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BRIEF COMMUNICATION

The mechanism of electrically stimulated adenosine release varies by brain region

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Abstract Adenosine plays an important role in neuromodulation and neuroprotection. Recent identification of transient changes in adenosine concentration suggests adenosine may have a rapid modulatory role; however, the extent of these changes throughout the brain is not well understood. In this report, transient changes in adenosine evoked by one second, 60 Hz electrical stimulation trains were compared in the caudate–putamen, nucleus accumbens, hippocampus, and cortex. The concentration of evoked adenosine varies between brain regions, but there is less variation in the duration of signaling. The highest concentration of adenosine was evoked in the dorsal caudate–putamen (0.34± 0.08 μM), while the lowest concentration was in the secondary motor cortex (0.06±0.02 μM). In all brain regions, adenosine release was activity-dependent. In the nucleus accumbens, hippocampus, and prefrontal cortex, this release was partly due to extracellular ATP breakdown. However, in the caudate–putamen, release was not due to ATP metabolism but was ionotropic glutamate receptor-dependent. The results demonstrate that transient, activity-dependent adenosine can be evoked in many brain regions but that the mechanism of formation and release varies by region.

Keywords Carbon-fiber microelectrode · Striatum · Hippocampus · Cortex · Electrical stimulation · Fast-scan cyclic voltammetry

Abbreviations

FSCV	fast-scan cyclic voltammetry
aCSF	artificial cerebrospinal fluid
EDTA	ethylenediaminetetraacetic acid
TTX	tetrodotoxin
AOPCP	α,β-methylene adenosine diphosphate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
AP5	D(−)-2-amino-5-phosphonopentanoic acid
CP	caudate-putamen

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Introduction

Adenosine is a neuromodulator that regulates many biological processes, including blood flow, energy availability, and cellular excitability. Traditionally, neuromodulatory effects of adenosine have been thought to occur via slow changes in extracellular adenosine concentrations during normal or pathological conditions such as sleep [1], presentation of novel stimuli [2], or ischemia [3]. Microdialysis studies have identified faster changes in adenosine that last only a few minutes after activation of ionotropic glutamate receptors, muscarinic acetylcholine receptors, or β-adrenergic norepinephrine receptors [4, 5]. Recently, electrochemical methods have revealed rapid, transient changes in extracellular adenosine that last only a few seconds [6]. Thus, adenosine may have important signaling function on multiple time frames from seconds to hours.

Transient adenosine release has been identified in the striatum [7, 8] and cerebellum [9] but has not been studied in other regions. Many factors that control adenosine signaling vary among regions, including receptor expression and basal levels, and these could affect rapid adenosine signaling. For example, inhibitory A_1 receptors have a relatively dense distribution throughout the brain but excitatory A_{2A} receptors are only densely distributed in the caudate–putamen (CP) and nucleus accumbens [10]. Basal adenosine levels also vary from 8–10 nM in the nucleus accumbens [2] to 60–70 nM in the striatum [11] and cortex [12] to a few hundred nanomolar in the hippocampus [13]. Differences in basal levels affect which receptors are tonically activated,

influencing long-term cellular excitability and possibly adenosine release.

The mechanism of adenosine release is debated and may vary by brain region. For slower adenosine changes, the accepted mechanisms of extracellular adenosine formation are transport of adenosine out of a cell by nucleoside transporters and the breakdown of ATP in the extracellular space after it is released by exocytosis or through hemichannels [14–16]. However, there is evidence that rapid adenosine release can also be due to downstream effects of activation of other neurons [6] or direct exocytosis of adenosine [17], but these mechanisms differ between species and brain regions.

In this report, we characterize transient adenosine release and its mechanism of formation in brain slices from the caudate–putamen, hippocampus, nucleus accumbens, and cortex. Adenosine was evoked by short electrical stimulations and measured using fast-scan cyclic voltammetry at carbon-fiber microelectrodes. While adenosine release varied between brain regions, all release was activity-dependent. Two main mechanisms of evoked release were elucidated and found to vary between regions: extracellular nucleotide breakdown and glutamate receptor activation. Thus, transient adenosine release is present throughout the brain but is formed by different mechanisms in different brain regions.

