PRP Pharmacology Research & Perspectives
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ORIGINAL ARTICLE

# Clearance of rapid adenosine release is regulated by nucleoside transporters and metabolism

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**Keywords**
Adenosine deaminase, adenosine kinase, equilibrative nucleoside transporter, voltammetry

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**Funding Information**
This research was funded by the National Institutes of Health (R01NS076875).

Received: 14 August 2015;
Accepted: 24 August 2015

***Pharma Res Per, 3(6), 2015, e00189, doi: 10.1002/prp2.189***

doi: 10.1002/prp2.189

## Abstract

Adenosine is a neuromodulator that regulates neurotransmission in the brain and central nervous system. Recently, spontaneous adenosine release that is cleared in 3–4 sec was discovered in mouse spinal cord slices and anesthetized rat brains. Here, we examined the clearance of spontaneous adenosine in the rat caudate-putamen and exogenously applied adenosine in caudate brain slices. The $V_{max}$ for clearance of exogenously applied adenosine in brain slices was 1.4 ± 0.1 μmol/L/sec. In vivo, the equilibrative nucleoside transport 1 (ENT1) inhibitor, *S*-(4-nitrobenzyl)-6-thioinosine (NBTI) (1 mg/kg, i.p.) significantly increased the duration of adenosine, while the ENT1/2 inhibitor, dipyridamole (10 mg/kg, i.p.), did not affect duration. 5-(3-Bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*]byrimidin-4-amine dihydrochloride (ABT-702), an adenosine kinase inhibitor (5 mg/kg, i.p.), increased the duration of spontaneous adenosine release. The adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (10 mg/kg, i.p.), also increased the duration in vivo. Similarly, NBTI (10 μmol/L), ABT-702 (100 nmol/L), or EHNA (20 μmol/L) also decreased the clearance rate of exogenously applied adenosine in brain slices. The increases in duration for blocking ENT1, adenosine kinase, or adenosine deaminase individually were similar, about 0.4 sec in vivo; thus, the removal of adenosine on a rapid time scale occurs through three mechanisms that have comparable effects. A cocktail of ABT-702, NBTI, and EHNA significantly increased the duration by 0.7 sec, so the mechanisms are not additive and there may be additional mechanisms clearing adenosine on a rapid time scale. The presence of multiple mechanisms for adenosine clearance on a time scale of seconds demonstrates that adenosine is tightly regulated in the extracellular space.

### Abbreviations

ABT-702, 5-(3-bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*]byrimidin-4-amine dihydrochloride; ANOVA, analysis of variance; CNT, concentrative nucleoside transporter; CV, cyclic voltammogram; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; ENT, equilibrative nucleoside transporter; KS, Kolmogorov–Smirnov; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; PBS, phosphate buffered saline; PCA, principal component analysis; SAH, *S*-adenosylhomocysteine.

## Introduction

Adenosine modulates neurotransmission in the brain and is neuroprotective during stressful conditions, such as ischemia and hypoxia. Adenosine appearance in the extracellular space is from the breakdown of adenosine triphosphate (ATP) and direct release through transporters (Wall and Dale 2008). Recently, rapidly released adenosine was discovered on a time scale of 3–30 sec following electrical stimulation (Klyuch et al. 2012; Pajski and Venton 2013), mechanical stimulation (Chang et al. 2009; Ross et al. 2014), and ischemia (Dale and Frenguelli

2009). In addition to stimulated release, spontaneously released adenosine was discovered in spinal cord slices of mice (Street et al. 2011) and in vivo in the rat brain (Nguyen et al. 2014). Adenosine is spontaneously released and removed from the extracellular space in less than 4 sec, implying adenosine is cleared on a rapid time scale. Although the mechanism of clearance for adenosine has been examined on a longer time scale (Pazzagli et al. 1995), the clearance of rapid adenosine has not been characterized.

Once released into the extracellular space, adenosine is typically cleared by nucleoside transporters or metabolism (Latini and Pedata 2001). There are two types of adenosine transporters: equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs). Adenosine follows a concentrative gradient through ENTs, while CNTs move nucleosides against a gradient (Thorn and Jarvis 1996). There are four types of equilibrative transporters, ENTs 1–4. The inhibitor nitrobenzylthioinosine (*S*-(4-nitrobenzyl)-6-thioinosine [NBTI]) is selective for ENT1 (Ward et al. 2000). There are only nonspecific inhibitors of ENT2, dipyridamole and dilazep (Visser et al. 2002). ENT3 is expressed in human and mouse tissue; however, ENT3 is located intracellularly (Baldwin et al. 2005) and is not involved in adenosine clearance. ENT4 has a low affinity for adenosine at physiological pH and could be involved in adenosine clearance during acidotic conditions from ischemia (Barnes et al. 2006); however, selective inhibitors are not widely available yet (Wang et al. 2013). There are three types of CNTs (1–3), however, there is no evidence that CNTs are regulating physiological levels of adenosine (Parkinson et al. 2011). While there are a few reports of specific inhibitors of human CNTs, they are not widely available (Damaraju et al. 2011). Thus, ENT1 and ENT2 were the primary focus for studying the regulation of transient adenosine release.

Adenosine is also cleared from the extracellular space by metabolism. Adenosine kinase phosphorylates adenosine monophosphate (AMP) to adenosine and adenosine deaminase breaks adenosine down to inosine. Both adenosine kinase and adenosine deaminase modulate adenosine concentrations; thus, they are expected to affect transient adenosine release. In the hippocampus, extracellular adenosine concentrations increased twofold with adenosine kinase inhibition (Pak et al. 1994). Studies on adenosine deaminase inhibition are mixed as one study showed increases in adenosine basal concentrations (Sciotti and van Wylen 1993), while another study found little change (Lloyd and Fredholm 1995). The effects of metabolism clearing adenosine on a faster time scale are unknown.

