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Radioligand Binding to Adenosine Receptors and Adenosine Uptake Sites in Different Brain Regions of Normal and Narcoleptic Dogs

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HAWKINS, M., S. O'CONNOR, M. RADULOVACKI, S. BOWERSOX, E. MIGNOT AND W. DEMENT. *Radioligand binding to adenosine receptors and adenosine uptake sites in different brain regions of normal and narcoleptic dogs.* PHARMACOL BIOCHEM BEHAV **38**(1) 1–6, 1991.—The present study compares the characteristics of radioligand binding to adenosine receptors and adenosine uptake sites in 100- and 50-day-old normal and narcoleptic dogs. Binding to A_1 receptors was quantified using a selective A_1 agonist ([^{3}H]N^6-[(R)-1-methyl-2-phenylethyl] adenosine, [^{3}H]R-PIA) and an antagonist ([^{3}H]dipropyl-8-cyclopentylxanthine, [^{3}H]CPX). Differences in the binding of [^{3}H]R-PIA and that of [^{3}H]5′-N-ethylcarboxamide adenosine ([^{3}H]NECA), which binds to both A_1 and A_2 receptors with similar affinities, were used to quantify A_2 receptors. Nucleoside transport sites were labeled with [^{3}H]nitrobenzylthioinosine ([^{3}H]NBTI), a potent inhibitor of nucleoside transport systems. The present study offered no evidence that either adenosine A_1 receptors and adenosine uptake sites in the frontal cortex or adenosine A_2 receptors in the putamen were altered in narcoleptic dogs. However, we found that adenosine A_1 receptors in the dog exist in different affinity states and that the affinity state in which the receptor is found depends on the brain region examined. A characterization of these low- and high-affinity sites was performed and results indicated that these sites cannot be explained by a single interaction of the A_1 receptor with a single G-protein population.

Canine narcolepsy Adenosine receptors G-proteins Adenosine uptake sites

A considerable amount of biochemical, physiological, pharmacological and behavioral evidence has been gathered over the past six decades suggesting that adenosine acts as an endogenous neuromodulator in the mammalian CNS (12). Iontophoretic application of adenosine has potent depressant effects on the responses of neurons in several brain structures (26), and administration of adenosine analogs to mice and rats produced marked sedation and hypothermia (11,31). In accordance, intracerebroventricular administration of adenosine (29) and intraperitoneal injections of analogs of adenosine (28) were shown to increase deep slow-wave and REM sleep in rats. Furthermore, an increase in adenosine A_1 receptor density in the rat cortex and corpus striatum, has been reported following 48 and 96 h of REM sleep deprivation (35).

In the genetically narcoleptic dog elevated levels of norepinephrine, dopamine and dopamine metabolites have been reported in discrete regions of the brain (13). Increased concentrations of dopamine D2, alpha-1 adrenergic and muscarinic M2 receptors have also been reported in these animals (8, 18, 23, 24). These findings are most likely indicative of an abnormality in some selective neurotransmitter system of the narcoleptic dog. Since adenosine inhibits the release and the turnover of many neurotransmitters in the CNS (12), we examined the potential role of adenosine receptors in canine narcolepsy.

Two types of extracellular adenosine receptors (A_1 and A_2) have been detected in many areas of the mammalian brain (6). Adenosine A_1 receptors mediate the inhibition of adenylate cyclase activity in most brain areas studied (21,34), whereas adenosine A_2 mediate the stimulation of adenylate cyclase activity only in membranes from striatum, nucleus accumbens and olfactory tubercle of the rat (27,30). In addition, high-affinity uptake sites for adenosine have also been characterized in the brain (14). This uptake may play a key role in termination of adenosine activity.

The aim of the present study was to examine the characteristics of radioligand binding to adenosine receptors and adenosine

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uptake sites in 100- and 50-day-old normal and narcoleptic dogs. These two groups of animals were selected because data from the Stanford University dog colony indicate that the severity of cataplexy observed in narcoleptic dogs gradually increases as animals approach maturity and then decreases in adulthood. For example, 36–60-day-old dogs have significantly fewer attacks than 100–180-day-old dogs, while 100–180-day-old animals have significantly more attacks than 2–4 years old (2). Thus it was of interest to establish the potential correlation between the age-dependent severity of cataplexy and the density of adenosine receptors and adenosine uptake sites in the brains of narcoleptic dogs.

