Characterization, Distribution, and Ontogenesis of Adenosine Binding Sites in Cat Visual Cortex

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In vitro autoradiographic techniques were used to characterize binding sites for $^3$H-cyclohexyladenosine (CHA) and $^3$H-$S$-N-ethylcarboxamidoadenosine (NECA) in cat and kitten visual cortex. $^3$H-CHA binding sites in adult cat have a $B_{max}$ of 1363 fmol/mg protein and a $K_I$ of 6.8 nM. Displacement experiments indicate that $^3$H-CHA binds to an adenosine receptor similar to the $A_1$-adenosine receptor described by other investigators. $^3$H-NECA binding sites in adult cat have a $B_{max}$ of 518 fmol/mg protein and a $K_I$ of 15.4 nM. Displacement experiments do not allow us to identify this binding site unambiguously. $B_{max}$ values increase during postnatal development for both binding sites, peaking in adulthood for $^3$H-CHA and at 30 d for $^3$H-NECA. $K_I$ values show neither consistent nor significant differences during postnatal development for either binding site. $^3$H-CHA and $^3$H-NECA binding sites are concentrated in layers 1-3 and upper layer 5 in the visual cortex of adult cats. These laminar patterns, however, change during postnatal development, showing the densest binding in the deep cortical layers (5 and 6) in kittens younger than 30 d of age and a fairly homogeneous binding in older kittens before achieving the adult distribution.

The visual cortex plays a critical role in the analysis of visual signals concerned with form, motion, and depth. The mechanisms by which the cortex processes visual information have been the subject of numerous investigations at the physiological (Hubel and Wiesel, 1962), anatomical (Lund, 1973), and neurochemical (Emson and Hunt, 1979) levels. In particular, the last few years have witnessed a dramatic increase in our understanding of the chemistry of the cortex. Accumulating evidence gathered with immunocytochemical, iontophoretic, and pharmacological (Emson and Hunt, 1979) have provided information on the characteristics and laminar distributions of the various receptor populations. Special attention has been directed towards the study of receptor alterations during normal postnatal development, since several receptor populations show differing characteristics and/or distributions in infant and adult animals (Shaw et al., 1984a, c, 1985). In these cases, receptor alterations take place during the physiologically defined critical period (Cynader et al., 1980; Hubel and Wiesel, 1970; Olson and Freeman, 1980), the period in the first few months of postnatal life of greatest neuronal plasticity. Our working hypothesis is that alterations of receptor distribution, number, and/or affinity may play a role in the mechanism by which visual exposure modifies cortical function during the critical period.

In the present study, we have examined adenosine binding sites in visual cortex using $^3$H-CHA and $^3$H-NECA as ligands (Bruns et al., 1980; Goodman et al., 1983). The adenosine binding sites are characterized and their postnatal development charted in terms of number, affinity, and laminar distribution. While this paper was being revised, a report by Aoki (1985) appeared describing adenosine binding site distribution in normal and dark-reared cats.

Materials and Methods

Fourteen male colony cats were maintained on a 14 hr/10 hr light/dark cycle and were sacrificed at approximately the same time of day (1600–1830). Following an overdose of sodium pentobarbital, they were perfused through the heart with cold (4°C) phosphate buffer solution (PBS; 0.1 M, pH 7.4). In some animals, the PBS was followed by a 0.1–0.2%...
formaldehyde/PBS solution in order to lightly fix the tissue. This procedure had no discernible effect on the characteristics of adenosine binding sites. The brains were quickly removed and frozen in liquid Freon. Sixteen micron coronal sections were cut on a cryostat and thaw-mounted onto subbed glass slides, then stored at -20 to -25°C until used. In experiments designed to compare animals of different ages, sections were cut within the same 24 hr period and stored for the same length of time before being used.

3H-CHA (New England Nuclear; specific activity, 25 Ci/mmol), an adenosine agonist, was used to study binding at A1-adenosine binding sites, and 3H-NECA (New England Nuclear; specific activity, 30 Ci/mmol) was employed in an attempt to label both A1 and A2 binding sites.

