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Differences in Adenosine A-1 and A-2 Receptor Density Revealed by Autoradiography in Methylxanthine-Sensitive and Insensitive Mice

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JARVIS, M. F. AND M. WILLIAMS. *Differences in adenosine A-1 and A-2 receptor density revealed by autoradiography in methylxanthine-sensitive and insensitive mice.* PHARMACOL BIOCHEM BEHAV 30(3) 707–714, 1988.—Two strains of inbred mice, CBA/J and SWR/J, have been identified which are, respectively, sensitive and insensitive to the behavioral and toxic effects of methylxanthines. Autoradiographic analyses of brain adenosine receptors were conducted with [^{3}H]CHA to label adenosine A-1 receptors and [^{3}H]NECA, in the presence of 50 nM CPA, to label adenosine A-2 receptors. For both mouse strains, adenosine A-1 receptors were most highly concentrated in the hippocampus and cerebellum whereas adenosine A-2 receptors were selectively localized in the striatum. CBA/J mice displayed a 30% greater density of adenosine A-1 receptors in the hippocampal CA-1 and CA-3 regions and in the cerebellum as compared to the SWR/J mice. The number of A-2 receptors (B_{max}) was 40% greater in the striatum and olfactory tubercle of CBA/J as compared to SWR/J mice. No significant regional differences in A-1 or A-2 receptor affinities were observed between these inbred strains of mice. These results indicate that the differential sensitivity to methylxanthines between these mouse strains may reflect a genetically mediated difference in regional adenosine receptor densities.

Adenosine receptors Caffeine Autoradiography CBA/J SWR/J

CAFFEINE is one of the most widely consumed psychoactive agents in the world. The stimulant effects of caffeine on the central nervous system are primarily due to the blockade of cell surface purinergic receptors [5, 29, 33]. These receptors have been divided into adenosine A-1 and A-2 subtypes based upon their different pharmacological profiles and effects on adenylate cyclase [4, 12, 29]. Adenosine exhibits nanomolar affinity for A-1 receptors and can mediate an inhibition of adenylate cyclase, whereas at A-2 receptors adenosine has micromolar affinity and receptor activation can result in a stimulation of adenylate cyclase [4, 29, 33].

Some important biochemical effects of caffeine include: release of catecholamines [5, 7, 8, 13, 20]; increased intracellular calcium [14]; increased glucose metabolism [19]; and at high concentrations ($>10^{-6}$), phosphodiesterase inhibition [15,20]. Caffeine has been shown to produce a wide variety of behavioral effects in laboratory animals. These include biphasic effects on locomotion [7, 8, 15, 16, 25–27, 30], schedule-controlled behavior [32], and core body temperature [2, 24]. At toxic doses, caffeine causes tonic and/or clonic seizures and death [5, 26, 27]. In humans, the psychoactive effects of caffeine include increased alertness [9], anxiety [3], insomnia [9], and at increasingly large doses, tremor, and seizures [22,36]. It is interesting to note that the behavioral sensitivity of caffeine varies greatly within species. For example, variability in caffeine sensitivity in humans ranges from individuals in whom a single cup of coffee causes mild tremors to others who can imbibe 10–15 cups a day with no apparent ill effects [11].

The variation in behavioral sensitivity to caffeine (and other methylxanthines) has received much recent attention [2, 11, 16, 25–27]. Using strains of inbred mice, the behavioral response to caffeine has been shown to reflect inherent neurologic rather than pharmacokinetic or metabolic differences [2]. Specifically, two strains of mouse, CBA/J and SWR/J, have been identified which markedly differ in their sensitivity to caffeine. CBA/J mice have been shown to be significantly more sensitive to the locomotor stimulating, hyperthermic, and lethal effects of caffeine as compared to SWR/J mice [2, 16, 25–27]. Interestingly, caffeine sensitivity in these mice has been demonstrated to be genetically determined with different genetic components controlling sen-

sitivity to different methylxanthines [25]. While a single pair of autosomal alleles appear to determine caffeine-induced lethality in these mice [26], genetic contributions to the locomotor stimulating effects of theophylline in the F-1 hybrid offspring of CBA/J and SWR/J mice do not fully determine caffeine-induced stimulation of locomotor activity [25].

