamic tract did not reduce the concentration of adenosine receptors in the thalamus.

Our results indicate an association of adenosine receptors with axon terminals of at least two major excitatory neuronal pathways in the central nervous system. This is not a universal feature of adenosine receptors since other prominent excitatory pathways, such as the corticothalamic and corticostriate pathways, do not appear to contain receptors associated with axon terminals.

The adenosine receptors on axon terminals may play a role in adenosine-mediated regulation of transmitter release. In biochemical studies, adenosine inhibits transmitter release (*11*); neurophysiologic inhibition by adenosine involves the blockade of excitatory transmitter release as well as some postsynaptic inhibition (*1*). The stimulatory effects of xanthines have been linked to the blockade of adenosine receptors (*2*), and caffeine-induced tremor may be associated with influences on adenosine receptors at cerebellar granule cells. The function of the adenosine receptors on retinal ganglion cells is unclear.



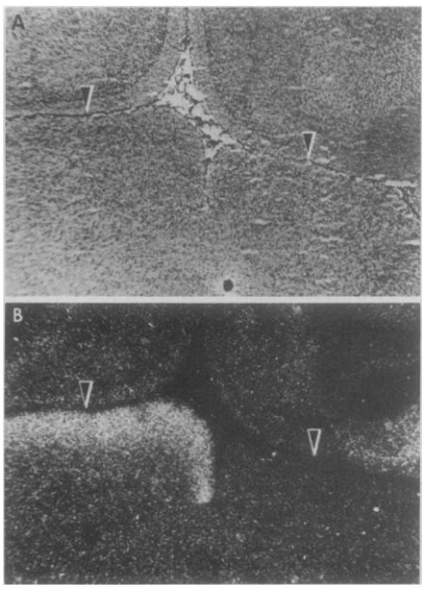


Fig. 1 (left). Loss of adenosine receptors in the cerebellum of Weaver mice. Mutant mice were purchased from the Jackson Laboratory and mated. Affected mice were identified as early as possible (at 6 to 8 days of age), and unaffected littermates were also identified and used as controls. (A) Bright-field micrograph showing a tissue section and (B) dark-field micrograph showing the autoradiographic grain pattern of the cerebellum in a Weaver mouse control. The white matter (*W*) has very few grains, whereas grain density is nearly uniform over the granule cell layer (*G*) and the molecular layer (*M*). (C) Bright field showing the tissue and (D) dark field showing the autoradiographic grain pattern in the cerebellum of an affected littermate. In (C), the remaining cells can be seen above the area of white matter. The grain density is reduced by 70 to 80 percent. The overall autoradiographic procedure used has been described (*4*). Briefly, mice were perfused with 0.1 percent formaldehyde in isotonic phosphate-buffered saline. Tissues were removed from the skull, cerebellar slices were rapidly frozen onto brass microtome chucks, and sections 8 μm thick were cut in a microtome (Harris Cryostat). Tissue sections were thaw-mounted onto subbed slides and stored at −20°C until used. Sections were incubated with 2 n*M* ^{3}H-labeled N^6-cyclohexyladenosine ([^{3}H]CHA) (11.5 Ci/mmole; New England Nuclear) for 90 minutes at room temperature with or without 5 μ*M* 1-N^6-phenylisopropyladenosine (Boehringer-Mannheim) in 170 m*M* tris-HCl buffer, *p*H 7.4. This procedure provides about 70 percent receptor occupancy (*5*). Drug displacement studies indicate that bound [^{3}H]CHA labels physiologic adenosine A_1 receptors (*5*). Before exposure with [^{3}H]CHA, all tissue sections were incubated at room temperature for 20 minutes in buffer with adenosine deaminase (Sigma), 1 IU/ml, to degrade endogenous adenosine. After incubation with [^{3}H]CHA, tissue sections were washed in buffer at 0°C (two 5-minute washes) to reduce nonspecific binding. The tissues were rapidly dried under a stream of cold, dry air. Cover slips coated with Kodak NTB-3 emulsion were apposed to the tissue sections and, after 6 to 8 weeks of exposure at 4°C, the autoradiograms were developed and the tissue was stained with Pyronine Y (*4*). For grain density quantification, autoradiograms were evaluated at ×1000 with a calibrated eye-piece grid, and mean grain counts were determined in six 6000-μm^2 areas from representative sections from each of two animals. Grain densities observed were within range of linearity with radioactivity (*12*). Fig. 2 (right). Loss of adenosine receptors in the contralateral superior colliculus after enucleation. (A) Bright-field micrograph of the superior colliculi and overlying cerebral cortex. The arrows point to the surface of the superior colliculus. (B) Dark-field micrograph of the same section. Only the autoradiographic grains are visible. The loss of binding on the right side where there is some shrinkage of tissue due to the enucleation is nearly total. One eye was removed from each of three adult male Sprague-Dawley rats 3 weeks before perfusion, sectioning, and exposure to [^{3}H]CHA (see legend to Fig. 1).

ROBERT R. GOODMAN
MICHAEL J. KUHAR
LYNDA HESTER
SOLOMON H. SNYDER*

Departments of Neuroscience, Pharmacology and Experimental Therapeutics, and *Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

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* To whom correspondence should be addressed.

