HIGH LEVEL OF ADENOSINE A1 RECEPTOR-LIKE IMMUNOREACTIVITY IN THE CA2/CA3a REGION OF THE ADULT RAT HIPPOCAMPUS

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Abstract—We describe the immunocytochemical distribution of adenosine A1 receptors in the rat hippocampus. Adenosine A1 receptor-like immunoreactivity was seen on the cell soma and dendrites of pyramidal cells and the cell soma and proximal part of dendrites of granule cells, but not on glial cells. Developmentally, adenosine A1 receptor-like immunoreactivity was diffuse on postnatal day 7 and increased in intensity in individual cells by day 21. In the CA2/CA3a region, the adult pattern of A1 receptor distribution was established by day 28. In the adult rat hippocampus, rostrocaudal inspection revealed that immunoreactivity in CA2/CA3a was greatest. Confocal microscopy revealed differences in the staining patterns for the adenosine A1 receptor and synaptophysin, a marker of presynaptic terminals. This result suggests that the adenosine A1 receptor might have postsynaptic physiological functions. Double-labeling of adenosine A1 receptors and anterogradely-labeled fibers from the supramammillary nucleus showed that the fibers from the supramammillary nucleus terminate directly on the cell soma of the A1 receptor-immunopositive neurons in CA2/CA3a and the dentate gyrus.

These results indicate that the adenosine A1 receptor in CA2/CA3a and the dentate gyrus are in a position to regulate hippocampal theta activity and that resultant strong synaptic depression in CA2/CA3a could play a role in regulating the intrinsic signal flow between CA3 and CA1. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: adenosine A1 receptor, immunohistochemistry, hippocampus, CA2/CA3a region, supramammillary nucleus.

Adenosine A1 receptors (A1Rs) have been implicated in synaptic inhibition in the CNS through a variety of pre- and postsynaptic mechanisms, including: a potent inhibitory effect on adenylyl cyclase activity,15,16 increase of K+ conductance,18,22,49,54 and suppression of Ca2+ influx.43,48,62 Although endogenous adenosine is thought to provide inhibitory tone to brain activity,1,31 its role in normal physiological function is still unclear. A precise description of the anatomical distribution of the A1Rs is necessary to understand the physiological roles of endogenous adenosine. In situ hybridization studies have shown A1Rs to be highly expressed in cortex and hippocampus.10,45,53 Autoradiographic studies also have indicated high levels of ligand binding to A1Rs in cortex and hippocampus.21,27,58 Evidence for both presynaptic and postsynaptic localization of A1 receptors has been derived from radioligand binding studies. Dense adenosine receptor ligand binding sites on hippocampal synaptic terminals have been demonstrated by lesions ofafferent system to hippocampus.12,14,20 Selective neuronal damage in the regions of CA3 and CA1 also offer evidence for the existence of A1R on postsynaptic structures.12,40 Electrophysiological studies have shown that A1Rs function both postsynaptically and presynaptically. However, recent immunohistochemical studies in rat hippocampus suggest that A1R may be predominantly localized to axons but not to synaptic terminals.47,51 The present studies were undertaken to resolve these apparent differences in the distribution of A1R.

Adenosine is also thought to act as an endogenous anti-convulsant.13,63,64 CA2 neurons are seizure-resistant in human epilepsy8,11,28 and in rat status epilepticus.63,64 Endogenous adenosine seems to be one of factors that protect CA2 neurons from cell damage. However, ligand binding analyses have not shown regional differences in adenosine binding which could explain the neuropathological asymmetries of hippocampal neurons. In this report, we describe the immunohistochemical and regional distribution of A1Rs in the adult rat hippocampus and their postnatal maturation. The distribution that we have observed suggests important roles for endogenous adenosine in the regulation of hippocampal function.

EXPERIMENTAL PROCEDURES

Anti-adenosine A1 receptor antibody

The anti-A1R antibody used in this study has been previously described.38 In brief, rat brain A1R was purified approximately 50,000-fold to homogeneity from brain membranes, by sequential processes including affinity chromatography on immobilized xanthine
amine congener-agarose, hydroxyapatite chromatography, and reaﬃnity chromatography. Purity of the protein was veriﬁed by immuno blotting, aﬃnity binding, silver staining and radiolodination. This antibody recognizes both the denatured and native forms of the receptor and shows no cross-reactivity against the puriﬁed human brain A1R.

