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ADENOSINE UPTAKE SITES IN RAT BRAIN: IDENTIFICATION USING [^{3}H]NITROBENZYLTHIOINOSINE AND CO-LOCALIZATION WITH ADENOSINE DEAMINASE

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The binding characteristics of [^{3}H]nitrobenzylthioinosine ([^{3}H]NBI) to rat brain membrane preparations was examined, and the autoradiographic distribution of this ligand in brain sections was compared with the immunohistochemical localization of adenosine deaminase (ADA). It was found that [^{3}H]NBI labels sites for which adenosine has far higher affinity than do other nucleosides, that these sites are heterogeneously distributed and that there is an exact correspondence between areas containing [^{3}H]NBI sites and ADA-immunoreactive neurons. Our results indicate that [^{3}H]NBI and ADA are potential markers for revealing anatomical sites at which actions of adenosine may be expressed.

There is a growing concensus that adenosine is fundamentally involved in interneuronal communication in the central nervous system [7, 11, 20, 23, 24]. A major issue is whether distinct neural systems exist which preferentially release adenosine. Currently, the localization of adenosine receptors [13, 14] and their associated adenylate cyclases [9, 12, 26] has been the only means available to define brain structures and neuronal pathways whose function may be selectively influenced by adenosine. In order to identify possible neuronal release sites of adenosine, we have focused on the processes of adenosine reuptake and metabolism since these are typical presynaptic functions of other neurotransmitters [10]. Adenosine uptake sites in the rat brain were examined using the well established nucleoside transport inhibitor, nitrobenzylthioinosine (NBI) [4, 15, 28], and the distribution of these sites was correlated with that of neurons containing the enzyme, adenosine deaminase (ADA) [19].

All experiments were performed using adult male Sprague-Dawley rats. For

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[^{3}H]NBI binding studies using membrane preparations, whole brains (minus the cerebellum and brain stem) were homogenized in 50 mM Tris-HCl buffer, pH 7.4 (Tris buffer) using a Polytron set at medium speed. The homogenate was centrifuged at 30,000 *g*, and the resultant membrane pellet was twice more resuspended by homogenization in Tris buffer and centrifuged. The final membrane pellet was resuspended in Tris buffer to a final concentration of 1.5–2.0 mg protein/ml as determined by the method of Lowry et al. [16] using bovine serum albumin (BSA) as standard. [^{3}H]NBI binding was conducted in a final volume of 1.0 ml of Tris buffer containing 0.6 nM [^{3}H]NBI (16 Ci/mmol; Moravek Biochemicals, Brea, CA, U.S.A.), 150–200 μg of membrane proteins in the absence (total binding) or presence of 5 μM NBI (non-specific binding). Incubations were conducted for 15 min at 24°C and terminated by rapid filtration through Whatman GF/B filters. The filters were washed twice with ice-cold Tris buffer, placed in vials containing 10 ml of scintillation fluid (ACS; Amersham) and counted in a scintillation counter.

For immunohistochemical analysis, tissues were processed following transcardiac perfusion with 4% paraformaldehyde containing 0.1 M sodium phosphate buffer, pH 7.4. Free floating frozen microtome sections were incubated with antibody to ADA for 48 h at 4°C in a solution containing 0.9% NaCl, 0.1 M sodium phosphate buffer (pH 7.4) and 0.3% Triton X-100. Sections including those serving as primary antibody absorption controls, were processed using the peroxidase-anti-peroxidase (PAP) method as previously described [19].

For [^{3}H]NBI autoradiography, unfixed cryostat sections were collected on cold gelatinized slides. The sections were washed twice in 50 mM Tris-HCl buffer (pH 7.4) for 15 min, incubated with 1 nM [^{3}H]NBI for 20 min at 25°C, washed twice for 5 min in the same buffer without [^{3}H]NBI and dried overnight at 4°C. The sections were exposed for 3 weeks to Ultrofilm which was then developed with Kodak D-76 developer and printed on Agfa-Gevaert No. 6 paper giving the final appearance of a dark-field photomicrograph.

Specific [^{3}H]NBI binding to brain membranes was saturable, reversible, reached equilibrium within 2 min and typically represented 85% of total binding (see legend to Fig. 1). In agreement with previous reports [18, 21], computer analysis of the data indicated that [^{3}H]NBI binds to a single class of sites with K_d and B_{max} values of 0.06 nM and 147 fmol/mg protein, respectively. As shown in Fig. 1, adenosine displaced [^{3}H]NBI binding with a potency 50–330 times greater than the other nucleosides tested. The affinities of the nucleosides (expressed as K_i values calculated from IC_{50} data, μM) for the [^{3}H]NBI site were: adenosine, 3.0; inosine, 164; thymidine, 245; uridine, 391; guanosine, 464; and cytidine, 1000. These K_i values agree favorably with the K_m (1–5 μM) of adenosine uptake into CNS tissues and the K_i (100–300 μM) with which other nucleosides inhibit this uptake [2, 25]. In contrast, the K_m for nucleoside transport into peripheral tissues is in the range of 100 μM for adenosine and 100–300 μM for the other nucleosides [22]. These findings together with evidence that [^{3}H]NBI binding sites are present only on cells with