Materials and methods

Electrodes Fifty-micrometer-long cylindrical carbon-fiber microelectrodes were made from T-650 carbon fibers (Cytec Engineering Materials, West Patterson, NJ) as described previously [18]. Fast-scan cyclic voltammograms were collected using a Chem-Clamp potentiostat (Dagan, Minneapolis, MN), using data acquisition software and hardware as described previously [7]. The triangular waveform was applied from −0.4 to 1.5 V and back at 400 V/s with a 10-Hz repetition rate. Prior to use in a brain slice, microelectrodes were calibrated in a flow injection apparatus with 4 s injections of 1 μM adenosine, using the same buffer as the brain slice experiments. Previous tests of the sensitivity of carbon-fiber microelectrodes to several interferents, including inosine and hypoxanthine, have indicated that other interferents are unlikely to be contributing to the brain slice signal [7, 8].

Animals and slice preparation Adult, male Sprague–Dawley rats (250–350, Charles River) were housed in a vivarium and were fed and watered ab libitum. All experiments were approved by the Animal Care and Use Committee of the University of Virginia. Rats were anesthetized and 400-μm-thick brain slices were collected as described previously [7]. Coronal slices were collected from the caudate–putamen, nucleus accumbens, and prefrontal cortex. Sagittal slices were collected from the hippocampus.

Brain slice experiment Slices were kept in artificial cerebral spinal fluid (aCSF): 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$ (dihydrate), 1.2 mM $MgCl_2$ (hexahydrate), 25 mM $NaHCO_3$, 11 mM glucose, and 15 mM tris(hydroxymethyl) aminomethane, with the pH adjusted to 7.4 (all from Fisher, Waltham, MA). Experiments were performed at room temperature following a 1-h incubation period after slicing and slices could be used up to 6 h following slicing. There was no apparent effect of incubation period on slice performance. Slices were perfused with aCSF at a rate of 1 mL/min. The working and stimulating electrodes were implanted at about a 45° angle, about 75 μm into the slice as previously reported [7]. The stimulation train was 5, 300 μA biphasic pulses, each 4 ms per phase, at 60 Hz.

Brain area comparison Four brain areas were tested: the caudate–putamen, nucleus accumbens, hippocampus, and cortex. The concentration of adenosine, time for the signal to drop from maximum to half maximum release ($t_{1/2}$), and the number of slices where adenosine was evoked were compared between regions. In some slices, more than one brain region was tested.

Pharmacological tests For all pharmacology tests, two stimulations were initially performed 15 min apart, then the perfusion solution was changed to aCSF containing drug for 30 min and another stimulation performed. Data were compared before and after drug in the same slice. Drugs were dissolved in aCSF and normally used on the same day but were occasionally frozen and used within 30 days. To test activity-dependence, slices were treated with 0.5 μM tetrodotoxin (TTX, IC_{50}<100 nM, Tocris Bioscience, Ellisville, MO) [19]. TTX was solubilized in 0.2 M citrate buffer (pH 4.8) and frozen as 50 μM aliquots that were later diluted. A safety protocol for TTX was approved by the Office of Environmental Health and Safety at the University of Virginia. To test the effect of ionotropic glutatmate receptors, slices were perfused with a combination of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, AMPA receptor antagonist, K_i 1 μM,Tocris Bioscience, Ellisville, MO) [20, 21] and 100 μM D(−)-2-amino-5-phosphonopentanoic acid (AP5, NMDA antagonist, K_i 0.2 μM, Tocris Bioscience) [21, 22]. The combination of CNQX and AP5 significantly decreases electrode sensitivity for adenosine (55 %, data not shown), and data were corrected to account for this decrease. To test for extracellular ATP breakdown, slices

were perfused with a combination of 50 μM ARL-67156 (ARL, K_i 0.3 μM, Sigma-Alrich, St. Louis, MO) [23] and 100 μM α,β-methylene adenosine diphosphate (AOPCP, K_i 5 nM, Sigma-Alrich, St. Louis, MO) [24]. This combination of drugs does not affect the sensitivity of carbon-fiber microelectrodes for adenosine (data not shown).