The exact mechanisms underlying the behavioral sensitivity to methylxanthines remain unknown; however, possible differences in central purinergic systems are indicated by recent observations of marked interspecies variation in the pharmacological profile of adenosine antagonists in both adenosine A-1 and A-2 receptor systems [6,31]. The present study was conducted to evaluate the possibility that differences in the behavioral sensitivity to caffeine in the CBA/J and SWR/J mouse strains are mediated by differences in regional adenosine receptor affinity and/or density. To this end, quantitative autoradiography was used to visualize the regional distributions of brain adenosine A-1 and A-2 receptors.

METHOD

Adult male CBA/J and SWR/J mice (Jackson Labs, Bar Harbor, ME) were housed in individual cages with free access to food and water. Mice were maintained in a temperature and humidity controlled colony room on a 12 hr light/dark cycle (lights on at 0700). Mice were sacrificed with carbon dioxide and transcardially perfused with phosphate-buffered saline. Twenty-micron horizontal and sagittal sections were thaw-mounted onto gelatin-coated slides and stored at −70°C.

On the day of the assay, tissue sections were warmed to room temperature and allowed to dry. Sections were preincubated in 50 mM Tris-HCl (pH=7.4) containing 2 IU/ml adenosine deaminase (Type III, Boehringer-Mannheim, Indianapolis, IN) for 30 minutes at 37°C. [^{3}H]Cyclohexyladenosine ([^{3}H]CHA) was used to label adenosine A-1 receptors. Saturation experiments were conducted by incubating tissue sections in 7–9 concentrations of ligand over the range of 0.12–40 nM. [^{3}H]5-N-ethylcarboxamidoadenosine ([^{3}H]NECA), in the presence of 50 nM cyclopentyladenosine (CPA), was used to label adenosine A-2 receptors [1]. Saturation experiments were conducted by incubating tissue sections in 7–9 concentrations of ligand over the range of 0.60–60 nM. For both ligands, nonspecific binding was determined in the presence of 20 μM 2-chloroadenosine (2-CADO). Incubations were conducted in 50 mM Tris-HCl buffer (pH=7.4) for two hours at 23°C. For [^{3}H]NECA, the incubation buffer also contained 10 mM $MgCl_2$ [1,31]. Experiments were terminated by washing tissue sections four times for five minutes each in ice-cold buffer. Tissue sections were then rinsed for two seconds in ice-cold distilled water to remove excess buffer. Tissue sections were rapidly dried under a stream of warm air and apposed to tritium-sensitive film (Ultrofilm, LKB, Bromma) for 28 days.

Quantitative analysis of the resulting autoradiograms was performed using an EyeCom II Image Analysis System (Spatial Data Systems, Melbourne, FL). Grey values obtained from each brain region were converted to a measure of radioactivity (DPM) per milligram of brain tissue based upon internal brain paste standards on each sheet of film. Saturation parameters (K_d and B_{max}) for each brain region were obtained by further analysis with the Lundon I iterative curve fitting program [18]. Statistical differences in binding parameters (K_d and B_{max}) were determined by ANOVA followed by Dunnett's t-test.

[^{3}H]CHA (25 Ci/mmol) and [^{3}H]NECA (20 Ci/mmol) were obtained from Du Pont-NEN, Boston, MA. 2-CADO and CPA were obtained from Research Biochemicals Inc., Natick, MA. All other chemicals and reagents were obtained from Fisher Scientific Inc., Springfield, NJ.

RESULTS

Both [^{3}H]CHA and [^{3}H]NECA specifically bound to mouse brain tissue sections in a heterogeneous pattern (Table 1 and Figs. 1A, B and 2A, B). Nonspecific binding amounted to less than ten percent of total binding for both ligands. Iterative computer analysis of the saturation data for both [^{3}H]CHA and [^{3}H]NECA, revealed that the binding data were best fit to a one-site model in all brain regions examined (Fig. 3).

The density of [^{3}H]CHA binding sites was greatest in the CA-1 region of the hippocampus (Table 1 and Fig. 1A). Lower binding densities were observed in other brain areas in the following order: hippocampus (CA-3) > molecular layer of the cerebellum > thalamic nuclei ≥ cerebral cortex = striatum. Similar regional distributions of [^{3}H]CHA binding sites were observed in both the CBA/J and SWR/J mice. The average affinity (K_d) of [^{3}H]CHA across the brain regions examined was 1.1±0.1 and 0.8±0.1 nM for CBA/J and SWR/J mice, respectively.

The density of [^{3}H]NECA binding sites was greatest in the striatum and olfactory tubercle with little or no quantifiable specific binding observed in other brain regions (Table 1 and Fig. 2A). The average affinity for [^{3}H]NECA across the brain regions examined was 6.9±0.3 and 6.3±1.1 nM for CBA/J and SWR/J, respectively.