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Maternal Coordination of the Fetal Biological Clock in Utero

Abstract. *Deoxyglucose labeled with carbon-14 was used in studying the utilization of glucose in the suprachiasmatic nuclei of fetal rats. The results showed that an entrainable circadian clock is present in the suprachiasmatic nuclei during fetal development and that the maternal circadian system coordinates the phase of the fetal clock to environmental lighting conditions.*

Circadian rhythmicity of biological processes is the overt expression of an endogenous timekeeping mechanism (*1*). The rhythms are normally coordinated (entrained) to the 24-hour day by periodic environmental time cues, the daily alteration in light and darkness being one of the most potent entraining stimuli. Entrainment ensures a state of internal temporal order whereby the rhythms are expressed in proper relation to each other and to the 24-hour day. This helps to optimize the economy of biological systems and prepares an organism to foresee and to cope with alterations in the environment.

In mammals, the hypothalamic suprachiasmatic nuclei (SCN) appear to function as an endogenous pacemaker (biological clock). This concept was based originally on the results of lesion studies (*2*) and has been strengthened by the findings that the metabolic and electrical activities of the SCN vary on a circadian basis (*3*, *4*). Photic information for entrainment reaches the SCN from the retina via a direct retinohypothalamic pathway (*5*).

Most work on the mammalian circadian timing system has been done with adult rodents, and little effort has been directed at elucidating the development of this system. A major reason for this deficiency is that most circadian rhythms are not overtly expressed in rodents until the third week of life (*6*), making study of the activity of an entrainable circadian oscillator before that time difficult. An ingenious approach to this problem was pioneered by Deguchi (*7*), who used the phase (timing) of an overt rhythm monitored under constant conditions during the postnatal period to infer what had happened to the central oscillator underlying the rhythm at an earlier developmental stage. With this method, he provided data in the rat to suggest that the developing biological clock might be functional and entrainable by the mother during fetal life (*7*). However, the possibility that some aspect of the birth process itself starts and sets the timing of the developing clock cannot be readily excluded by this indirect approach (*8*). Proving that the fetal circadian clock functions before birth requires a method that could measure an intrinsic, functionally relevant property of the clock itself.

An autoradiographic technique in which ^{14}C-labeled deoxyglucose is injected intravenously allows for the simultaneous determination in vivo of the rates of glucose utilization of individual brain structures (*9*). Since brain structures are dependent on a continuous supply of glucose for energy (*10*) and since brain energy utilization and functional activity are closely linked (*11*), the amount of glucose utilized by an area reflects the overall functional activity of that area. Glucose utilization has provided an effective assay for oscillatory activity of the SCN in the adult rat (*3*). We applied this strategy to study the SCN in the fetal rat and now report that the fetal nuclei manifest a clear daily rhythm of glucose utilization and that the maternal circadian system coordinates the phase of the fetal clock to environmental lighting conditions (*12*).

In our first experiment, two pregnant albino rats were subjected to daily cycles of 12 hours of light and 12 hours of darkness, with lights on from 0700 to 1900 (designated LD) beginning early in pregnancy (*13*). On day 10 of gestation, each animal was outfitted with an intraatrial Silastic catheter (*14*). On day 19 of gestation, both pregnant rats were placed in and thereafter maintained in constant darkness. While the animals were in darkness, deoxyglucose (145 μCi/kg) (Amersham; specific activity 60 Ci/mole) was rapidly injected through the venous catheter. One rat was given the injection during her subjective night at 2300 hours on day 20 of gestation, and the other was given the injection during her subjective day at 1100 hours on day 21 of gestation; the injection times were,

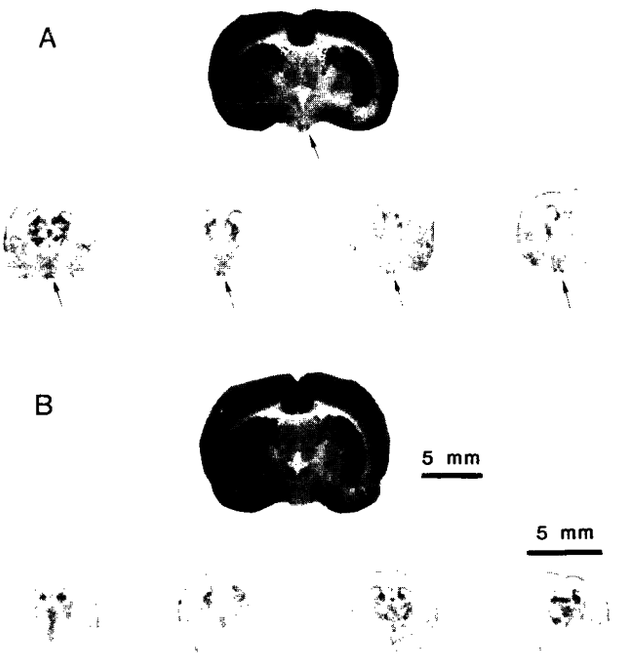

Fig. 1. Autoradiographs of coronal brain sections from mother and four of her fetuses after maternal injection during (A) subjective day and (B) subjective night. The metabolically active SCN appear as a pair of dark spots in the mother and fetuses during the subjective day (arrows), while the nuclei are relatively inactive and no longer visible during the night. The location of the SCN in the sections used to generate the autoradiographs in the lower panel was verified by cresyl violet staining.