The slide-mounted sections were allowed to warm to 4°C. The slides were then preincubated for 10 min in dishes containing 4°C Tris-HCl buffer (50 mM, pH 7.7). A small amount of formaldehyde (0.2%) was added to lightly fix the tissue. Two additional 5 min washes in buffer alone removed the formaldehyde. Reduction of endogenous adenosine was accomplished through the addition of adenosine deaminase, Sigma type VIII (Goodman and Snyder, 1982), to all 3 preincubation rinses at a concentration of 0.06 IU/ml. In preliminary experiments we determined that raising the preincubation concentration of adenosine deaminase to 1 IU/ml gave, at most, 20% higher binding. For this reason, preincubation times contained the lower concentration. All incubation media, however, contained adenosine deaminase at a concentration of 1 IU/ml (Goodman and Snyder, 1982). After the preincubation washes, the slides were laid out on a black plastic tray to aid visualization of the sections. The incubation medium containing the tritiated ligand (0.4-52 nM for both ligands) was dripped onto the sections. Nonspecific binding was always performed in duplicate. All experiments for these characterizations were performed at least twice. All time course and displacement curves thus represent average values for 2 or more experiments.

Characterization of adenosine binding sites using 3H-CHA and 3H-NECA

Figures 1 and 2 illustrate biochemical experiments in which we characterized the binding sites for 3H-CHA and 3H-NECA, respectively. For each experiment, each point represents at least 3 separate determinations for total binding. Determination of nonspecific binding was always performed in duplicate. All experiments for these characterizations were performed at least twice. All time course and displacement curves thus represent average values for 2 or more experiments.

Figure 1. Top, Measurements of association and dissociation time constants for 3H-CHA binding sites in the visual cortex (areas 17-19) of an adult cat. Incubation was at 23°C with 9 nM 3H-CHA. Total binding values (TB) are given for the association points, while total, specific (SB), and nonspecific (NSB) values are shown for the dissociation points. For association rate measurements, the rinse was for 1 min. Arrow indicates the beginning of the postincubation rinse. Two 5 min rinses were adopted for subsequent autoradiographic studies. This rinse time left the specific binding quite high while virtually eliminating nonspecific binding. Center, Saturation binding for 3H-CHA in the visual cortex (areas 17-19) of an adult cat. The 3H-CHA incubation was for 120 min at 23°C. Nonspecific binding was measured by coincubation of alternate sections with 1 x 10^-4 M unlabeled CHA. Filled circles, total binding; open circles, specific binding; triangles, nonspecific binding. Inset, Eadie-Hofstee plot. Bottom, Displacement studies for 3H-CHA in the visual cortex (areas 17-19) of an adult cat. IC50 values are given in Table I. Incubation in each case was in 20 nM 3H-CHA for 120 min at 23°C with the appropriate displacers present.
Figure 2. Top. Time constant measurements for $^3$H-NECA binding sites in the visual cortex (areas 17-19) of an adult cat. Incubation was at 23°C using 8 nM $^3$H-NECA. All other details as in Figure 1. Center, Saturation binding for $^3$H-NECA in the visual cortex (areas 17-19) of an adult cat. The $^3$H-NECA incubation was for 120 min at 23°C. Nonspecific binding was measured by coincubation of alternate sections with $1 \times 10^{-4}$ M L-PIA. Filled circles, total binding; open circles, specific binding; triangles, nonspecific binding. Inset, Eadie-Hofstee plot. Bottom, Displacement studies for $^3$H-NECA in the visual cortex (areas 17-19) of an adult cat. $IC_{50}$ values are given in Table 1. Incubation in each case was in 20 nM $^3$H-NECA for 120 min at 23°C with the appropriate displacers present.