Significant and selective differences were observed in the regional distributions of [^{3}H]CHA and [^{3}H]NECA binding sites between these inbred mouse strains (Table 1 and Figs. 4 and 5). CBA/J mouse brain was found to contain an approximately 30% greater density of [^{3}H]CHA binding sites in the CA-1 and CA-3 regions of the hippocampus as compared to SWR/J mouse brain. Significantly greater densities of adenosine A-1 receptors were also observed in the molecular layer (15%) and granular layer (30%) of the cerebellum of CBA/J mice as compared to SWR/J mice. Essentially equivalent amounts of [^{3}H]CHA binding were found in the other brain regions examined from these inbred mice. With respect to adenosine A-2 receptors, CBA/J mouse brain was found to contain an approximately 40% greater density of [^{3}H]NECA binding sites as compared to SWR/J mouse brain. No significant differences in adenosine receptor affinity between brain regions or mouse strain were observed.

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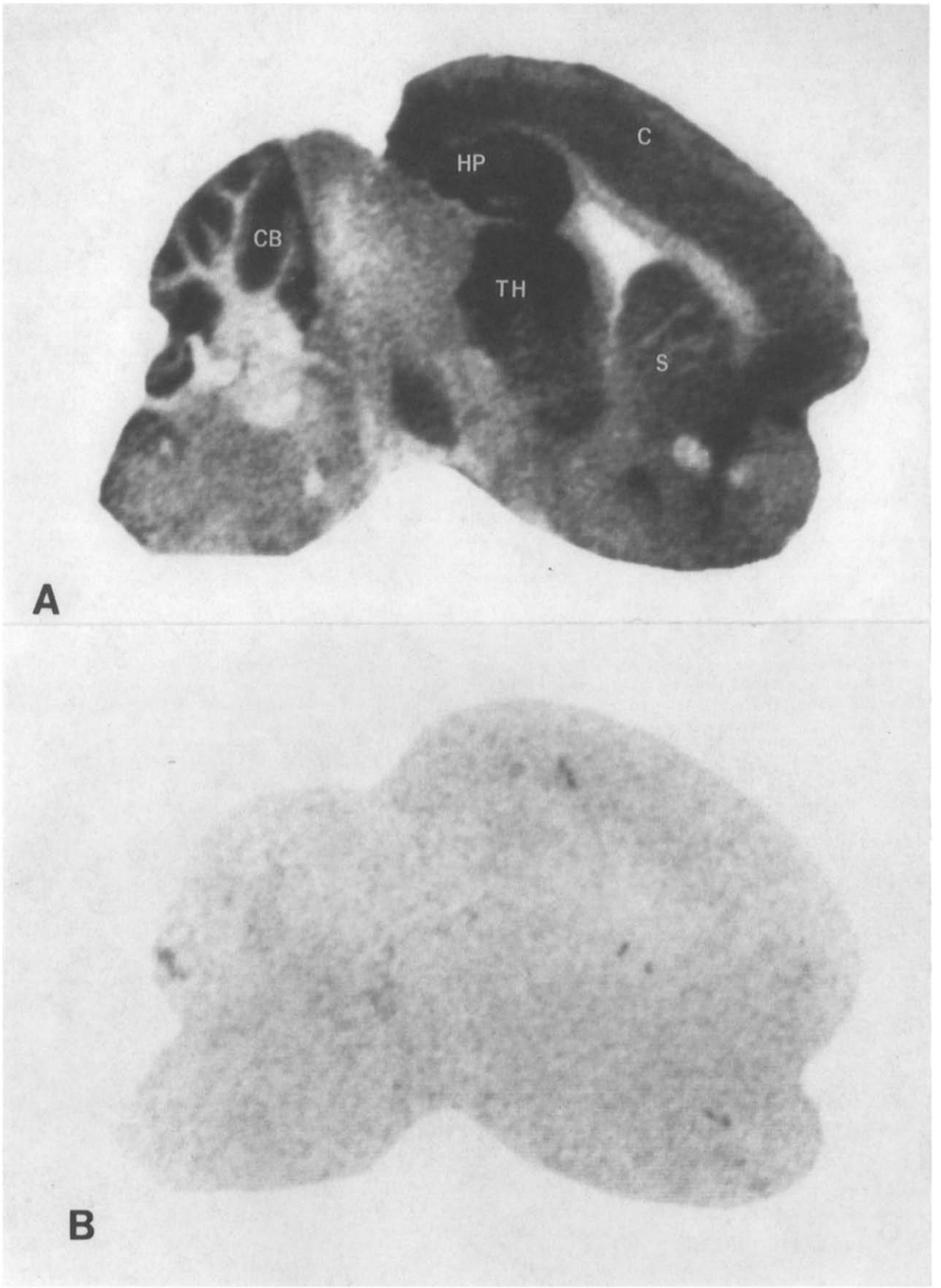
FIG. 1. Digitized autoradiographic images of (A) total and (B) nonspecific [^{3}H]CHA (1 nM) binding in CBA/J sagittal brain section. Abbreviations are: striatum (S), cortex (C), thalamus (TH), hippocampus (HP) and cerebellum (CB).