**Speciﬁcity of the adenosine A1 receptor antibody**

Because we were concerned about problems of antibody cross-reactivity, the speciﬁcity of the antibody was further characterized by Western blotting, expression in Chinese hamster ovarian (CHO) cells and preadsorption tests using the puriﬁed antigen.

**Western blotting.** The A1R preparation (2 μg) was partially puriﬁed from rat brain membranes by aﬃnity chromatography, separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred electrotheretically to a nitrocellulose membrane. The membrane was incubated successively with: 5% skim milk (overnight, 4˚C), anti-A1R with 3% bovine serum albumin (1:500; 2h) followed by anti-rabbit IgG antibodies (1:7,000; 1 h); all sera were diluted in Tris–HCl buffered saline (pH 7.4) containing 0.1% Tween 20. Immunoreactive bands were detected using an enhanced peroxidase complex (Amer sham) according to the manufacturer’s protocol.

**Immunohistochemistry of adenosine A1 receptor cDNA-transfected Chinese hamster ovarian cells.** The ability of the antibody to directly recognize the rat A1R proteins was evaluated using a CHO cell line to express rat A1R protein. CHO cells were stably transfected with A1R cDNA; the stable transfectants, expressing about 2 pmol/mg of A1R, were maintained in Ham’s F12 medium supplemented with 10% fetal bovine serum and 0.01% kanamycin under normal culture conditions. Cells from this line and non-transfected cells were cultured in Lab-Tek tissue culture chambers (Nunc). When cells were 30–50% conﬂuent they were washed with phosphate-buffered saline (PBS; Dulbecco’s PBS ICN Biomedicals, Inc.), ﬁxed in cold ethanol (−20˚C, 5 min) and then cold ethanol: acetone (80:20; 5 min). The slides were dried for 10 min and then processed for immunochemistry. The immunochemical staining procedure was the same except for the concentration of anti-A1R (1:50) and the incubation time and temperature (37˚C, 1 h).

**Preadsorption test.** As a further test of speciﬁcity, immunochemistry was performed using an antibody which had been preadsorbed with the rat brain A1R puriﬁed to homogeneity (0.3 μg and 3.0 μg).

**Immunohistochemistry of hippocampal adenosine A1 receptor**

**Animals.** Female Wistar rats were used in the study. Adult animals (n=40) were approximately three months old; for the developmental study, animals were used on postnatal days 7, 14, 21, and 28 (n=10 at each age). Animals were deeply anesthetized by chloral hydrate (3.5 mg/10 g body weight, i.p.) and perfused transcardially with PBS in 0.1 M Tris–HCl (pH 7.4), was injected into four sites of the SuM and the anti-A1R was visualized using rhodamine-conjugated goat anti-rabbit IgG (TAGO) (Fig. 3). Sections were cleared in xylene, coverslipped and photographed under a ﬂuorescence microscope (Olympus: AX70).

To determine the relationship of axon terminals from the supra mammillary nucleus (SuM) to the distribution of A1R-IR in the CA2/CA3a region and the dentate gyrus (DG), we combined axonal transport of the anterograde tracer dextran–biotin to label the SuM–hippocampal pathway with A1R immunohistochemistry. The tracer was pressure-injected bilaterally into the SuM in four male Wistar rats (nine weeks old, 270–280 g). Animals were anesthetized by i.p. injection of sodium pentobarbital (40–50 mg/kg) and placed on a stereotaxic apparatus. A glass micropipette with a ﬁne tip (tip size: 30–40 μm) was made from a disposable micropipette (volume: 50 nl/mm, Drummond) and connected to an air pressure system with a polyethylene tube that was used for stereotaxic microinjection. A total of 0.25–0.3 μl of 15% dextran–biotin (Molecular Probes, Inc.), dissolved in 0.1 M Tris–HCl (pH 7.4), was injected into four sites of the SuM (70–75 nl at each site) at following stereotoxic coordinates: (i) 3.7 mm anterior to the ear bar, −0.5 mm lateral from the midline, 8.3 mm ventral from the surface of cortex; (ii) 3.7 mm anterior to the ear bar, +0.5 mm lateral from the midline, 8.3 mm ventral from the surface of cortex; (iii) 4.2 mm anterior to the ear bar, −0.5 mm lateral from the midline, 8.4 mm ventral from the surface of cortex; (iv) 4.2 mm anterior to the ear bar, +0.5 mm lateral from the midline, 8.4 mm ventral from the surface of cortex.