The time course of association and dissociation was examined for $^3$H-CHA binding (Fig. 1, top panel). At 23°C, equilibrium binding was achieved by 120 min, remaining stable for at least another hour. The association rate constant ($K_a$) was calculated to be $2.06 \times 10^{-3}$ min$^{-1}$ nM$^{-1}$. Rinsing the sections in an "infinite" dilution led to a rapid decline in specific binding over 10 min, followed by a slower decline. The dissociation rate constant ($K_d$) was calculated to be $1.93 \times 10^{-2}$ min$^{-1}$. The
Figure 3. Laminar binding patterns for $^3$H-CHA (A) and $^3$H-NECA (B) in the visual cortex of adult cats. In both cases, incubation was for 120 min at 23°C. Ligand concentrations were 26 nM (A) and 30 nM (B); exposure period for LKB Ultrofilm was 14 d (A) and 21 d (B). Adjacent sections in each instance were processed for cytochrome c oxidase activity (Wong-Riley, 1979). Photographs of the autoradiograms and the cytochrome c-stained sections were made to the same magnification. A portion of a photograph of a cytochrome c-stained section (medial bank of area 17) has been superimposed on the photograph of the autoradiogram. Dark arrows indicate layer 4 which stains most densely for cytochrome c oxidase; white arrow in A points to the labeled sublayer in layer 5. Calibration bar, 1 mm. Dorsal (D) and medial (M) directions are as indicated.

Table 1. $IC_{50}$ values (M) of various adenosine analogs displacing $^3$H-CHA and $^3$H-NECA

| Displacer | $^3$H-CHA binding sites | $^3$H-NECA binding sites |
|-----------|-------------------------|--------------------------|
| CHA       | $5 \times 10^{-9}$       | $1.6 \times 10^{-9}$     |
| L-PIA     | $1 \times 10^{-8}$       | $8 \times 10^{-10}$      |
| D-PIA     | $4 \times 10^{-8}$       | $5.6 \times 10^{-9}$     |
| NECA      | $1.6 \times 10^{-7}$     | $1.4 \times 10^{-8}$     |
| DPX       | $1 \times 10^{-6}$       | $1 \times 10^{-6}$       |
| Theophylline | $3 \times 10^{-4}$     | $4 \times 10^{-5}$       |

Displacement experiments were performed for $^3$H-CHA and $^3$H-NECA using various adenosine analogs. The numbers shown represent $IC_{50}$ values (the concentration required to displace 50% of total binding) for each compound. PIA, phenylisopropyl adenosine; DPX, 1,3 diethyl-8-phenylxanthine.
Figure 4. Distribution of $^3$H-CHA binding sites in the visual cortex of cats of different postnatal ages: A, 3 d; B, 15 d; C, 30 d; D, 60 d; E, 95 d; F, adult. Ligand concentration, 26 nM. Photographs are from LKB Ultrofilm autoradiograms photographed for actual contrast; film exposure, 14 d. Note the changes in laminar distribution during development, from deep layers in the young kittens to superficial layers in adults. For each cat the different laminae were identified in alternate sections stained with cresyl violet or cytochrome c oxidase. Calibration (all panels), 1 mm. Dorsal is up, medial is left for all panels.

Impervious to additions of up to $1 \times 10^{-6} \text{M}$ dipyridamole, a blocker of facilitated diffusion of adenosine into cells (Wu et al., 1981). Neither the number of binding sites, nor their laminar distributions, was altered for either ligand. These data suggest that neither $^3$H-CHA nor $^3$H-NECA is labeling high-affinity adenosine transport sites in cat visual cortex.