FIG. 2. Digitized autoradiographic images of (A) total and (B) nonspecific [^{3}H]NECA (4 nM) binding, in the presence of 50 nM CPA, in CBA/J in sagittal brain section. Abbreviations are: striatum (S) and olfactory tubercle (OT).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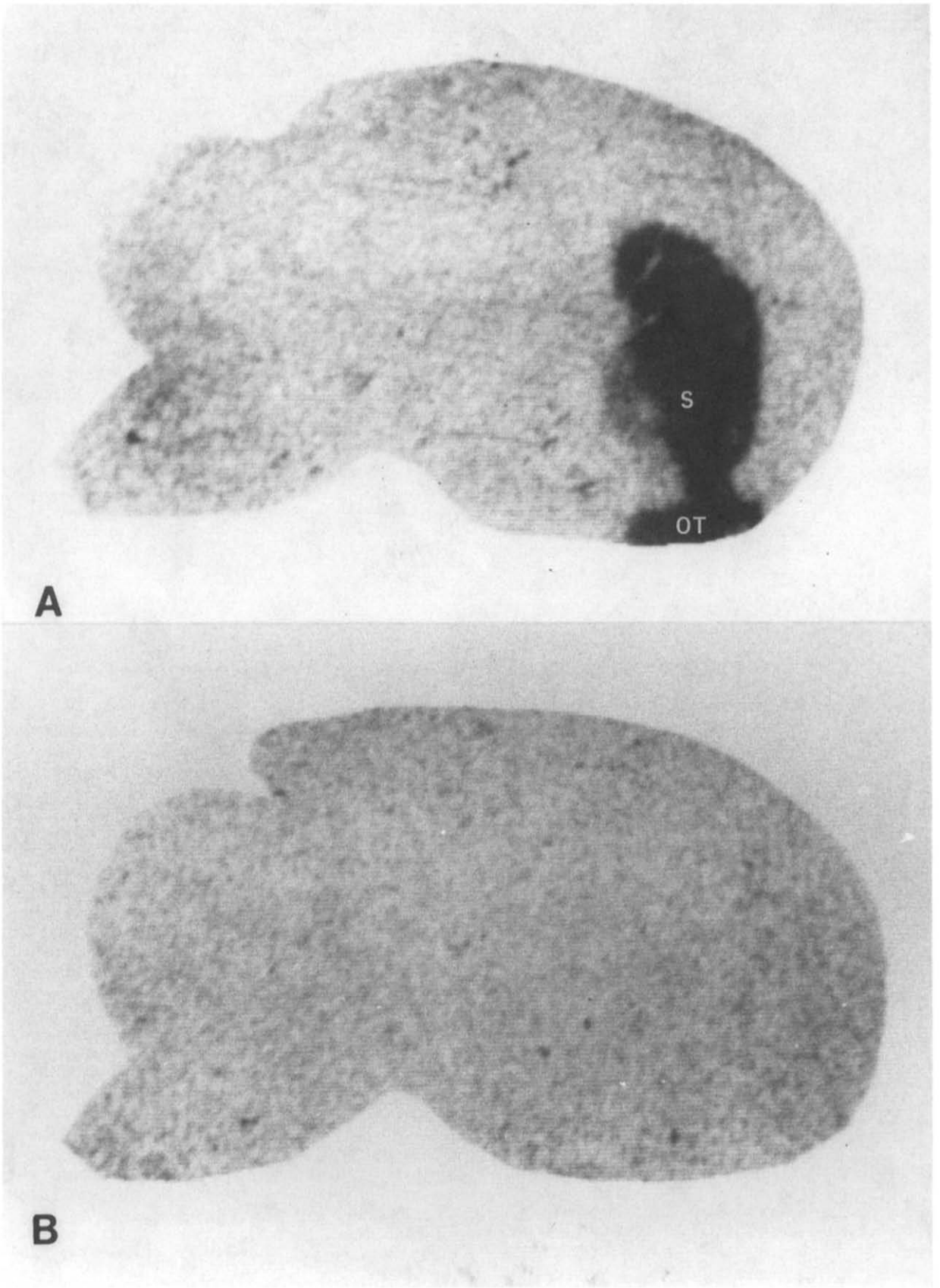
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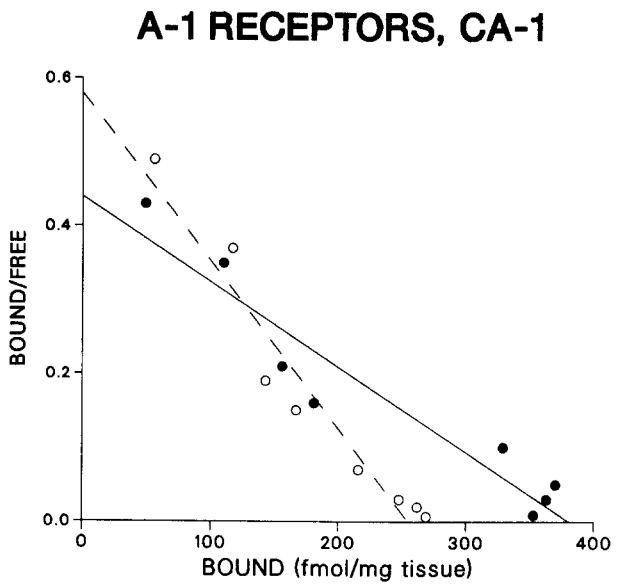