**Abbreviations used in ﬁgures**

| cc | corpus callosum |
| cg | cingulum |
| CA1 | field CA1 of the hippocampus |
| CA2 | field CA2 of the hippocampus |
| CA3 | field CA3 of the hippocampus |
| CA3a | field CA3a of the hippocampus |
| D3V | dorsal third ventricle |
| Hb | habenular |
| mf | mossy fiber |
| Oc1L | occipital cortex, area 2, lateral |
| Oc2M | occipital cortex, area 2, medial |
| Par1 | parietal cortex, area 1 |
| RSA | retrosplenial agranular cortex |
| RSG | retrosplenial granular cortex |
Seven to 10 days after the tracer injection, animals were anesthetized deeply and perfused transcardially. Sections were made as described above, and incubated with anti-A1R (1:800 in PBS with 1% normal goat serum and 0.2% Triton-X 100; overnight; 4°C), then with rhodamine-conjugated goat anti-rabbit IgG (1:200; 2 h, Amersham). Dextran–biotin was detected directly by incubation with streptavidin–fluorescein (1:200; 2 h, Amersham). After washing with PBS, sections were mounted on slides, coverslipped in PermaFluor containing 0.1% p-phenylenediamine (Sigma) and visualized using a confocal laser scanning microscope (Leica). Confocal images were obtained for a series of optical sections covering a distance of 1 μm on the Z-axis. The injection sites of dextran–biotin were visualized histochemically using the avidin–biotin–peroxidase complex method (Vector).

RESULTS

Specificity of the anti-adenosine A1 receptor antibody

On Western blots, our antibody recognized only one band which corresponded in apparent molecular mass to the A1R protein (35,000 mol. wt) (Fig. 1A, lane A). The preparation also contained other proteins which could be detected with silver staining (Fig. 1A, lane B). In the CHO cells, the rat A1R were detected only in the transfected cells (Fig. 1Ba); no immunoreactivity was seen in non-transfected cells (Fig. 1Bb) or when the primary antibody was omitted (data not shown). Staining in brain sections were reduced by incubating the antibody with 0.3 μg of the purified A1R and eliminated completely with 3.0 μg of the protein. These results indicate that the antibody can be used as a selective probe of the location of A1R.

Distribution of the adenosine A1 receptor immunoreactivity in the adult rat hippocampus

A1R immunoreactivity was intense in the adult hippocampal formation (Fig. 2A). Immunoreactivity was seen in the CA1, CA2, CA3 regions and in the DG. Almost all pyramidal neurons and granule cells were positive (Fig. 2B–E). A1R immunoreactivity was not detected on glial cells.

In CA1 (Fig. 2B), pyramidal cell bodies, apical dendrites and some of their branches were stained faintly in the strata radiatum (SR) and lacunoso-molecular (SLM), but basal dendrites in the stratum oriens (SO) were not stained. A1R-IR on cell bodies and dendrites of all neurons in CA2 were stronger than in other fields. In CA2 (Fig. 2C), intense immunoreactivity was observed in large pyramidal cells adjacent to the faintly stained cells in CA1. The areas surrounding pyramidal neurons were stained more strongly than the cytoplasm of these neurons. In CA3 (Fig. 2D), A1R-IR was present on cell bodies, thick dendritic shafts of apical dendrites, and some of dendritic branches in the DG (Fig. 2E), cell bodies and proximal parts of dendrites in the granule cell layer (GrDG) were immunolabeled, but the granule cell axons, the mossy fibers, were not stained. In addition, the molecular (Mol) and polymorphic (PoDG) layers were weakly stained. Large A1R-immunopositive fusiform neurons were scattered in the PoDG.