METHOD

Brain Tissue

Frozen brain tissue from five normal and five narcoleptic dogs 100 days old, and from four normal and four narcoleptic dogs 50 days old, were used. The dogs were either male or female Doberman pinchers derived from at least two separate litters per group. Animals were sacrificed by intravenous injection of thiopental sodium (55 mg/kg). We have used the same dissection procedure to extract frontal cortex and putamen as reported by Bowersox et al. (8). Extracted tissue samples were stored at −80°C until assayed.

Ligand Binding Assays

Crude membrane fractions isolated from the frontal cortex (posterior sigmoid gyrus, coronal gyrus, frontal pole) and putamen were prepared by a modification of the method of Anand-Srivastava and Johnson (1) as described by Yeung and Green (36). Preliminary binding studies were performed in order to establish assay parameters under equilibrium conditions. Radioligand binding parameters for adenosine A_1 receptors were determined by Scatchard analysis, using incubation conditions similar to those described by Yeung and Green (36). Concentration of [^{3}H]N6-(R)-1-methyl-2-phenylethyladenosine ([^{3}H]R-PIA, 38.5 Ci/mmol, Du Pont NEN, Boston, MA) ranged from 0.2 to 50 nM and concentrations for 1,3[^{3}H]dipropyl-8-cyclopentylxanthine ([^{3}H]CPX, 103 Ci/mmol, Amersham, Arlington Heights, IL) ranged from 0.6 to 70 nM. Radioligand binding assays performed on membranes isolated from 50-day-old dogs were conducted in the presence and absence of 100 μM Gpp(NH)p. Nonspecific binding was defined in the presence of 10 μM unlabeled R-PIA or 100 μM unlabelled N^6-cyclopentyladenosine (CPA). To assess adenosine A_1 and A_2 receptors in the putamen of 100-day-old dogs we used a single saturating concentration (50 nM) of [^{3}H]R-PIA to assess A_1 receptor binding, and a single saturating concentration (200 nM) of [^{3}H]5′-ethylcarboxamide adenosine ([^{3}H]NECA, 20.8 Ci/mmol, Du Pont NEN) to assess the sum total of A_1 and A_2 receptor binding as described by Yeung and Green (36). An estimate of A_2 receptor binding was determined by subtracting the amount of [^{3}H]R-PIA bound from the amount of [^{3}H]NECA bound. The assays were performed in triplicate and 50 μM unlabelled R-PIA was used to define nonspecific binding. Reactions were incubated at 37°C for 30 min in a shaking water bath to attain equilibrium. In preliminary studies saturation isotherms for [^{3}H]NECA were performed, in membranes isolated from the putamen of two normal dogs, using eight concentrations of the radioligand ranging from 0.4 to 89 nM. Scatchard analysis of the binding indicated a dissociation constant (K_d) of 5.30 nM and a maximal number of binding sites (B_{max}) of 686 fmol/mg of protein, saturability was reached at 25 nM (data not presented). Adenosine transport sites in the posterior sigmoid gyrus and coronal gyrus of 100-day-old dogs were labeled with [^{3}H]nitrobenzylthioinosine ([^{3}H]NBTI, 26 Ci/mmol, Du Pont NEN), a potent inhibitor of nucleoside transport systems. Parameters for [^{3}H]NBTI binding were determined by Scatchard analysis, using incubation conditions similar to those described for adenosine receptor binding. Radioligand binding reactions contained one of 8 concentrations of [^{3}H]NBTI, ranging from 0.4 to 20 nM. Nonspecific binding was defined in the presence of 15 μM unlabeled NBTI. Reactions were incubated at 24°C for 30 min to attain equilibrium.