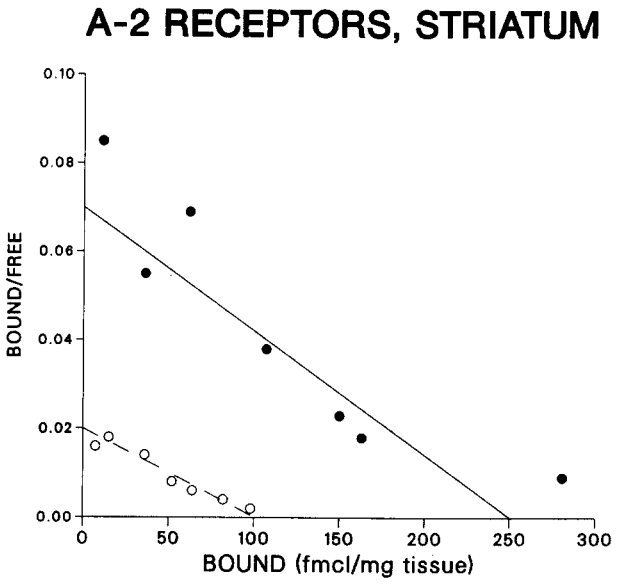

FIG. 3. Representative Scatchard plots of specific [^{3}H]CHA and [^{3}H]NECA binding in the hippocampal CA-1 region and striatum, respectively. Closed circles represent data from CBA/J mice and open circles represent data from SWR/J mice.

TABLE 1

SATURATION PARAMETERS OF [^{3}H]CHA AND [^{3}H]NECA BINDING IN XANTHINE-SENSITIVE CBA/J AND XANTHINE-INSENSITIVE SWR/J MICE

	K_d		B_{max}	
Brain Region	CBA/J	SWR/J	CBA/J	SWR/J
[^{3}H]CHA (adenosine A-1)				
Cortex Outer Layer	0.9 (0.2)	0.7 (0.1)	177 (18)	187 (14)
Inner Layer	0.9 (0.2)	0.6 (0.1)	291 (19)	277 (6)
Hippocampus				
CA-1	1.4 (0.3)	0.9 (0.4)	404 (33)*	283 (30)
CA-3	1.2 (0.2)	0.8 (0.2)	376 (28)*	256 (19)
Striatum	1.5 (0.2)	1.1 (0.1)	200 (8)	181 (5)
Thalamus	1.1 (0.1)	0.9 (0.1)	220 (11)	206 (3)
Cerebellum				
Molecular Layer	0.8 (0.2)	0.6 (0.1)	264 (3)*	230 (9)
Granular Layer	1.3 (0.5)	0.8 (0.1)	207 (6)*	148 (9)
[^{3}H]NECA (50 nM CPA) (adenosine A-2)				
Striatum	6.7 (1.1)	7.9 (1.3)	213 (25)*	135 (14)
Olfactory Tubercle	7.2 (1.1)	4.6 (1.5)	204 (18)*	121 (2)

Values represent mean (± 1 SEM) K_d (nM) and B_{max} (fmol/mg tissue) from triplicate values of brain images from 4–6 animals. *Represents significant strain differences as determined by Dunnett's t-test, $p<0.05$.