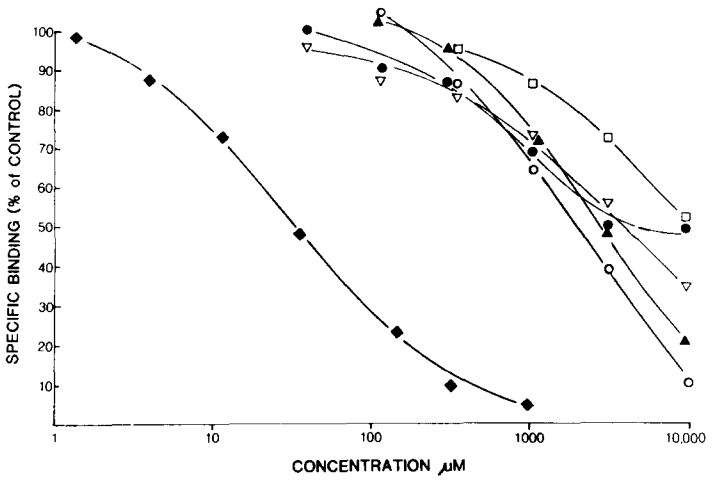

Fig. 1. Displacement of [^{3}H]NBI binding to rat brain membranes by various nucleosides. Displacement curves for the nucleosides adenosine (◆), inosine (○), thymidine (▲), uridine (▽), guanosine (●), and cytidine (□) were constructed using 8–10 concentrations of these agents. IC_{50} values were calculated on the basis of percent of control binding levels which was determined as that amount of [^{3}H]NBI binding displaceable by 5 μM NBI. All samples were assayed in duplicate, and these results are representative of experiments performed at least twice with similar results.

with functional nucleoside transport sites, indicate with reasonable certainty that [^{3}H]NBI labels a physiologically relevant, high-affinity nucleoside transport system in rat brain. However, this system in brain, with its preference for adenosine, appears to exhibit a more restricted substrate specificity compared to peripheral tissues. This is the likely basis for the greater affinity and selectivity of [^{3}H]NBI for what appears to be primarily an adenosine transporter in CNS tissue.

The autoradiographic localization of [^{3}H]NBI binding sites in two brain regions, the superior colliculus and hypothalamus, is shown in Fig. 2. In the superior colliculus, the highest density of sites was found in its most superficial layer, the stratum griseum superficiale (SGS) (Fig. 2c). Substantially less [^{3}H]NBI labeling was found in the overlying cortex, the inferior colliculus (ic) and deeper collicular layers. In the hypothalamus (Fig. 2d), the heaviest accumulation of silver grain occurred in its posterior basal portion in a band immediately flanking the mammillary nucleus (mn) both anteriorly and posteriorly. These qualitative results are in agreement with quantitative determinations of the regional distribution of [^{3}H]NBI sites in the CNS where, of the 30 discrete areas we examined, the superior colliculus and hypothalamus exhibited among the highest levels of [^{3}H]NBI binding sites (data not shown). Fig. 2e shows a tissue section similar to that in Fig. 2c but incubated with [^{3}H]NBI in the presence of 5 μM unlabelled NBI. The low level of background labelling is consistent with the high proportion of specific binding obtained using membrane preparations.