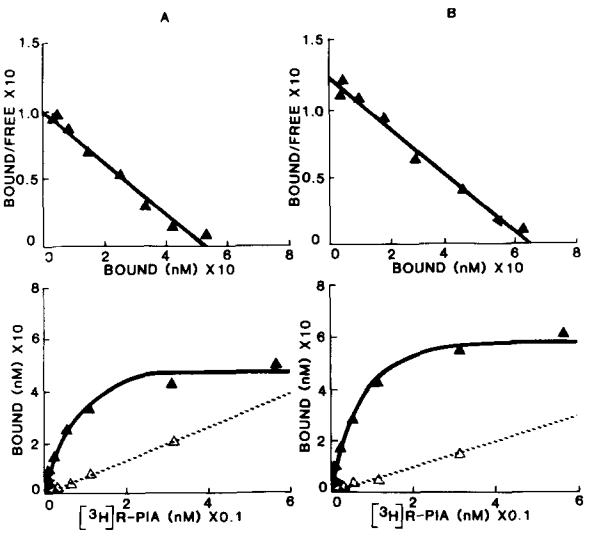

FIG. 1. Representative saturation isotherms of specific (-) and nonspecific (- - -) binding of [^{3}H]R-PIA to cortical membranes isolated from the posterior sigmoid gyrus of normal (A) and narcoleptic (B) dogs 100 days old. Scatchard plots of the specific binding are illustrated on the top panel of the figures as determined by least squares analysis of the saturation isotherms. The B_{max} values obtained in these membrane preparations were 418 fmol/mg protein (A) and 645 fmol/mg protein (B). The respective K_d values were 5.6 and 5.2 nM.

Saturation isotherms were analyzed by least squares analysis of Scatchard plots and by the nonlinear curve fitting program LIGAND (25). Statistical comparisons of binding data in 100-day-old dogs were done by the nested analysis of variance technique with unequal sample size (32). This analysis was chosen since assays were carried out blindly and multiple samples of the posterior sigmoid gyrus and coronal gyrus were examined. Student's *t*-test (two-tailed) was used for all other comparisons. When the curve fitting program LIGAND indicated two affinity states of the adenosine A_1 receptor, binding parameters were constrained to normal values or were varied independently by LIGAND in order to decide the significance of any changes observed.

RESULTS

Representative saturation isotherms and Scatchard plots of [^{3}H]R-PIA binding to cortical membranes isolated from normal and narcoleptic dogs 100 days old are shown in Fig. 1. The results indicate that [^{3}H]R-PIA bound to cortical membranes with similar high affinities in both normal and narcoleptic animals (5.24 ± 0.39 and 6.46 ± 0.86 nM, respectively). In both groups of animals cortical membranes isolated from the posterior sigmoid gyrus and coronal gyrus appeared to contain a single population of high-affinity receptors. Hill coefficients were near unity in both brain regions (1.06 ± 0.02 for normal dogs and 1.05 ± 0.04 for narcoleptic dogs).

Since the assays were carried out blindly and multiple samples of each brain area of the same dog were examined, the nested analysis of variance technique with unequal sample size (32) was

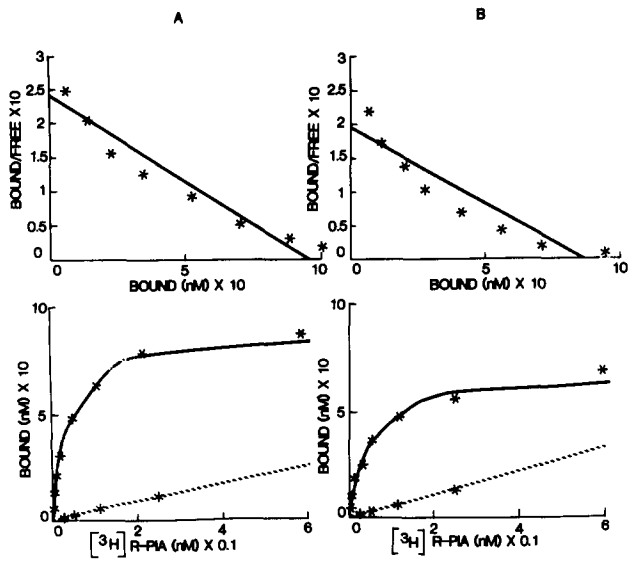

FIG. 2. Representative saturation isotherms of specific (-) and nonspecific (- - -) binding of [^{3}H]R-PIA to cortical membranes isolated from the frontal pole of normal (A) and narcoleptic (B) dogs 50 days old. Scatchard plots of the specific binding are illustrated on the top panel of the figures as determined by least squares analysis of the saturation isotherms. The results show that in both normal and narcoleptic animals Scatchard plots were not linear. Binding parameters were determined by the nonlinear curve fitting program LIGAND (Table 1).

used to analyze the amount of ligand specifically bound. A significant effect among dogs within groups, $F(8,24)=3.31$, $p<0.05$, with no significant effect between normal and narcoleptic dogs, $F(1,8)=3.11$, $p<0.1$, was observed. These two groups were also compared using the mean measurements for each dog for a two sample t-test. Though the mean of the five dogs in the narcoleptic group was higher than that of the normal group (696 ± 94 vs. 594 ± 95 fmol/mg protein), the difference was not significant ($t=1.7$, $p=0.1252$).