Statistics All values are reported as mean±standard error of the mean (SEM). Error bars are plotted as the SEM. Experiments were repeated using slices from different rats, with a different electrode for each slice. Paired *t* tests determined drug effects, comparing stimulated release before and after a drug in the same slice. One-way ANOVA statistics with Bonferroni post-tests were performed to compare all brain areas. Statistics were performed in GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA) and considered significant at the 95 % confidence level. A χ^2 test was performed to compare frequency data.

Results

Regional variation in adenosine release The first goal was to determine the extent to which transient adenosine release could be evoked in the caudate–putamen, nucleus accumbens, hippocampus, and cortex. Coronal slices were tested for all regions except the hippocampus, where sagittal slices were tested. Each brain region was divided into smaller areas and Fig. 1 shows example evoked adenosine in the different brain regions. High frequency stimulations (5 pulses, 60 Hz, duration marked by red bar in Fig. 1) were chosen because they evoke the greatest levels of adenosine release in the striatum; the frequency may affect the mechanism of formation [7]. In all regions, peak release occurs between 1 and 2 s after stimulation. Frequently, the decrease in current following adenosine release falls below baseline due to large ionic changes starting around 5 s after stimulation. When all the data are examined (such as using 3D color plots [7]), the adenosine signal is easily differentiated from later ionic background changes. Evoked release varied widely in the caudate–putamen, with low signals in the lateral CP and larger signals in the medial CP. In the nucleus accumbens, adenosine release is slightly greater in the shell than the core (Fig. 1b). In the hippocampus (1C) and cortex (1D), release was generally low, although it was larger in the prefrontal cortex than other cortical areas.

The example cyclic voltammograms (CVs) in Fig. 1 are from the subregion with the largest stimulated release. The CVs are characteristic of adenosine oxidation, with a main oxidation peak at 1.5 V [8]. In the nucelus accumbens, the CV shows another peak at about 0.6 V, corresponding to dopamine release. Dopamine is also detected in the caudate–putamen but the dopamine current is low compared to adenosine [7].

Table 1 gives average stimulated release (calculated as the increase above basal levels), number of slices where