Distributions of $^3$H-CHA and $^3$H-NECA binding sites in cat visual cortex

The laminar binding patterns for $^3$H-CHA and $^3$H-NECA are illustrated in Figure 3. These photographs of the autoradiograms have been combined with inserted slices of photographs from...
Figure 5. Distribution of $^3$H-NECA binding sites in the visual cortex of cats of different postnatal ages: A, 3 d; B, 15 d; C, 30 d; D, 60 d; E, 95 d; F, adult. Ligand concentration, 30 nM. Photographs are from LKB Ultrofilm autoradiograms photographed for actual contrast; film exposure, 21 d. Changes in laminar distribution are similar to those seen for $^3$H-CHA binding sites (see Fig. 4). Calibration (all panels), 1 mm. Dorsal is up, medial is left for all panels.
alternate sections processed for cytochrome c oxidase. Cytochrome c oxidase labels layer 4 most densely in cat visual cortex (Wong-Riley, 1979), offering a convenient laminar landmark with which to identify laminar patterns of receptor binding. In the adult cat, 'H-CHA (Fig. 3A) showed the densest binding in the supragranular layers (layers 1–3). Moderate binding was also seen in the upper portion of layer 5. 'H-NECA labeled binding sites with essentially the same laminar distribution as that of 'H-CHA in the adult cat cortex (Fig. 3B).

**Postnatal development of 'H-CHA and 'H-NECA binding patterns**

Figure 4 illustrates the laminar distribution of 'H-CHA binding sites in kittens of various postnatal ages. The illustrations shown here have been photographed from the original autoradiograms at the same contrast level in order to allow direct comparisons among the different panels of Figure 4. At 3, 15, or 30 d after birth (panels A–C), layers 5–6 were most densely labeled, while the superficial cortical layers were relatively lightly labeled. By 60 d of age, the deep cortical layers were still labeled, but moderate binding had appeared in the superficial layers as well, resulting in a relatively laminarly homogeneous, albeit slightly patchy, binding pattern. By 95 d postnatal (panel E), the adult binding pattern became more apparent: Layers 1–3 exhibited dense binding, with layers 4–6 much lighter in comparison. 

'H-CHA and 'H-NECA binding in other cortical and subcortical areas

Studies of other receptors in cat and raccoon cortex reveal that receptor binding in primary sensory cortices is often denser and can show different lamination patterns than that in adjacent association cortices (Sampson et al., 1984; Shaw et al., 1984a). 'H-CHA and 'H-NECA binding in cat visual cortex likewise follows this pattern: Areas 17 and 18 have denser binding and more distinct lamination than nearby association cortex. Similarly, auditory cortex shows a highly laminar-specific binding pattern, with the highest binding densities in the supragranular laminae (data not shown). Binding also becomes less dense and more homogeneous across cortical layers in nonsensory cortical areas. Figure 8 illustrates a clear transition between the binding pattern in the ventral-most part of the striate cortex and that of the cingulate cortex.

'3H-CHA and '3H-NECA have similar distributions in subcortical structures. Stratum oriens and stratum radiatum of the CA1 to CA3 fields of the hippocampus label densely. The subiculum and the inner molecular layers of the dentate gyrus also label densely for both ligands. The lateral geniculate nucleus (LGN) shows moderate and homogeneous binding in the cell layers, with the interlaminar zones relatively free of label. The medial geniculate nucleus labels moderately with some inhomogeneities. Both inferior and superior colliculi show low-moderate binding in the superficial layers only. Figure 8 shows '3H-CHA binding sites in the cat diencephalon midbrain to illustrate adenosine receptor distribution in some of the regions mentioned above.

**Discussion**

The identification of the '3H-CHA binding site in the cat visual cortex as an A1-adenosine receptor seems relatively straightfor-
Figure 8. Montage of 2 coronal sections of cat diencephalon midbrain and cortex are used to illustrate \(^{3}H\)-CHA binding in the visual cortex and other cortical and subcortical areas. The sections were incubated with 26 nm \(^{3}H\)-CHA for 120 min at 23°C. Note in general the dense binding in cortex compared with most of the diencephalon. A. The most densely labeled diencephalic areas are the hippocampus (hipp.; see text for further description) and the lateral and medial geniculate bodies (lgn, mgn). A clear laminar difference is observable between the binding in visual cortex (vc) and cingulate cortex (cing). B. Low to moderate binding is observed in the superficial layers of the superior colliculus (sc). Film exposure, 14 d. Calibration bar, 1 mm. Dorsal is up, medial is right in A and left in B.