Representative Scatchard plots of [^{3}H]R-PIA binding to membranes isolated from the frontal pole of normal and narcoleptic dogs 50 days old are presented in Fig. 2. The results show that in both groups of animals Scatchard plots were not linear. Analysis by the nonlinear curve fitting program LIGAND shows that the binding is best described by a 2 site fit, where the high-affinity dissociation constant (KH) was 0.32 ± 0.09 nM and the low-affinity dissociation constant (KL) was 6.40 ± 0.57 nM (Table 1). Results in Table 1 also show that nearly all the binding (92%) was to a lower affinity site, while only 8% was to a high-affinity site. A similar pattern was also observed in narcoleptic dog membranes. When we compared the goodness of fit for [^{3}H]R-PIA with and without a set of fixed values, the second model was not significantly worse ($p>0.1$), indicating that [^{3}H]R-PIA binding was not different in narcoleptic membranes. In addition, when both membranes were exposed to 100 μM Gpp(NH)p, [^{3}H]R-PIA binding to the high-affinity site was significantly decreased in both normal and narcoleptic dogs (Table 1). The low-affinity dissociation constant was also significantly increased by the addition of Gpp(NH)p in both normal and narcoleptic dogs, while no changes were observed in the high-affinity dissociation constant.

Agonist ([^{3}H]R-PIA) and antagonist ([^{3}H]CPX) radioligand binding were examined in the posterior sigmoid gyrus and coronal gyrus of normal and narcoleptic dogs 50 days old (Table 2). The data show that [^{3}H]R-PIA bound to these membranes with similar high affinities in both normal and narcoleptic dogs. K_d values were similar to those obtained in membranes isolated from 100-day-old dogs. Furthermore, analysis by LIGAND shows that the binding of [^{3}H]R-PIA was best described by a one-site model. Therefore, posterior sigmoid gyrus and coronal gyrus membranes isolated from 50-day-old dogs appear to contain a single population of A_1 receptors. [^{3}H]CPX, an adenosine antagonist which binds to both the high- and low-affinity coupling states of the A_1 receptor (7), bound similar amounts of A_1 sites as the agonist [^{3}H]R-PIA, suggesting again the presence of a single population of A_1 receptors in these brain areas.

Although [^{3}H]R-PIA bound to a single high-affinity A_1 site (Table 2), these sites were regulated by guanine nucleotides. In the presence of Gpp(NH)p the majority of the high-affinity agonist binding states in the posterior sigmoid gyrus were converted to low-affinity agonist binding states. Analysis of [^{3}H]R-PIA saturation isotherms in the presence of Gpp(NH)p showed a reduction in receptor affinity for [^{3}H]R-PIA in both normal and narcoleptic dogs. Dissociation constants (K_d) determined by LIGAND analysis were 25 ± 5 and 21 ± 4 nM, with B_{max} values of 800 ± 82 and 696 ± 66 fmol/mg protein for normal and narcoleptic dogs. Interestingly, a two-site model gave a significantly better fit of the data in both groups of animals. The K_d and B_{max} values estimated for the high-affinity site in normal dogs was 0.22 ± 0.21 nM and 21 ± 10 fmol/mg protein. Values in narcoleptic dogs were 0.12 ± 0.16 nM and 17 ± 8 fmol/mg protein. The presence of curvilinear Scatchard plots in the presence of Gpp(NH)p indicates an incomplete conversion of the high-affinity ag-

TABLE 1

[^{3}H]R-PIA BINDING IN THE FRONTAL POLE OF NORMAL AND NARCOLEPTIC DOGS 50 DAYS OLD IN THE PRESENCE AND ABSENCE OF 100 μM Gpp(NH)p

	Normal		Narcoleptic	
	−Gpp(NH)p	+Gpp(NH)p	−Gpp(NH)p	+Gpp(NH)p
KH	0.32 ± 0.09	0.62 ± 0.09	0.35 ± 0.08	0.60 ± 0.02
KL	6.40 ± 0.57	18.0 ± 2.10*	6.40 ± 0.69	20.0 ± 3.10*
RH	75 ± 15	21 ± 4.5*	91 ± 12	26 ± 0.75*
RL	831 ± 25	706 ± 99	895 ± 135	895 ± 112

The data represent analysis of 4 saturation isotherms per group by LIGAND. Membranes were simultaneously prepared from normal and narcoleptic dogs. Values of K_d and B_{max} were mean ± S.E.M., KH (nM): high-affinity dissociation constant, KL (nM): low-affinity dissociation constant, RH (fmol/mg protein): receptors in high-affinity state, RL (fmol/mg protein): receptors in low-affinity states. *$p<0.05$ vs. absence of Gpp(NH)p.