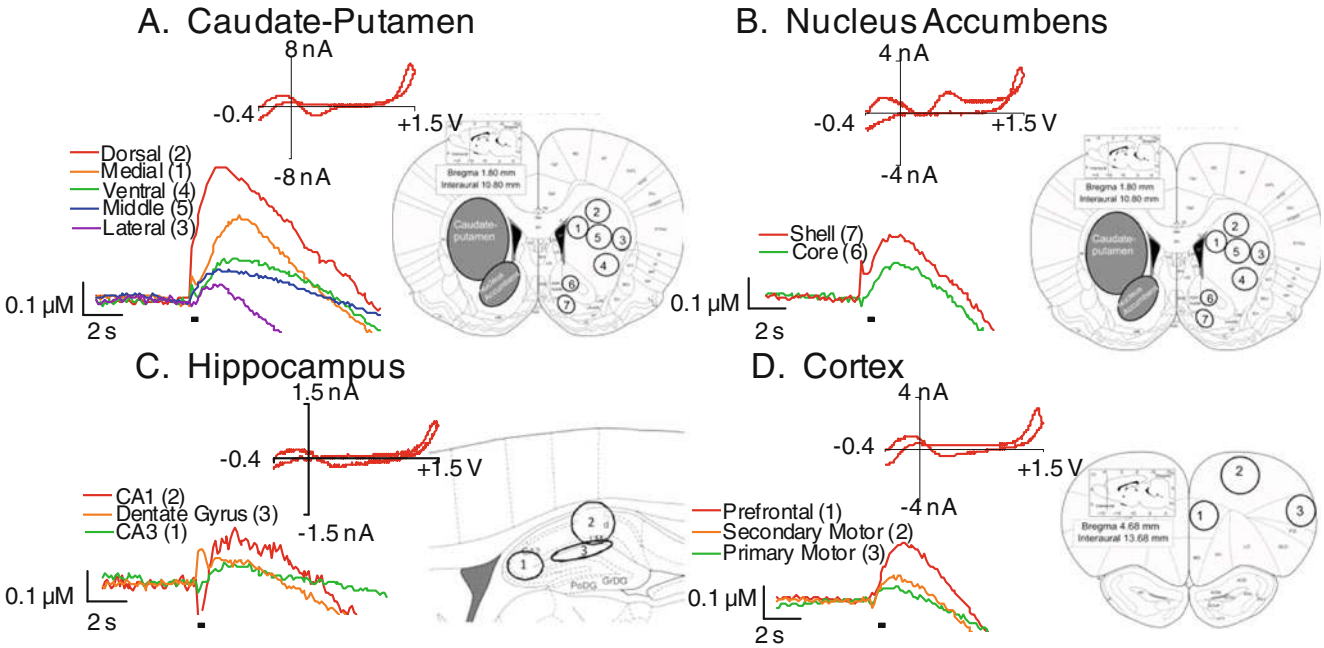

Fig. 1 Adenosine release in different brain regions. Sample current vs. time plots and cyclic voltammograms plots (*insets*) are shown for the **a** caudate–putamen, **b** nucleus accumbens, **c** hippocampus, and **d** cortex. *Black bars underneath each plot* indicate the length of electrical stimulation. The *pictures of brain slices* indicate where electrodes were implanted for different subregions (numbers on diagram refer to numbers after subregion given in the graph legend). Slice diagrams modified from [41]

Table 1 Stimulated adenosine release in the brain

Region	Subregion	Adenosine			
		Concentration (μM)	$t_{1/2}$ (s)	No. of slices detected	Frequency of slices detected (%)
Caudate–putamen (n=15)	Medial	0.25±0.07	2.0±0.7	3 out of 15	20
	Dorsal	0.34±0.08	2.2±0.7	4 out of 15	27
	Lateral	0.10±0.07	2±1	2 out of 15	13
	Ventral	0.2	4	1 out of 15	7
	Middle	0.2±0.1	3.6±0.9	4 out of 15	27
Nucleus accumbens (n=12)	Core	0.18±0.04	1.6±0.1	3 out of 12	25
	Shell	0.24±0.05	1.3±0.2	6 out of 12	50
Hippocampus (n=14)	CA1	0.13±0.03	1.8±0.2	10 out of 14	71
	CA3	0.07±0.02	0.9±0.2	7 out of 14	50
	Dentate gyrus	0.10±0.02	1.4±0.3	6 out of 14	43
Cortex (n=13)	Prefrontal	0.20±0.03	1.7±0.1	12 out of 13	92
	Primary motor	0.054±0.008	1.1±0.2	11 out of 13	85
	Secondary motor	0.07±0.02	1.1±0.1	10 out of 13	77

See text which denotes the significant differences

adenosine was detected, and $t_{1/2}$ data (a measure of adenosine clearance). Within the caudate–putamen, release was highest in the dorsal area and lowest in the lateral area but these trends were not significant. $t_{1/2}$ was significantly longer in the middle CP than the dorsal CP ($p<0.05$), but was otherwise similar across the caudate. In the CP, evoked adenosine was detected infrequently, only about a quarter of the time. In the nucleus accumbens, there were no significant differences in the concentration of stimulated release or $t_{1/2}$ for shell and the core, although adenosine was more frequently detected in the shell. Within the hippocampus, there were also no significant differences in evoked adenosine or $t_{1/2}$ and adenosine was most frequently detected in the CA1 area. In the cortex, the concentration of evoked adenosine was significantly greater in the prefrontal cortex than either the primary or secondary cortex ($p<0.05$) but $t_{1/2}$ was not significantly different. Evoked adenosine was most frequently detected in the prefrontal cortex. Release was lower, but more consistently evoked in the cortex and hippocampus, and was larger, but more variably evoked in the nucleus accumbens and caudate. When all subregions were averaged for a given region, the cortex, hippocampus, and nucleus accumbens had significantly higher frequency of detecting release than the caudate–putamen (χ^2 test, $p<0.05$).