DISCUSSION

The regional distributions of adenosine A-1 receptors found in the present study, involving inbred mouse strains, are consistent with other autoradiographic studies of A-1 receptors in rodent brain [10, 17, 28]. Similarly, the regional localization of adenosine A-2 receptors demonstrated in these mice is consistent with previous data from homogenate binding, electrophysiological, and adenylate cyclase studies [1, 5, 35]. The saturation parameters (K_d and B_{max}) for [^{3}H]CHA and [^{3}H]NECA binding in both the CBA/J and SWR/J mice are also in agreement with homogenate binding data obtained in a variety of mammalian species [6,31].

A selective localization of high affinity adenosine A-2 receptors in striatal tissue is indicated by the present results (Fig. 2). These data represent the first autoradiographic visualization of adenosine A-2 receptors using the nonselective adenosine agonist [^{3}H]NECA in the presence of 50 nM CPA [1], which effectively masks [^{3}H]NECA binding to adenosine A-1 receptors [1,31]. The utility of this approach is illustrated by the present identification of adenosine A-2 receptors in the olfactory tubercle (Fig. 2).

These results regarding the regional distributions of both A-1 and A-2 receptors indicate a regional specificity of adenosine receptor subtypes in mammalian brain. While saturation experiments have shown that striatal tissue contains approximately equal proportions of A-1 and A-2 receptors ([1], Table 1), there appear to be brain regions in which only one type of high affinity adenosine receptor predominates. Brain regions which contain high densities of A-1 receptors (i.e., hippocampus, cortex, and cerebellum) contain little or no high affinity A-2 receptors (Figs. 1 and 2). Similarly, the olfactory tubercle was found to contain a high density of A-2 receptors and no measurable specific [^{3}H]CHA binding sites.

While the functional significance of brain adenosine receptor organization remains largely unknown, the present results indicate that the differential behavioral sensitivity to methylxanthines, seen in CBA/J and SWR/J mice, may be related to differences in adenosine A-1 and A-2 receptors in specific brain regions. With respect to A-1 receptors, these differences were particularly evident in the cerebellum and hippocampus, and for A-2 receptors, in the striatum. Saturation experiments revealed that such differences were manifest as significant differences in receptor density (B_{max})