TABLE 2

[^{3}H]R-PIA AND [^{3}H]CPX BINDING IN THE POSTERIOR SIGMOID GYRUS AND CORONAL GYRUS OF NORMAL AND NARCOLEPTIC DOGS 50 DAYS OLD

		Posterior Sigmoid Gyrus		Coronal Gyrus	
		[^{3}H]R-PIA	[^{3}H]CPX	[^{3}H]R-PIA	[^{3}H]CPX
Normal	K_d	5.6 ± 0.45	28 ± 5.5	4.8 ± 0.76	20 ± 1.4
	B_{max}	759 ± 120	880 ± 157	592 ± 42	674 ± 49
Narcoleptic	K_d	6.6 ± 0.87	22 ± 1.7	4.6 ± 0.71	18 ± 1.3
	B_{max}	923 ± 131	976 ± 80	610 ± 42	723 ± 66

[^{3}H]R-PIA and [^{3}H]CPX binding were assayed on the same membrane preparation. K_d (nM) and B_{max} (fmol/mg protein) values are mean ± S.E.M. of 4 dogs per group. There was no statistical difference between normal and narcoleptic dogs.

onist binding states to low-affinity agonist binding states. The detection of small amounts of residual binding under this conditons has been recently reported by Leung et al. (20), suggesting that agonist-antagonist binding to adenosine A_1 receptors is more complicated than previously thought.

Cortical membranes contained a single class of high-affinity uptake binding sites in both normal and narcoleptic dogs, K_d values for normal dogs was 2.7 ± 0.35 nM and 2.6 nM for narcoleptic dogs. The concentration of uptake sites in normal and narcoleptic dogs were also similar, B_{max} values were 268 ± 21 and 273 ± 14 fmol/mg protein, respectively. The nested analysis of variance technique showed that there was no significant effect among dogs within groups nor between groups.

We examined A_2 receptor binding in the putamen because previous studies in the rat have shown that high-affinity adenosine A_2 receptors are localized mainly in the striatum (6,36). Similar to previous findings in the rat, the number of sites specifically bound by [^{3}H]NECA (869 ± 70 fmol/mg protein) in normal dogs was approximately twice that of [^{3}H]R-PIA (460 ± 45 fmol/mg protein), the difference was ascribed to be binding to A_2 receptors. No significant changes were observed in [^{3}H]R-PIA nor in the difference in the binding between these two ligands in narcoleptic animals as compared to normal animals.

DISCUSSION

Characterization of A_1 Receptors in the Frontal Cortex of Narcoleptic Dogs

A detailed characterization of the binding of [^{3}H]R-PIA to adenosine A_1 was performed in three regions of the frontal cortex (frontal pole, posterior sigmoid gyrus and coronal gyrus) of normal and narcoleptic dogs 50 days old. Our data show that [^{3}H]R-PIA labels two sites in membranes isolated from the frontal pole ("high and low affinity") and only one site in membranes isolated from the posterior sigmoid gyrus and coronal gyrus.

The two sites obtained with the A_1 agonist [^{3}H]R-PIA could represent either several conformational states of the same A_1 receptor protein or multiple receptor proteins. The A_1 receptor is coupled to the adenylate cyclase and most of the receptors of this family exist in several affinity states, depending whether or not they are associated with a G-protein (33). One of the receptor conformations, i.e., the association with the G-protein, usually shows a high affinity for agonists and is the active form of the receptor [RH; (4)]. The other form of the receptor (RL) is uncoupled with the G-protein and has a lower affinity for agonists, whereas both forms (RH and RL) have a similar affinity for antagonists. In our case, the two sites observed had different affinity for the agonist PIA, a result consistent with this theory. We further tested this hypothesis by examining the effect of guanine nucleotide on radioligand binding characteristics of PIA. In most adenylate cyclase associated receptors, the binding of these nucleotides such as GTP or Gpp(NH)p to the G-proteins produces a dissociation of the receptor with its G-protein and a disappearance of the high-affinity binding site. In our case, when we examined [^{3}H]R-PIA binding in the presence of 100 μM Gpp(NH)p, the percentage of receptors in the high-affinity state was significantly decreased in both the normal and narcoleptic membranes (Table 1), in all three regions of the frontal cortex. Again, this result is consistent with the existence of two states of affinity for a G-protein coupled receptor.