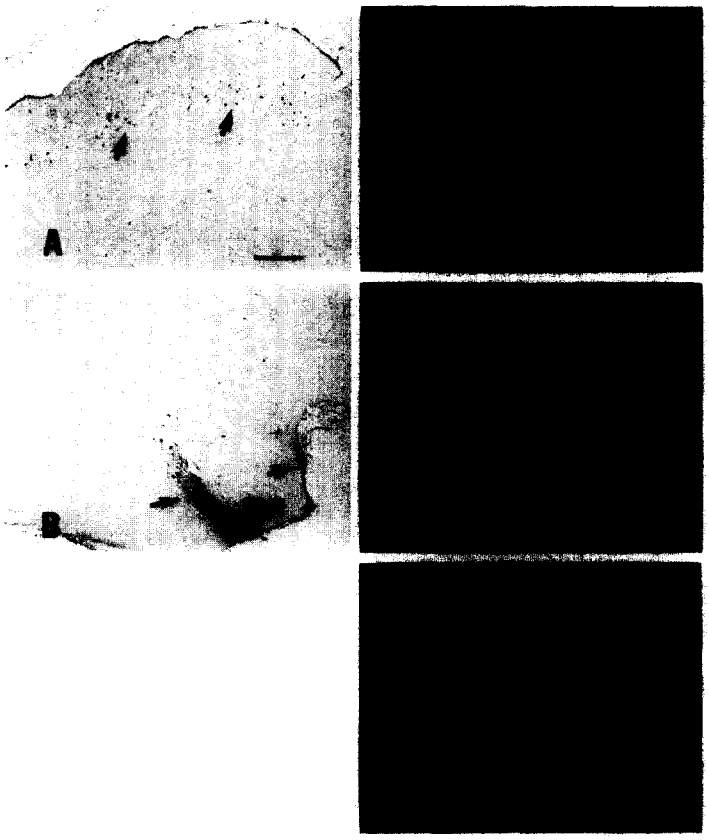

Fig. 2. Parasagittal sections of rat brain showing the distribution of neurons stained immunohistochemically for the enzyme adenosine deaminase (ADA) (A, B) and the autoradiographic localization and ^{3}H-nitrobenzylthioinosine ([^{3}H]NBI) binding sites (C, D). Note the close correspondence between areas containing the greatest concentration of ADA-immunoreactive neural elements and the highest density of autoradiographic silver grains. These areas include the superficial layers of the superior colliculus (sc) (arrows) and hypothalamic regions surrounding the mammillary nucleus (mn) (arrows). Non-specific binding of [^{3}H]NBI (shown in E) was determined in adjacent sections incubated with [^{3}H]NBI in the presence of 5 μM non-radiolabeled NBI. The level of non-specific binding thus obtained was similar to areas on the glass slides devoid of tissue. Scale bars: A, 500 μm; B–E shown in D = 500 μm.

That the binding of [^{3}H]NBI to sites in tissue homogenates and sections collected on slides represent the same sites was confirmed by comparing the kinetics of [^{3}H]NBI binding in the two preparations. Following incubation of sections in a manner identical to that used for whole brain membrane homogenates and autoradiographic studies, the sections were scraped from glass slides for scintillation counting. Scatchard analysis gave K_d and B_{max} values of 0.16 nM and 126 fmol/mg protein, respectively, which agree favorably with the above values observed in whole brain homogenates.

The distribution of [^{3}H]NBI sites in brain was compared with the location of neurons previously described to have high levels of the enzyme ADA as demonstrated immunohistochemically [19] using specific antibody to purified ADA [6]. Fig. 2a shows that ADA-immunoreactive neurons in the superior colliculus are concentrated in a zone straddling the deep portion of SGS and the subjacent collicular layer, the stratum opticum. A few ADA-containing neurons, however, can be seen scattered throughout SGS. In the hypothalamus, just anterior to the mammillary nucleus, ADA-immunoreactive neurons and their processes were located in a narrow transverse plane which in the parasagittal section shown in Fig. 2b is seen as a band of ADA-positive cells. ADA-containing neurons are also distributed in a region posterior to mn.

These results show that in the superior colliculus and hypothalamus there is an exact coincidence of the location of ADA-rich neurons with areas exhibiting the highest density of [^{3}H]NBI binding sites. However, it is clear from our more extensive regional analysis that this correlation extends beyond these two structures and the parykarya of ADA-rich neurons. For example, the SGS of the superior colliculus and several other CNS structures contained dense networks of ADA-immunoreactive axons and correspondingly high levels of [^{3}H]NBI sites. In some cases ADA-containing fiber pathways were seen clearly delineated in autoradiographs by their high levels of [^{3}H]NBI sites. These results strongly suggest that the cell bodies, axons and terminals of ADA-rich neurons contain an abundance of [^{3}H]NBI sites.

It is apparent from several observations that reuptake and catabolism may be important regulatory events governing the bioavailability of adenosine at its receptor. Blockers of adenosine uptake and inhibitors of ADA potentiate the sedative and antinociceptive actions of adenosine and its stable analogues in animals [5, 27] and the inhibitory effects of adenosine on neuronal electrical activity in the CNS [20]. These findings together with the existence of central neurons rich in both ADA and adenosine uptake sites suggest a relationship between these neurons and the neuronal source of calcium-dependent adenosine release which has been demonstrated in vitro [3, 8, 17] and in vivo [1]. It is reasonable to propose that neurons which have a high capacity to accumulate and metabolize adenosine may also exhibit the greatest propensity to release this substance. In support of this possibility, it is compelling to draw an analogy with other small molecule putative neurotransmitter agents which are contained in and released from neurons having high synthetic, degradative and reuptake capabilities for their transmitter. If ADA and [^{3}H]NBI are associated with sites at which adenosine is released then (1) these may be valuable markers in locating structural units for studying the actions of adenosine at the synaptic level and (2) the present results would suggest that adenosine release in the CNS is not a ubiquitous phenomenon but rather may be restricted to specific pathways.

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