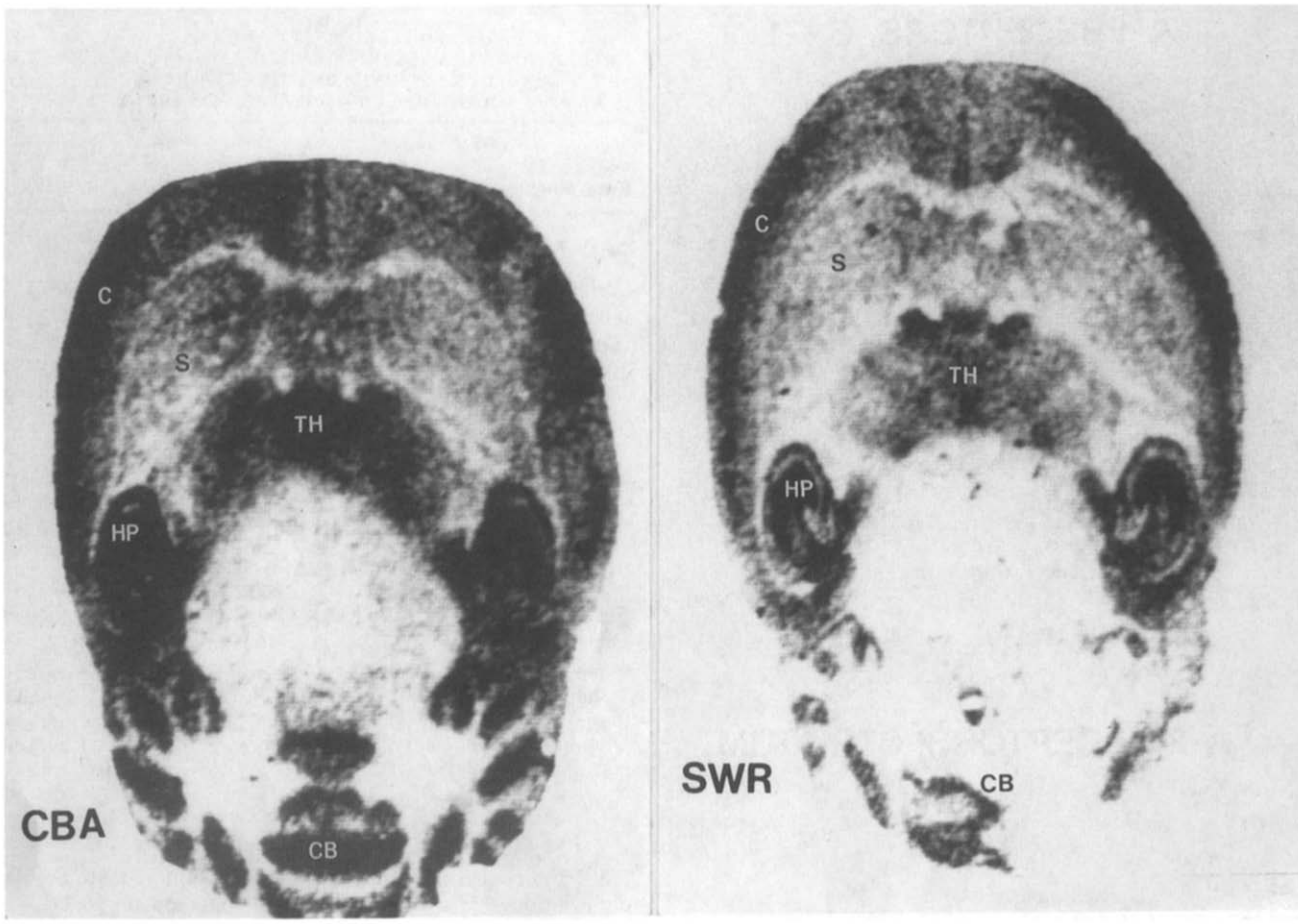

FIG. 4. Representative autoradiographic images of specific [^{3}H]CHA (1 nM) binding to (a) CBA/J and (b) SWR/J horizontal brain sections. Specific binding was revealed by digital subtraction autoradiography in which the image of specific binding was obtained through subtraction of the linearized nonspecific image from the total binding image. Abbreviations are: striatum (S), cortex (C), thalamus (TH), hippocampus (HP) and cerebellum (CB).

rather than differences in receptor affinity (K_d). Additionally, the differential behavioral sensitivity to methylxanthines seen in these mice does not appear to be due to different relative proportions of purinergic receptors since a high correlation between the regional distributions of A-1 and A-2 receptors was observed in these mice ($r=.86$, $p<0.05$). It is interesting to note that the differences in purinergic systems seen in these inbred mice may be neurochemically selective, since preliminary experiments revealed no differences in striatal dopamine D-1 or D-2 receptors between mouse strains using the selective ligands [^{3}H]SCH-23390 and [^{3}H]spiperone, respectively (unpublished observations). This apparent neurochemical specificity is further indicated by the observation that within the striatum, which was found to contain equal densities of A-1 and A-2 receptors, only significant differences in the density of A-2 receptors were seen in this brain region.

The stimulant effects of caffeine may be mediated by a variety of mechanisms including interactions with a variety of neurotransmitters [5, 7, 8, 13, 20], calcium mobilization [14] and/or phosphodiesterase inhibition [21]. However, the present results clearly indicate the possible involvement of cell surface purinergic receptors in the behavioral and toxic responses to methylxanthines seen in these strains of inbred mice [2, 16, 25–27]. This conclusion is further supported by the demonstration of a high correlation between the locomotor-stimulating effects of methylxanthines in rodents and their affinity for adenosine A-1 receptors in vitro [30]. Additional work is still required, however, to determine what contribution purinergic receptors, within specific brain regions, have in the mediation of the different behavioral effects of methylxanthines.

