AUTORADIOGRAPHIC LOCALIZATION OF ADENOSINE UPTAKE SITES IN RAT BRAIN USING [3H]NITROBENZYLTHIOINO freshwater

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Abstract

The adenosine uptake site has been localized in rat brain by an in vitro light microscopic autoradiographic method, using [3H]nitrobenzylthioinosine ([3H]NBI) as the probe. The binding characteristics of [3H]NBI on slide-mounted sections are comparable to those seen in studies performed on brain homogenates. A very high density of uptake sites occurs in the nucleus tractus solitarius, in the superficial layer of the superior colliculus, in several thalamic nuclei, and also in geniculate body nuclei. A high density of sites are also observed in the nucleus accumbens, the caudate putamen, the dorsal tegmentum area, the substantia nigra, and the central gray. The localization of the adenosine uptake site in brain may provide information on the functional activity of the sites and suggests the involvement of the adenosine system in the central regulation of cardiovascular function.

Materials and Methods

The autoradiographic procedure involves incubating slide-mounted tissue sections with [3H]NBI (1 Ci/mmol, Moravek Biochemicals) to label the binding sites and apposing tritium-sensitive film (LKB Ultrofilm). The details of this procedure have been reported previously (Herkenham and Pert, 1982). Male Sprague-Dawley rats (250 to 300 gm) were decapitated, and the brains were removed quickly and frozen at 40°C in 2-methylbutane, then fixed onto a brass Microtome chuck with embedding media (Tissue Tek II, Lab Tek). Sections of 20 µm were cut at 4°C in an American Optical cryostat, thaw-mounted on gelatin-coated glass slides, and stored at 20°C until needed. A set of intercalated sections was saved from each rat brain for staining with thionine.

Receptor labeling procedure.Binding was performed by incubating slide-mounted sections at room temperature for 30 min with [3H]NBI (0.7 nM) in 50 mM Tris-HCl (pH 7.4) with or without other compounds of interest. Following incubation with [3H]NBI, the tissue section was washed in buffer alone at 0°C (three 1-min washes). Blank values were generated by adding an excess of NBI (20 µM final concentration). In the preliminary biochemical studies the tissue sections were wiped off the slide with a Whatman GF-B filter and placed in a scintillation vial with 15 ml of Ready-Solv (Beckman). The radioactivity was measured in a scintillation counter (Searle) using a single-channel ratio for calculation of the efficiency.

Receptor autoradiographic studies. For the autoradiographic studies sets of 120 sections from four different animals were incubated under optimized conditions using 0.7 nM [3H]NBI for 30 min. Nonselective binding was obtained on adjacent sections by adding to the incubation medium an excess of NBI. Following the incubation and the rinses the sections were placed on racks and dried under a stream of cold, filtered, dry air.
and then placed in apposition to LKB Ultrascan in a light-proof cassette (Wolf Picker). After 9 weeks of exposure, the films were developed with Kodak D-19 (4 min at 22°C), then rinsed in running water for 30 min, cleaned with deionized water, and dried at room temperature. The autoradiograms were screened and optical density measurements were performed using a computer image-processing system previously described (Gooch et al., 1980). The identification of the brain microregions was made with the aid of adjacent stained sections, using as reference the atlases of Paxinos and Watson (1982) and Koenig and Klippel (1963). For further comparison, one set of rat brain sections was incubated with \[^{3}H\]cyclohexyladenosine, using conditions previously described (Lewis et al., 1981).

**Results**

**Biochemical properties of \[^{3}H\]NBI binding to brain slices.** \[^{3}H\]NBI binding to rat brain sections is saturable (Fig. 1). Nonspecific binding represents 30% of the total binding for \[^{3}H\]NBI concentrations below 1 nM.

Scatchard analysis of the binding shows a dissociation constant (K_d) of 0.39 nM and a maximal number of sites (B_max) of approximately 150 fmol/mg of protein (Fig. 1). These values are comparable to those observed in rat brain homogenates (Marangos et al., 1982). To ensure that the binding in brain sections involved the same site that had been characterized previously in brain membranes, we evaluated the displacement of \[^{3}H\]NBI by NBI, N^6-cyclohexyladenosine (CHA), a specific ligand of the adenosine receptor, and dipyridamole. The IC_{50} values (data not shown) obtained were 2 nM for NBI, 6 μM for dipyridamole, and 15 μM for CHA, all of which are comparable to those observed with membrane preparations (Marangos et al., 1982). The binding to brain sections reached an equilibrium
after 20 min, and more than 70% of the initial specific binding remained after a 20-min wash in 500 ml of 50 mM Tris buffer (data not shown).

Regional distribution of \(^{3}H\)NBI-binding sites. The optical densities of the various brain areas measured are summarized in Table I. In the forebrain several structures show high NBI binding density, among which is the pyriform cortex. All other cortical areas show a very low density of binding sites with a more elevated level in layer IV, with the exception of cingulate cortex which shows a moderate to low density of sites (Fig. 2, B and D). Caudate putamen and nucleus accumbens show a homogeneous high density. In the septum only the dorsal part of the lateral septum displays a high density of binding sites (Fig. 3a). In the amygdala, moderate optical densities are observed in medialis and cortical nuclei. The hippocampus shows a very low density of sites (Fig. 2D). In the midbrain, high densities are observed in the superficial layer of the superior colliculus, the central gray matter in the substantia nigra, the dorsal tegmentum area, where both nuclei parabrachialis superior and inferior and locus ceruleus show higher density than does the nucleus tegmentum dorsalis of Gudden (Figs. 3 and 4). In the hindbrain the localization of NBI-binding sites is very heterogeneous. The nucleus tractus solitarius shows the highest density of sites in the whole brain. A very high density of sites is observed in the commissural part of that nucleus, the rostral part shows lower density. (Fig. 5, a and b). The substantia gelatinosa of the nucleus of the spinal tract of the trigemini nerve also shows a high density of NBI sites (Fig. 5b). Moderate levels of binding are observed in the superior olivary complex (Fig. 5b). The cerebellum has a low homogeneous level of sites (Fig. 2F). All white matter areas seem to have a negligible amount of NBI-binding sites.