Double-immunofluorescence labeling of adenosine A1 receptor and synaptophysin

The border between CA1 and CA2 can be easily identified because of the characteristic dense packing of the CA1 pyramidal neurons. In contrast, the border between CA2 and CA3 is unclear in sections stained with only the A1R antibody (Fig. 3A). We were able to define this border by staining the sections with monoclonal antibodies that recognize synaptophysin, a synaptic vesicle protein. These antibodies intensely stained mossy fibers, which are found in the CA3 region but do not innervate CA2. In Fig. 3B, the intensely immunoreactive mossy fiber tract in the stratum lucidum (SL) of CA3 is clearly labeled among strongly labeled synaptic terminals in the SR and SO of all fields. This allowed us to document that intense A1R-IR was observed throughout CA2, and in the distal part of CA3, i.e. CA3a (Fig. 3A, B).

A1R-IR seemed to be located predominantly in postsynaptic structures, because the mossy fibers were not recognized by the A1R antibody (Fig. 2A, E and Fig. 3). Confocal microscopic observations of dual-labeled brain sections revealed different staining patterns for A1R and synaptophysin. Fluorescence images of FITC and rhodamine labeling were sequentially acquired on the same fields. Each fluorescence image was composed of three images taken at 0.18 μm steps (0.54 μm thickness). The results are shown in Fig. 4, where FITC-labeled A1R is shown in green and rhodamine-labeled synaptophysin is shown in red. Whereas A1R immunofluorescence was seen in the pyramidal cell bodies in the stratum pyramidale of all fields (SP in Fig. 4), dendrites and spine-like protrusions in the SR of CA1 and CA2 and the SL of CA3, synaptophysin was detected mainly in the SR (Fig. 4A and B) and SL (Fig. 4C). There was rare co-labeling of both signals (yellow) in the SR of CA1 and CA2 and in the SL of CA3, indicating the differential distribution of these proteins. In the SP of CA2 (Fig. 4B), synaptophysin-positive structures were found more than in the SP of other fields and a few yellow signals were observed. In CA3 (Fig. 4C), mossy fiber terminals in the SL were labeled only for synaptophysin. These results indicate that the antibody used in this study detected A1R expressed in the postsynaptic regions of hippocampal neurons, but not in their axons or presynaptic terminals.

The rostrocaudal and dorsoventral distribution of adenosine A1 receptor-positive neurons

A camera lucida was used to map the distribution of neurons containing A1R immunoreactivity in both coronal (Fig. 5A) and horizontal (Fig. 5B) planes throughout the adult hippocampus. Intense immunoreactivity (red dots) was detected along the rostrocaudal and dorsoventral axes in CA2/CA3a throughout the hippocampal formation. Neurons in CA1 and CA3 and in the DG were weakly labeled (black dots).

Postnatal development

We analysed the level of A1R-IR in the hippocampus during postnatal development (Fig. 6). There was an increase in the level of hippocampal A1R-IR with development and thus was especially pronounced in the CA2/CA3a region. At postnatal (P) day 7, homogeneous immunoreactivity was observed in the GrDG and in all pyramidal cell layers; while the SR and SLM were weakly stained (Fig. 6A) and individual cell bodies and dendrites could not be clearly recognized. At P14, immunopositive CA1–CA3 pyramidal cells and granule cells could be identified (Fig. 6B). Up to P21, all of the fields of the hippocampal formation were homogeneously stained, and regional...
differences in the levels of A1R-IR were not seen (data not shown). However, the adult pattern of intense A1R-IR in CA2/CA3a was established by P28 (Fig. 6C) and then increased in the adult (Fig. 6D). These results indicate that the distribution of A1R-IR changes over the course of development.