Several recent behavioral studies have shown that sensitivity to the locomotor-stimulant effects of methylxanthines is governed by complex genetic determinants [25]. Similarly, species differences in the relative activity of arylxanthines [6,31] and individual sensitivity to the behavioral effects of caffeine [11] support a potential dynamic role of purinergic neuromodulatory processes in the expression of xanthine-mediated effects at the molecular level. The results of the present study indicate that these genetic determinants are expressed, at least in part, by selective differences in the number of purinergic receptors in discrete brain regions. In this regard, the expression of adenosine A-1 receptors in the hippocampus and cerebellum and A-2 receptors in the striatum may be particularly sensitive to genetic variation.

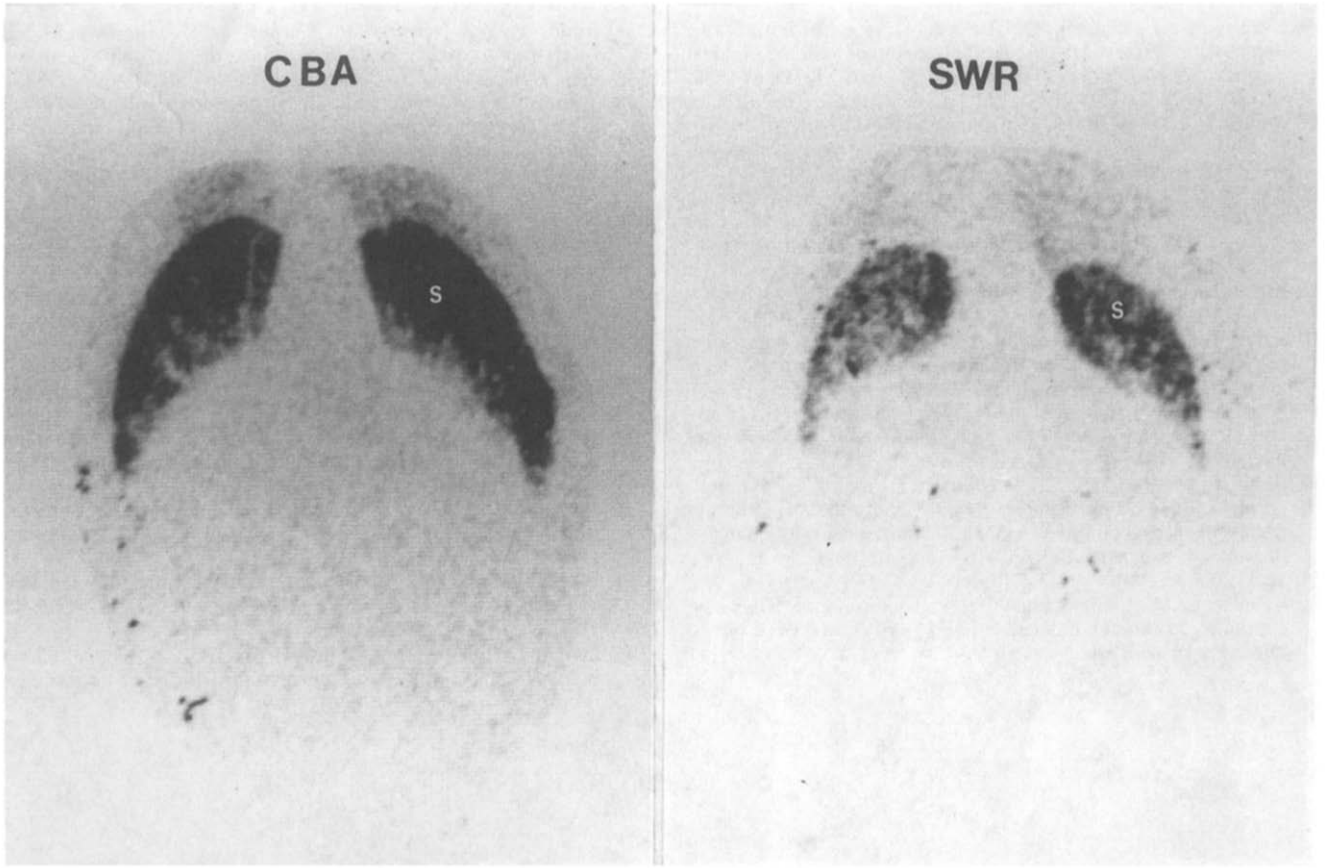

FIG. 5. Representative autoradiographic images of specific [^{3}H]NECA (4 nM) binding to (a) CBA/J and (b) SWR/J horizontal brain sections. Specific [^{3}H]NECA binding was revealed as described in Fig. 4 legend. Abbreviation is striatum (S).

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