The amount and duration of stimulated adenosine release varied from region to region. A one-way ANOVA of the averaged data in Table 1 revealed a significant effect of brain region for evoked adenosine concentration ($p<0.0001$) and $t_{1/2}$ ($p<0.0001$). Bonferonni post-tests were used to compare individual brain regions. Overall, release was greater and $t_{1/2}$ longer in the caudate–putamen than in all other brain regions ($p<0.05$). The concentration of evoked adenosine was significantly greater in the dorsal and middle caudate–putamen than the CA1 or CA3 regions of the hippocampus ($p<0.05$) or the primary or secondary motor cortex ($p<0.05$). $t_{1/2}$ tended to be longer in areas with higher concentrations of evoked adenosine, likely because more adenosine requires more time to be cleared. $t_{1/2}$ was significantly longer in the dorsal and middle areas of the caudate–putamen than all other areas of the brain ($p<0.05$) and was longer in the ventral caudate–putamen than the CA3 area of the hippocampus and the primary and secondary cortex ($p<0.05$).

Mechanism of release The dorsal caudate–putamen, the nucleus accumbens shell, the CA1 area of the hippocampus and the prefrontal cortex were chosen for pharmacological studies because adenosine was detected most frequently in these subregions. Previous results indicate that transient adenosine release in the caudate–putamen is a calcium-dependent downstream effect of ionotropic glutamate reception and not dependent on ATP breakdown [7]. Thus, we tested slices for activity-dependence, dependence on glutamate receptor activity, and effect of ATP breakdown inhibitors.

In all slices, an initial stimulation was performed, then the slice was perfused with the drug, and stimulation was repeated 30 min later. In Fig. 2, asterisks denote significant differences determined from paired t tests comparing evoked concentrations before and after drug for each slice. The saline control data, where saline was used instead of drugs, prove repeated stimulations were stable. Activity

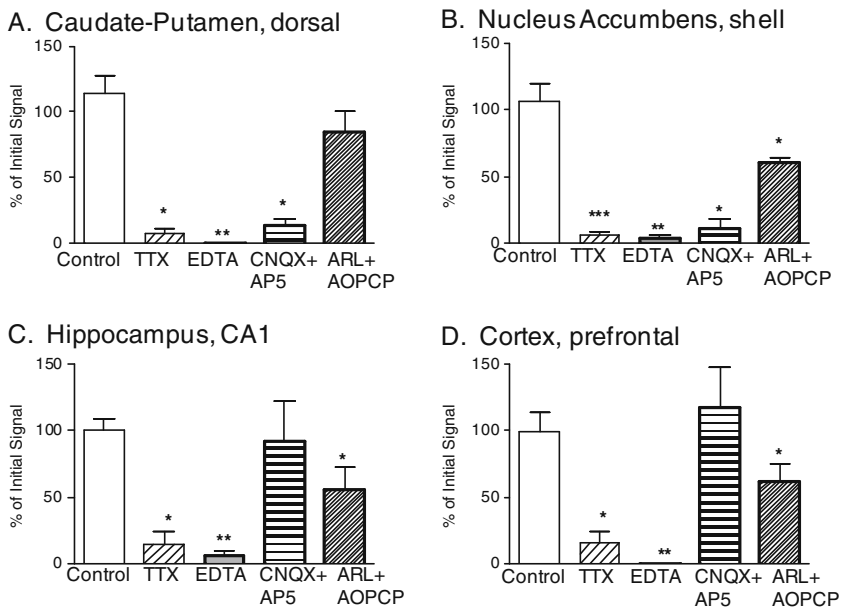

Fig. 2 Pharmacological tests of the mechanism of release. The *y* axis is the percentage of the predrug stimulation. Data were collected in the **a** dorsal caudate–putamen, **b** shell of the nucleus accumbens, **c** CA1 area of the hippocampus, and **d** prefrontal cortex. Tetrodotoxin (*TTX*, 0.5 μM) was administered to inhibit action potentials. EDTA (1 mM) chelated calcium which is necessary for exocytosis. A combination of CNQX (10 μM) and AP5 (100 μM) were added to block ionotropic glutamate receptors. A combination of ARL-67156 (50 μM) and AOPCP (100 μM) were added to prevent extracellular ATP breakdown into adenosine. $n=5$ for the caudate–putamen, nucleus accumbens, and cortex. $n=6$ for the hippocampus. *$p<0.05$; **$p<0.01$; ***$p<0.001$

dependence was tested using 0.5 μM tetrodotoxin (TTX), which blocks Na^+ channels and prevents action potential propagation and EDTA, which chelates extracellular Ca^{2+}, inhibiting exocytosis. TTX reduced stimulated adenosine release significantly in all brain regions as did EDTA (Fig. 2).