Fig. 1. Specificity of the anti-A1R antibody. (A) Lane (A) Immunoblotting of partially purified A1Rs from rat brain membranes. The antibody detected only a single, broad band of approximately 35,000 mol. wt. Lane (B) Silver staining of the proteins from the same preparation, indicating the presence of many other proteins. (B) (a) A1R-IR in CHO cells stably transfected with rat A1R cDNA. (b) Non-transfected CHO cells. No immunoreactivity was observed in these cells. Scale bar=100 μm.
Fig. 2. Typical pattern of A1R-IR in the rat hippocampus. (A) Low-power micrograph showing A1R-IR in the adult rat forebrain. Immunoreactivity was strongly observed in the pyramidal cell and the granule cell layers, particularly in CA2 (arrows). (B, C, D, E) High-power micrographs of CA1, CA2, CA3 and DG, respectively. (B) In CA1, pyramidal cells and apical dendrites are faintly stained. (C) In CA2, staining is seen in pyramidal cells and also in the SO, SR and SLM. Immunoreactivity was very intense in the cell layer. (D) In CA3, A1R-IR was seen on pyramidal cell bodies and on thick shafts and their branches of apical dendrites. (E) In the DG, staining is seen in the GrDG and fusiform cells of the PoDG. Scale bars: (A) 500 μm; (B–E) 50 μm.
Fig. 3. Double labeling of sections with a polyclonal antibody to A1R (A) and a monoclonal antibody to synaptophysin (B). Synaptophysin, localized in presynaptic terminals, strongly marks the mossy fiber tract. The A1R-positive neurons in the pyramidal cell layer extend from the border between CA1 and CA2 (large black arrow) through CA2 (no mossy fibers) to the CA2/CA3a border (white arrow; the tip of synaptophysin-labeled mossy fiber tract). The small black arrow and asterisk indicate the same blood vessel in A and B. Scale bars=200 μm.

Fig. 4. Scanning confocal microscopic analysis of double labeling of A1R and synaptophysin in CA1 (A), CA2 (B) and CA3 (C). Digital composed images of FITC (green) and rhodamine (red) fluorescence of the same field are shown. Each fluorescent image was acquired sequentially and is composed of three optical sections at 0.18 μm intervals. A1R immunoreactivity (green) is localized on pyramidal cell bodies, their dendrites in the SR and SL. Immunoreactivity of synaptophysin localized mainly in the presynaptic terminals (red) is detected in SR and SL. The paucity of overlapping labeling (yellow) indicates differential distribution of the two proteins. Scale bars=10 μm.
Implications of high adenosine A1 receptor in CA2/CA3a for hippocampal circuitry

The principal cells in CA2/CA3a are known to be one of specific targets of afferent fibers from the SuM in the hypothalamus. However, the physiological mechanisms that allow activity in SuM to control the discharge of hippocampal neurons is not clear. An anterograde tracer, dextran–biotin, was injected into the supramammillary nucleus to label SuM fibers innervating the hippocampus (Fig. 7A). Labeled fibers were detected in the GrDG, the SO and the SP of CA2/CA3a (Fig. 7B). In CA2/CA3a, a dense network of labeled fibers appeared to surround the pyramidal cell bodies (Fig. 7C). Figure 8 shows confocal microscopic observations of dual labeling of SuM fibers and A1R-IR in the hippocampus. SuM fibers (green) densely innervated the SP of CA2/CA3a, surrounding A1R-positive pyramidal cell bodies (red) (Fig. 8A). SuM fibers were also found surrounding the A1R-IR positive granule cells in the DG (Fig. 8B). Yellow signals indicating double labeling of SuM fibers suggests these fibers had A1R-IR or that these fibers synapsed upon A1R-IR positive neurons (Fig. 8).
Fig. 6. Postnatal development of A1R-IR. Micrographs in A, B, C and D show the anti-A1R immunohistochemical profiles in the P7, P14, P28 and adult rat hippocampi, respectively. The immunoreactivity is detected in P7 but the mature distribution patterns of A1R are not established until P28. At this time, CA2 shows intense immunoreactivity (field between the two arrows in C) and these immunohistochemical characteristics are greatly enhanced in the adult (D). Scale bars = 500 μm.
DISCUSSION