### Discussion

In this study we show that the binding of \(^{3}H\)NBI to brain tissue sections has characteristics of the adenosine uptake site in brain homogenates (Marangos et al., 1982). The binding is saturable with kinetic constants compatible to those observed in homogenate preparations and shows comparable pharmacological properties. The autoradiographic study shows a heterogeneous distribution of adenosine uptake sites throughout the brain that is basically consistent with the regional distribution observed in brain homogenate studies (Marangos et al., 1982). In order to evaluate the meaning of this distribution, we have compared the localization of the adenosine uptake site with the localization of the adenosine A-1 receptor (Table II). For this comparison we have used previous reports describing the anatomical distribution of adenosine receptor (Lewis et al., 1981; Goodman and Snyder, 1982), and we have performed an autoradiographic study on a set of sections adjacent to those used for \(^{3}H\)NBI binding. The results of this comparison are summarized in Table I and illustrated in Figure 2. Some structures that show a very high density of both sites are the caudate putamen, nucleus accumbens, dorsal septum, pyriform cortex, substantia nigra, superficial layer of the superior colliculus, and substantia gelatinosa of the nucleus of the spinal tract of the trigeminal nerve. In the thalamus both the adenosine receptor and the adenosine uptake site are also present at high densities, but some differences exist in their relative distribution among the different thalamic nuclei. Higher adenosine receptor densities are observed in the anterior nuclei, gelatinosa, and medial nuclei. Higher levels of adenosine uptake sites are seen in paraventricular nucleus and the rhomboid and reuniens nuclei (see Fig. 2). In contrast, some structures which possess a very high density of adenosine receptors show low amounts of aden-
The relative density of both the receptor and uptake site could represent one index of functional activity of the adenosine system, high activity being associated with high number of adenosine receptor and uptake sites. In light of this, an active adenosinergic system might be expected in caudate putamen, nucleus accumbens, pyriform cortex, septum, substantia nigra, superior colliculus (superficial layer), and substantia gelatinosa of the trigeminal nerve. In contrast, cerebellum and hippocampus, although rich in the adenosine receptor, might display a lower level of adenosinergic function. Further physiological studies are of course required to substantiate these speculations.

A third group of structures containing a high density of the adenosine uptake site with very low levels of adenosine receptor also exists. In this group are found the nucleus of the tractus
Figure 3. Distribution of [3H]NBI-binding sites in various brain areas. a is section A7474 µ (Koenig and Klippel, 1963). Moderate to high densities of receptor are observed in the dorsal part of the lateral septum (ds), in the caudate putamen (c), in the nucleus of stria terminalis (*), in the medial preoptic area (mpo), and in the pyriform cortex (P). b is one intermediate section between −5.3 and −5.8 mm bregma (Paxinos and Watson, 1982). The superficial layer of the superior colliculus (SC) shows a very high receptor density. The substantia nigra (sn), the central gray matter (cgm) (specifically, its dorsal part), and the dorsal part of the geniculate body (G) show moderate to high density of [3H]NBI sites. Only a low [3H]NBI receptor density is present in gyrus dentatus (GD).

Figure 4. [3H]NBI-binding site distribution in the dorsal tegmentum area of the rat brain. The locus ceruleus (LC) shows higher [3H]NBI site density than do the surrounding nuclei: nucleus dorsal tegmentum of Gudden (dg), nucleus parabrachialis dorsal (dpb) and ventral (vpb).

Figure 5. [3H]NBI site distribution in the rat brainstem. In both a and b appear the very high density [3H]NBI site localization of the nucleus of tractus solitarius (nts) in its medial and then rostral part. A low receptor density is present in the inferior olivary complex (io). In a, high site density is observed on the substantia gelatinosa (Vg) of the nucleus of the spinal tract of the trigeminal nerve (sv). The hypoglossal nucleus (12) has a low but significant level of [3H]NBI receptor.

It is also possible that the [3H]NBI-binding sites seen in some brain areas function to take up other nucleosides, since this has been shown to be possible (Hammond and Clanachan, 1983). However, it has been shown that the adenosine uptake site does favor adenosine (Bender, 1980).

The presence of a very high density of adenosine uptake sites in the nucleus of the tractus solitarius could be related to some central effect of adenosine. This nucleus, particularly its commissural part, represents the main central control structure of blood pressure. The closely related nucleus parasolitarius is also involved in the control of respiration (Palkovits and Za-
The highest density of adenosine uptake site is in the pars commissuralis of the nucleus tractus solitarius. BZ receptors are only present in very low density in that area.

The adenosine uptake site is, therefore, highly localized in rodent brain with a regional distribution that is different from that of the adenosine receptor labeled by \(^{3}H\)CHA and the BZ receptor. It appears, therefore, that certain brain nuclei have a greater density of adenosine uptake sites, and it is possible that these areas may represent brain regions which contain adenosinergic neurons.

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