The binding pattern is similar to that reported for \(A_1\)-adenosine receptors in the cat (Aoki, 1985); in rat cortex the binding pattern differs (Goodman and Snyder, 1982; Goodman et al., 1983). Additionally, the \(K_d\) and displacement characteristics are generally similar to those reported for \(A_2\)-adenosine receptors previously described (Aoki, 1985; Bruns et al., 1980; Goodman and Snyder, 1982), although Aoki (1985) reports 2 distinct binding sites in cat visual cortex using \(^{3}H\)-CHA.

The identification of the cortical \(^{3}H\)-NECA binding site is more problematic. \(^{3}H\)-NECA binds with lower affinity than \(^{3}H\)-CHA, in agreement with reports of \(A_2\)-adenosine receptor binding in other preparations (Bruns et al., 1980). A Hill plot of the saturation binding data suggests the existence of a single population of binding sites, although \(^{3}H\)-NECA in other preparations is reported to bind to both \(A_1\) and \(A_2\) adenosine sites (Fredholm, 1982; Snyder, 1985). The higher \(K_d\) for \(^{3}H\)-NECA is attributable to an almost 3-fold slower association rate constant. The dissociation rate constants for the 2 ligands are approximately the same, suggesting that the differences in \(B_{max}\) (\(^{3}H\)-CHA nearly 3 times that of \(^{3}H\)-NECA) are due to different numbers of distinct binding sites. If \(^{3}H\)-NECA were binding to both \(A_1\) and \(A_2\) sites, we would expect a higher \(B_{max}\) than for \(^{3}H\)-CHA, unless the \(K_d\) were appreciably faster, which is not the case. In addition, the postnatal time course of variation in \(B_{max}\) is quite different for the 2 ligands. Taken together, these data argue for a cortical \(^{3}H\)-NECA binding site distinct from the \(A_1\) site labeled by \(^{3}H\)-CHA. The difficulty with this interpretation is that the order of effectiveness for the various displacer substances tested is very similar, although not identical, for the 2 ligands (Figs. 1; 2; Table 1). Previous reports in other systems have indicated major differences in displacer potencies at the 2 sites. Another difficulty is the nearly identical laminar distribution of \(^{3}H\)-CHA and \(^{3}H\)-NECA binding patterns. These 2 reservations, especially the first, raise questions concerning the identification of the \(^{3}H\)-NECA binding site in the cat cortex as the \(A_1\)-adenosine receptor described by other investigators. Further experiments are required to establish definitively whether \(^{3}H\)-NECA binds to the same sites as does \(^{3}H\)-CHA or to a distinct subclass of adenosine receptors in cat visual cortex.

The binding patterns exhibited for adenosine receptors in the
adult cat resemble those previously described for muscarinic ACh receptors (Shaw et al., 1984a), cholecystokinin receptors (Shaw and Cynader, 1985), \( \beta \)-adrenergic receptors (Shaw et al., 1984c), and, to a lesser extent, binding sites labeled by \( ^{3}H \)-l-glutamate and \( ^{3}H \)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) (C. Shaw and M. Cynader, unpublished observations).

Both \( ^{3}H \)-CHA and \( ^{3}H \)-NECA binding sites show alterations in their laminar binding patterns during postnatal development. Similar changes, or even reversals, of the original neonatal laminar binding pattern have been found in the majority of cortical receptor populations we have studied to date (Shaw and Cynader, 1985; Shaw et al., 1984a, b, 1985). These data are summarized in schematic form for young kittens and adult cats in Figure 9.