Patterns of adenosine A1 receptor labeling

When sections were double-labeled for A1R and synaptophysin and viewed with a confocal microscope, the receptor was present mainly in cell bodies, apical dendrites and their protrusions, but not in axons, such as mossy fibers and Schaffer collaterals. Our results differ from previous immunohistochemical studies where A1R-IR was identified in axons. Differences in the molecular properties of A1Rs have been reported among species and tissues. For example, A1 receptor preparations differ in their molecular mass and immunoreactivity, mainly due to variations in glycosylation. Additional variants of A1Rs could also be generated by differences in RNA processing. The human A1 receptor gene has two types of transcripts containing distinct exons, which are expressed in a tissue-specific manner. One reason for the difference between our results and earlier reports may be that the various antibodies used recognize different epitopes. Previous studies used a polyclonal antibody raised against synthetic peptides corresponding to a portion of the third cytoplasmic loop and the carboxyl terminus; there are identical in the rat and human A1 receptors. We used an antibody against the purified A1R of rat brain, which poorly reacts with human A1R. Immunoblot analysis of our antibody revealed a broad band characteristically representing a glycoprotein, at a position of approximately 35,000 mol. wt (Fig. 1A). This is slightly different from the pattern of the
Fig. 8. Scanning confocal microscopic analysis A1R-IR and anterograde-labeled SuM efferents in CA2/CA3a (A) and the DG (B). Each optical image was taken at a focal plane close to the surface of the section. SuM fibers are green, A1R-IR is red, and dual-labeled structures are yellow. The fibers are found in close proximity to the A1R receptors. Scale bars = 25 μm.
38,000 mol, wt protein stained by the antibody raised against the synthetic A1R peptide used in previous immunohistochemical studies. Since our antibody recognized A1Rs specific to the cell soma and dendrites of neurons, it is possible that A1R in these domains have different sugar residues or other molecular differences from A1Rs expressed in axons.

In situ hybridization studies of A1R mRNA and autoradiographic observations of ligand binding reveal regional variations in A1R that differ from those we report in this study. In situ hybridization studies have shown that A1R mRNA is abundantly expressed in the cell bodies of pyramidal and granule cells, while autoradiographic studies have shown that A1R ligand binding is low in cell layers and high in dendritic layers. In the present study, we detected A1R-IR both in cell somata and in the dendrites. Some discrepancies between the results of studies based on ligand binding and immunocytochemistry of A1Rs are to be expected. Unlike immunohistochemistry, ligand binding studies cannot identify the cells or cell structures involved; the binding affinities of ligands are also affected by environmental factors such as the concentrations of endogenous adenosine, guanine nucleotides and magnesium ions. Thus, one possible reason for the discrepancy is that the A1Rs in hippocampal cell bodies may not bind adenosine ligands under the conditions usually employed in binding experiments. Another prominent difference in localization is in the dendritic layer, where our results showed weak immunoreactivity. Intense ligand binding activity in the dendritic layers of the DG is due to A1R binding sites in presynaptic terminals, as indicated by reduced ligand binding following lesions of the perforant path. As can be seen in our study of the co-localization of A1R and synaptophysin, our antibody apparently does not recognize A1R in presynaptic terminals. This would be expected to yield weaker immunoreactivity in the dendritic layer compared to that reported by previous ligand binding studies. Our results are consistent with observations that selective neuronal damage in the CA1 and CA3 regions reduces ligand binding activity by approximately half, suggesting a contribution of postsynaptic A1R in those fields. It also seems that the ratio of presynaptic and postsynaptic localization of A1 receptors may vary in different regions of the hippocampus. For example, the high level of immunoreactivity of A1R indicated in our study suggests that postsynaptic sites in CA2/CA3a are higher than in other regions. Another possible cause of the discrepancy is that our A1R-IR antibody does not detect receptors expressed in glial cells, while ligand binding studies may detect these receptors. Rat brain astrocytes respond to an A1 specific agonist and the ligand binding activity of glial cells is probably much higher in the dendritic layer. Thus, it appears that our antibody specifically recognizes receptors that mediate postsynaptic functions in neurons and excludes other A1R, on presynaptic terminals and glial cells, that have been examined in other studies.