To determine if adenosine release is a downstream effect of ionotropic glutamate receptor activation, CNQX (an AMPA receptor antagonist) and AP5 (an NMDA receptor antagonist) are applied to the slices. In the caudate–putamen and the nucleus accumbens, CNQX and AP5 significantly decreased evoked adenosine release, similar to the effect of TTX administration (Fig. 2). However, in the hippocampus and cortex, CNQX and AP5 had no effect on stimulated adenosine release.

Because ATP is released by exocytosis [14], extracellular breakdown of ATP is a possible source for activity-dependent adenosine release. Slices were treated with a combination of ARL-67156, a selective ATPase inhibitor, and AOPCP, an inhibitor of ecto-nucleotidase to block extracellular ATP breakdown. This combination of drugs should also inhibit adenosine formation from the breakdown of extracellular cyclic AMP. In the caudate–putamen, there is no significant effect of ARL-67156 and AOPCP (Fig. 2a). However, inhibition of ATP breakdown reduced adenosine release significantly by about 40 % in the nucleus accumbens (Fig. 2b), hippocampus (Fig. 2c), and cortex (Fig. 2d). While pharmacological agents to block ATP breakdown are not always fully effective, extracellular breakdown of nucleotides contributes at least in part to transient adenosine release in these three brain regions.

Discussion

Short trains of electrical stimulation pulses in rat brain slices can evoke transient adenosine changes in multiple brain regions. In all regions, stimulated adenosine release was activity-dependent and two mechanisms of release were identified: release after ionotropic glutamate receptor activation and extracellular breakdown of ATP. The amount of transient adenosine release and the frequency that it was detected depended on the mechanisms of formation, demonstrating that transient adenosine release may be differently regulated in various brain regions.

Transient adenosine release varies by brain region Stimulated adenosine release was lowest in the hippocampus and cortex and highest in the caudate–putamen. When comparing evoked adenosine levels with basal levels reported in the literature, the rank order of evoked adenosine release is not the same as the trends for regional differences in basal levels. The caudate–putamen and nucleus accumbens have the highest evoked release but the lowest basal adenosine levels [25–27]. In the cortex and hippocampus, trends for stimulated and basal release are better correlated as basal

adenosine and evoked adenosine are both lower in the motor cortex and hippocampus but higher in the prefrontal cortex [25, 27]. Higher stimulated adenosine levels were found in the nucleus accumbens and caudate, where high levels of A_{2a} receptors are expressed [10]. A_1 receptors are expressed in every brain region and do not correlate as well to high levels of stimulated release.

Mechanism of release In this study, we identified two mechanisms of transient adenosine formation: breakdown of extracellular nucleotides and downstream release after glutamate receptor activation. All release was activity-dependent, as chelation of Ca^{2+} or application of tetrodotoxin nearly eliminated adenosine efflux. Adenosine breakdown after activity-dependent ATP release has been widely demonstrated to influence basal adenosine levels in many regions, including the cerebellum, hippocampus, cortex and brain stem [28, 29]. In our study, breakdown of extracellular nucleotides accounts for about half of the evoked adenosine release in the hippocampus, prefrontal cortex, and nucleus accumbens, but not in the caudate–putamen. Extracellular breakdown of ATP occurs within 200 ms [30] so breakdown of ATP in the extracellular space could give rise to transient release. Downstream effects of ionotropic glutamate receptor activation were the predominant mechanism of formation for evoked adenosine in the caudate–putamen and nucleus accumbens. Thus, exocytosis of another neurotransmitter such as glutamate causes downstream release of adenosine, although the mechanism of that release is unknown. Using enzymatic biosensors, a portion of evoked adenosine release in the cerebellum is AMPA receptor dependent in mice [17] but not in rats [31]. Therefore, the dependence of evoked adenosine release on glutamate receptors varies by brain region and species.