A surprising result was the finding of a significant difference between the low-affinity dissociation constant determined in the absence and in the presence of 100 μM Gpp(NH)p. This result cannot be explained if one considers this site as the uncoupled form of the A_1 receptor. Thus it appears that the low-affinity state determined in the absence of Gpp(NH)p does not represent binding to the uncoupled A_1 receptor. We therefore think that our results will be better explained by the existence of several G-protein coupled to A_1 receptor subtypes with respectively high and low affinity for PIA but equal affinity for CPX. Interestingly, only the low-affinity receptor type was found in the posterior sigmoid gyrus and coronal gyrus, suggesting regional differences in the distribution of these receptors subtypes. Clear definition of the regional localization of these receptor subtypes will be possible when the appropriate G-protein coupled receptor genes have been identified and cloned.

Comparative Studies of A_1 and A_2 Receptors As Well As Adenosine Uptake Sites in Narcoleptic and Control Dogs 100 Days Old

Although an increase in A_1 receptors was reported in rat cerebral cortex and corpus striatum following REM sleep deprivation (35), in the present study we found no significant changes in adenosine A_1 receptor density in the brains of narcoleptic dogs. One explanation may be that although narcolepsy can be characterized as a REM sleep dysfunction, it is not REM sleep deprivation. However, the nested analysis of variance demonstrates a significant effect among dogs within groups. Therefore, it is possible that factors such as sex, litter and symptom severity together with a small sample size could account for the variance and statistical

nonsignificance between groups. When we examined both [^{3}H]R-PIA and [^{3}H]NECA binding in membranes isolated from the putamen, we found that the B_{max} for [^{3}H]NECA were twice that of [^{3}H]R-PIA in both normal and narcoleptic dogs. A similar pattern is observed in the caudate-putamen of the rat brain (9,36). No significant changes were observed in the binding between these two ligands in the putamen between normal and narcoleptic dogs.

In the present study we have utilized the paired determination of [^{3}H]R-PIA and [^{3}H]NECA B_{max}'s to arrive at an estimate of the A_2 receptor number. The validity of this method has previously been determined (9, 15, 36). Although [^{3}H]NECA has been used successfully to specifically labeled adenosine A_2 receptors in the rat brain (5, 9, 15, 19, 36), various studies have revealed a complex binding profile of NECA in both membrane homogenate and autoradiographic studies. Bruns et al. (5) have reported different affinity states for [^{3}H]NECA binding in the striatum that include two coupling states of the A_1 receptor, two coupling states of the A_2 receptor and a low-affinity, high-capacity "nonreceptor" site. In addition, Lee and Reddington (19) have used autoradiographic methods to characterize [^{3}H]NECA binding sites. The first is sensitive to displacement by nM concentrations of A_1-selective agonists (R-PIA, CHA, CPA) and antagonists, this site appears to be the A_1 receptor. The second site is sensitive to displacement by μM concentrations of R-PIA and CPA, this site appears to be the A_2 binding site. The third site is not sensitive to R-PIA (mM), this site has been called the "PIA-insensitive" site. The latter site appears to correspond to the "nonreceptor" site described by various authors in both the CNS and peripheral tissues (5, 19, 22, 36). Under the conditions used in the present experiments, the "PIA-insensitive" site was included in the determination of nonspecific binding and excluded from the determination of specific [^{3}H]NECA binding. However, quantitative analysis of [^{3}H]NECA binding to membranes isolated from dog brain should be looked at with caution since species difference are bound to exist. The advent of newer compounds such as CGS 21680 (16), with high affinity and selectivity for the adenosine A_2 receptor, will provide a better characterization of these receptors in the future.

An interesting finding in our study is that [^{3}H]NBTI binding did not show any significant difference among dogs within groups even though assays were conducted in the same membrane preparation as [^{3}H]R-PIA binding. These data indicate that adenosine A_1 receptors are more variable than adenosine uptake sites among dogs within groups, possibly because adenosine uptake sites are not only located on neurons but also on glial cells (3). On the other hand, although we did not find significant differences between narcoleptic and normal dogs with respect to [^{3}H]NBTI binding parameters, there is a possibility that specific changes in other adenosine transporters could have occurred since there are at least three types of nucleoside carriers (10,17).

The present study offered no evidence that either adenosine A_1 receptors in the frontal cortex or adenosine A_2 receptors in the putamen were altered in narcoleptic dogs. This conclusion is tentative, however, and requires further studies since there was a significant difference among dogs within groups that could mask any changes between groups.

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