Most of the previously studied receptors that have exhibited changes in developmental laminar binding patterns (muscarinic/cholinergic, cholecystokinin, and \( \beta \)-adrenergic binding sites) have shown dense concentrations in layer 4 initially, and subsequent alterations to favor the superficial and/or deep layers. Other receptors (GABA, benzodiazepine) are concentrated in layer 4 at all postnatal ages. Adenosine binding sites differ in that layers 5 and 6 show the densest binding initially, layers 1-3 are most heavily labeled in the adult, and layer 4 is not a zone of highest concentration at any stage of development. The pattern of alteration of binding sites (from deep to superficial cortical layers) parallels that previously reported for the ontogeny of neurons in the different cortical layers (Rakic, 1974). The relative absence of adenosine receptors in layer 4 at all ages suggests that adenosine does not exert its effects at the first stage of cortical information processing, but rather that it acts on higher-order cortical functions. This reduced presence in layer 4 also characterizes muscarinic acetylcholinergic binding sites of the adult cat (Shaw et al., 1984a), and it is interesting to note that these 2 "modulatory" substances seem to have similar effects on cortical EEG and behavioral arousal. It has long been known that administration of the muscarinic antagonist scopolamine results in cortical EEG synchronization in the absence of behavioral sleep (Steriade and Hobson, 1976). Administration of L-PIA, an adenosine agonist, results in behavioral manifestations of sleep, but with a desynchronized cortical EEG (Meltzer et al., 1984).

As was the case for several other receptors studied in cat visual cortex (Jonsson and Kasamatsu, 1983; Shaw and Cynader, 1985, unpublished observations; Shaw et al., 1984a-c; Wilkinson et al., 1983) and the cortex of other species (Candy and Martin, 1979; Pittman et al., 1980), adenosine receptor density \( (B_\text{max}) \) was initially low but increased during postnatal development. \( ^{3}H \)-CHA binding density peaked in adulthood, while \( ^{3}H \)-NECA binding density peaked at 30 d postnatally. Geiger et al. (1984) have reported an increase in the number of \( \alpha \)-adenosine receptors during postnatal development in rat cerebral cortex.

The issue of the cellular location of adenosine receptors is a subject of some controversy. Goodman et al. (1983) have sug-
gested a presynaptic locus for A1-adenosine receptors in rat superior colliculus but not for the LGN or visual cortex. In other preparations, adenosine receptors are probably postsynaptic (Geiger et al., 1984). We have examined these issues in a preliminary way in adult cats subjected to unilateral enucleation (3 month survival), unilateral decortication (2–3 week survival), or unilateral LGN lesion (2 week survival). In no case have we noted a significant change in adenosine receptor density or pattern of binding in either the LGN (following enucleation), superior colliculus, or visual cortex. Results were the same whether [3H]-CHA or [3H]-NECA was employed as a ligand. These results suggest that if adenosine receptors are associated with the retninogeniculate, retinocollricular, geniculocortical, or callosal pathways, then they are not presynaptic in location, at least not in the cat. Any presynaptic loci for adenosine receptors in cat visual cortex are thus likely to arise from intracortical circuits.

The functional role of adenosine receptors within the cortex remains unclear. The evidence that adenosine modulates the synaptic release of a wide variety of different substances (Harms et al., 1979; Hollins and Stone, 1980; Michaelis et al., 1979; Snyder, 1985) allows for a number of different roles. It is interesting, in view of recent findings, that adenosine blocks release of glutamate from cortical slices (Dolphin and Archer, 1983), and that adenosine receptors and binding sites labeled by L-glutamate (Monaghan et al., 1985) show somewhat similar laminar distributions within the visual cortex (C. Shaw and M. Cynader, unpublished observations). We note, however, that the distributions of adenosine receptors and glutamate-related binding sites both alter their laminar distribution during development and that these patterns of changes are clearly different. The developmental patterns, which are specific and different for these, and other, receptors may allow for maximum modulation of the functions of any particular neurotransmitter system by adenosine at a specific time during postnatal development.

The identification of adenosine receptors in cat visual cortex and the description of their laminar binding pattern and ontogenesis add important information to the broadening picture of the chemical circuitry of this region of the brain. In conjunction with the description of the other receptor populations thus far studied, these data reveal a complex system with the possibility of multiple interactions among different neurotransmitters. Unraveling these interactions promises to be a major task for the future.

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