While portions of evoked adenosine release were dependent on extracellular ATP breakdown and ionotropic glutamate receptor activation, there was not a strong correlation between those two mechanisms. In studies of $CD73^{-/-}$ mice deficient in ectonucleotidase, dependence on ATP breakdown and ionotropic glutamate receptors was correlated [17], indicating they were the same mechanism. In our studies, evoked adenosine was both ATP and glutamate receptor dependent in the nucleus accumbens, demonstrating that more than one mechanism can occur in a region. Because blocking glutamate receptors nearly eliminated release, some of the ATP release in the nucleus accumbens was likely a downstream effect of glutamate receptor activation. However, our results indicate that in the hippocampus and cortex, stimulated adenosine release was ATP dependent but not glutamate receptor dependent, and in the CP, release was glutamate receptor dependent but not ATP dependent. Therefore, our overall work shows little correlation between ionotropic glutamate receptor dependence and breakdown ATP.

Adenosine release could also be the downstream effect of other neurotransmitters such as dopamine or GABA. While we have previously shown that activity-dependent adenosine in the caudate–putamen is not dopamine-dependent [7], this may not be the case in other brain regions. There is evidence of a correlation between high levels of evoked adenosine and higher densities of GABAergic neurons [32, 33]. However, the Dale group has previously found that GABA, as well as several other downstream neurotransmitters, is unlikely to be a cause of downstream adenosine release in Purkinje cells of the rat cerebellum [21]. Future studies could examine downstream effects of other neurotransmitters in these various regions.

One trend evident from the mechanism of release study was that regions with lower stimulated release and more frequent detection of release were dependent on ATP breakdown. In the two regions with the lowest adenosine release, the hippocampus and cortex, adenosine was partially formed by extracellular ATP breakdown. The caudate–putamen had the highest release and was more dependent on ionotropic glutamate receptor activation. These differences show that different mechanisms of adenosine formation might lead to different levels of adenosine available to activate receptors and modulate neurotransmission. Evoked adenosine was detected more frequently in regions where it was formed after the breakdown of ATP, which suggests that ATP breakdown may produce more widespread changes in adenosine that are easily measured. Because local stimulations in slices cause global neural firing, the failure to detect adenosine in many of the slices in the caudate and nucleus accumbens may point to a site-specific aspect of downstream glutamate effects, especially in the striatum which has areas of high and low glutamate receptor density [34, 35]. This may further explain why areas where adenosine release was more frequently detected, such as the cortex and hippocampus, showed less variance in the concentration of adenosine evoked, since reliable stimulation would result in more consistent pathway activation.

Possible effects of transient adenosine release Adenosine signaling is mediated through G-protein coupled adenosine receptors which modulate cellular excitability. A_1 receptors are highly expressed in the hippocampus, cerebellum, caudate, and cerebral cortex with more moderate levels in the nucleus accumbens [36, 37]. A_{2A} receptors are found in high density in only a few areas of the brain, including the caudate–putamen and nucleus accumbens [38]. Activation of different receptors could lead to different downstream effects of transient adenosine release. For example, the high abundance of A_1 receptors in comparison to A_{2A} receptors in the hippocampus and cortex would lead to stronger inhibitory effects. Transient adenosine release observed in the nucleus accumbens may be more excitatory due to more

A_{2A} receptors. Both A_1 and A_{2A} receptors are found at relatively high densities in the caudate, thus the high variation in evoked adenosine in this region may point to regional differences in adenosine production and regulation. A_1 receptors self-regulate transient adenosine release [39] so areas with high densities of A_1 receptors may have less stimulated release. Transient adenosine may reach concentrations high enough to activate lower affinity adenosine receptors such as the A_{2B} and A_3 receptors [40]. The physiological effects of adenosine could vary due to receptor density and future studies to correlate release sites and receptor locations may provide more information about the function of transient adenosine release.

Conclusions

Using short trains of electrical stimulations, we characterized adenosine release in the caudate–putamen, nucleus accumbens, hippocampus, and cortex. Evoked adenosine was found to be activity-dependent in all brain areas. Two mechanisms of formation were determined: breakdown of extracellular nucleotides and downstream actions of ionotropic glutamate receptors. Adenosine release was highest in areas where it was formed as a downstream effect of glutamate receptor activation, but it was also the most heterogeneous in these regions and not as frequently detected. These findings suggest that transient adenosine is found in many brain regions but that different mechanisms of formation can lead to differences in signaling between brain regions.

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