Implications of the distribution of hippocampal adenosine A1 receptor

Many physiological studies for hippocampal neurons have indicated a postsynaptic distribution of the A1R. Our results present the first immunohistochemical confirmation of the presence of the A1R in postsynaptic structures, thus supporting a postsynaptic function for the receptors. Furthermore our data lend supports to the idea that some posttranscriptional modifications are related to different functions in different locations.

In contrast to the restricted distribution of A1R-IR in the adult hippocampus, the distribution of the A1R was uniform at P7. Immunoactivity on individual cells can be distinguished at P14; the intense staining in CA2/CA3a was first established at P28. Previous biochemical studies have shown that A1R agonist binding increases during postnatal development in the rat nervous system. AIR binding activity can be detected as early as embryonic day 14 and a rapid increase in receptor binding was seen between postnatal days 6–28 in subcortical structures—thalamus, hippocampus, striatum and hypothalamus. Thus, the changes in staining patterns we detected with the anti-A1R antibody during postnatal development appear to be consistent with the results of ligand binding studies.

In order to accurately describe the anatomical distribution of the intensely-staining A1R hippocampal fields, we double-labeled our sections with the polyclonal antibody to A1R and either a monoclonal antibody to synaptophysin or to calbindin (data not shown). Synaptophysin antibodies strongly labeled the tract of mossy fibers, whereas calbindin labeled pyramidal neurons in CA1 and CA2 in addition to the mossy fibers. These studies indicated that the most intense A1R-IR occurred in CA2/CA3a (Fig. 2C). Given that A1R are inhibitory, our results suggest that there is a strong inhibition of synaptic transmission in CA2/CA3a.

A novel intrahippocampal pathway, that goes from CA3 to CA2 and then to CA1, has recently been described. This led to the suggestion that synaptic activity in the CA2 region was more suppressed than in other regions and, thereby, acts as a gate controlling signal flow from CA3 to CA1. The high level of A1R-IR in CA2/CA3a supports the hypothesis that CA2 can serve as such a gate and further suggests that endogenous adenosine may mediate this gating affect. Hippocampal adenosine levels change with behavior, with increases in “sleep-like” or quiet behavior and decreases in motion and eating behaviors linked to such changes in the level of adenosine. The A1 receptors in the ‘CA2’ gate are thus in a perfect position to regulate these behaviorally-linked changes in signal propagation.

CA2 neurons show a decreased vulnerability to neuronal damage in status epilepticus and to glutamate toxicity. The high level of inhibitory A1R in CA2/CA3a may be a key factor in the resistance of these neurons to seizure and ischemia-induced neuronal damage. In hippocampal slices, penicillin or bicuculline cause a synchronized burst discharge which originates from CA2 neurons and CA1. This suggest that CA2 neurons could be involved in the generation of epileptic discharges. Adenosine blocks experimentally-induced seizure activity. It is therefore possible that endogenous adenosine and densely concentrated A1Rs could be factors protecting CA2 neurons from epileptogenesis. However, other unique features of CA2/CA3a neurons, such as high levels of calcium-binding proteins and several growth factor proteins, could also contribute to the decreased vulnerability of these neurons as compared to CA1 and CA3.

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In contrast to the restricted distribution of A1R-IR in the adult hippocampus, the distribution of the A1R was uniform at P7. Immunoactivity on individual cells can be distinguished at P14; the intense staining in CA2/CA3a was first established at P28. Previous biochemical studies have shown that A1R agonist binding increases during postnatal development in the rat nervous system. AIR binding activity can be detected as early as embryonic day 14 and a rapid increase in receptor binding was seen between postnatal days 6–28 in subcortical structures—thalamus, hippocampus, striatum and hypothalamus. Thus, the changes in staining patterns we detected with the anti-A1R antibody during postnatal development appear to be consistent with the results of ligand binding studies.

In order to accurately describe the anatomical distribution of the intensely-staining A1R hippocampal fields, we double-labeled our sections with the polyclonal antibody to A1R and either a monoclonal antibody to synaptophysin or to calbindin (data not shown). Synaptophysin antibodies strongly labeled the tract of mossy fibers, whereas calbindin labeled pyramidal neurons in CA1 and CA2 in addition to the mossy fibers. These studies indicated that the most intense A1R-IR occurred in CA2/CA3a (Fig. 2C). Given that A1R are inhibitory, our results suggest that there is a strong inhibition of synaptic transmission in CA2/CA3a.

A novel intrahippocampal pathway, that goes from CA3 to CA2 and then to CA1, has recently been described. This led to the suggestion that synaptic activity in the CA2 region was more suppressed than in other regions and, thereby, acts as a gate controlling signal flow from CA3 to CA1. The high level of A1R-IR in CA2/CA3a supports the hypothesis that CA2 can serve as such a gate and further suggests that endogenous adenosine may mediate this gating affect. Hippocampal adenosine levels change with behavior, with increases in “sleep-like” or quiet behavior and decreases in motion and eating behaviors linked to such changes in the level of adenosine. The A1 receptors in the ‘CA2’ gate are thus in a perfect position to regulate these behaviorally-linked changes in signal propagation.

CA2 neurons show a decreased vulnerability to neuronal damage in status epilepticus and to glutamate toxicity. The high level of inhibitory A1R in CA2/CA3a may be a key factor in the resistance of these neurons to seizure and ischemia-induced neuronal damage. In hippocampal slices, penicillin or bicuculline cause a synchronized burst discharge which originates from CA2 neurons and CA1. This suggest that CA2 neurons could be involved in the generation of epileptic discharges. Adenosine blocks experimentally-induced seizure activity. It is therefore possible that endogenous adenosine and densely concentrated A1Rs could be factors protecting CA2 neurons from epileptogenesis. However, other unique features of CA2/CA3a neurons, such as high levels of calcium-binding proteins and several growth factor proteins, could also contribute to the decreased vulnerability of these neurons as compared to CA1 and CA3.

Supramammillary nucleus fiber terminals exhibit adenosine A1 receptor immunoreactivity

Hippocampal function is strongly influenced by inputs
arising from the medial septum, SuM and the diagonal band of Broca.\textsuperscript{3,5} The SuM projects mainly to CA2/CA3a and the DG (Fig. 7).\textsuperscript{2,3,29,57} Neurons of the SuM have been shown to fire in phase with hippocampal theta discharges and these are thought to generate or relay theta activity.\textsuperscript{25,26,33} Hippocampal synaptic activity is modulated according to behavior stages as well as neural activity originating in the SuM. However, physiological mechanisms that allow activity in SuM to control the discharge of hippocampal neurons in a behavior-dependent manner is not clear. In this study, A1R-IR was found near the SuM fibers in the cell layers of CA2 and the DG. Theta activity from the SuM may regulate adenosine-mediated inhibition in the hippocampus. This hypothesis is consistent with the findings that electrical prestimulation or glutamate stimulation of the SuM facilitates perseverant path synaptic activity in the DG.\textsuperscript{5,35} Furthermore, hippocampal theta activity has been recorded during exploration and other behaviors associated with information acquisition.\textsuperscript{3,26,59} Changes in adenosine levels might be one factor that regulates hippocampal theta discharges. In addition, experimental stimulation designed to mimic theta rhythm (theta burst stimulation) produced long-term potentiation (LTP) at apical dendritic synapses on CA1 pyramidal neurons\textsuperscript{4} and this type of LTP is enhanced by A1R antagonists.\textsuperscript{2} These results allow us to speculate that A1Rs in the CA2/CA3a contributes to the mechanisms of hippocampal plasticity.

**CONCLUSIONS**

In this study, our polyclonal antibody recognized a neuron-specific adenosine A1 receptor; these receptors were located in sites that might have postsynaptic functions. The intense immunoreactivity of the CA2/CA3a suggests an important role for adenosine in the regulation of certain hippocampal activities. In addition, the results of this study suggest that adenosine A1 receptors in CA2/CA3a could protect these neurons from epileptogenesis and neuronal damage.

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