Here, we investigated the mechanism of release and clearance of transient adenosine. We examined rapid clearance of spontaneously released adenosine in vivo and of exogenously applied adenosine in brain slices. ENTs of adenosine were blocked with NBTI and dipyridamole, and ENT1 was responsible for rapid clearance of adenosine. Similarly erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an inhibitor of adenosine deaminase, and 5-(3-bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*] byrimidin-4-amine dihydrochloride (ABT-702), an adenosine kinase inhibitor, significantly decreased the clearance rate of adenosine. Spontaneous, transient adenosine is cleared by nucleoside transporters (ENT1), adenosine kinase, and adenosine deaminase; thus, adenosine is tightly regulated in the extracellular space by multiple clearance mechanisms.

## Materials and Methods

### Chemicals and drugs

All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Phosphate buffered saline (PBS) was composed of (in mmol/L): NaCl (131.25), $NaH_2PO_4$ (10.0), KCl (Fisher Scientific, Fair Lawn, NJ) (3.0), $Na_2SO_4$ (Fisher) (2.0), $CaCl_2$ (1.2), $MgCl_2$ (Fisher) (1.2). The pH of PBS solution was adjusted to 7.4. Adenosine was prepared daily in PBS from a 10 mmol/L stock solution in 0.1 mol/L $HClO_4$.

NBTI was dissolved in saline (Baxter, Deerfield, IL) and dimethyl (DMSO) (Amresco, Solon, OH) injected i.p. at 1 mg/kg. 2,2′,2″,2‴-(4,8-di(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidine-2,6-diyl)bis(azanetriyl)tetraethanol (Dipyridamole) (Tocris Bioscience, Ellisville, MO) dissolved in DMSO and injected i.p. at 10 mg/kg. EHNA was dissolved in saline and injected i.p. at 10 mg/kg. ABT-702 was dissolved in DMSO and injected i.p. at 5 mg/kg. For the cocktail, ABT-702, EHNA, and NBTI were dissolved at the same doses as given above in DMSO and injected i.p.

### Electrodes and electrochemistry

Carbon-fiber cylinder microelectrodes were prepared as previously described (Huffman and Venton 2009). T-650 carbon-fibers (7 $\mu$m diameter, Cytec Engineering Materials, West Patterson, NJ) were pulled in glass capillaries (A-M Systems Inc., Seqium, WA) and cylinders were cut between 75–125 $\mu$m in length. Cyclic voltammetry data were collected with TarHeel CV software and High-Definition Cyclic-Voltammetry (gift from Mark Wightman, UNC) using a Dagan ChemClamp (Dagan Corporation, Minneapolis, MN). Electrodes were scanned from −0.40 to 1.45 V and back at 400 V/sec at 10 Hz against a Ag/AgCl reference electrode. Electrodes were postcalibrated with 1.0 $\mu$mol/L adenosine after each experiment.

### Data collection and analysis

Electrodes were allowed to equilibrate in vivo for at least an hour and a half in order to obtain a steady baseline. If transients were not observed or the frequency was too low (less than five per hour) within the first hour of equilibration, another electrode was inserted until transients were found. Two hours of pre-drug and 2 h of postdrug were collected, however only the second hour of predrug and first hour of postdrug were used due to electrode stability. To calculate concentration and duration, principal component analysis (PCA) was used in the high definition cyclic voltammetry analysis software (Nguyen et al. 2014). Using PCA, the smallest adenosine transient that can be detected is 40 nmol/L.

We examined the exponential decay rates of transient adenosine release in vivo in the 150–250 nmol/L range. Transients less than 150 nmol/L have small peak signals, which can be obscured by baseline noise. The upper limit was set at 250 nmol/L so the predrug and postdrug have similar numbers of transients for comparison. Curves were fit with a one-phase exponential decay with the equation:

$$[\mathrm{AD}](t) = [\mathrm{AD}]_{\max e^{-kt}}$$

Curves were fit in GraphPad PRISM 6 (GraphPad Software Inc., San Diego, CA). For the velocity versus concentration graph, the concentrations were placed into bins (Sabeti et al. 2002).

### Fast-scan cyclic voltammetry of adenosine

Adenosine was measured with sub-second temporal resolution, using fast-scan cyclic voltammetry (Swamy and Venton 2007) and the clearance times and rates were investigated before and after pharmacological agents were administered. To measure adenosine, the electrode potential was swept from −0.40 to 1.45 V and back at 400 V/sec at 10 Hz (Nguyen and Venton 2015). Adenosine was identified by its unique, background subtracted cyclic voltammogram (CV) which has a primary oxidation peak at 1.4 V followed by a secondary oxidation at 1.0 V (Cechova and Venton 2008). A concentration versus time plot was made using PCA and was used to track how long adenosine was elevated in the extracellular space (Nguyen et al. 2014). We examined spontaneous, transient adenosine release in vivo as well as exogenously applied adenosine in brain slices to determine the mechanism of rapid adenosine clearance.

### Statistics

Statistics were performed using MatLAB (The MathWorks, Inc., Natick, MA) and GraphPad PRISM 6. Data are shown as mean ± SEM with $n$ number of animals. One-way analysis of variance (ANOVA) with Bonferroni post-test was used to determine significance in the stability test. Unpaired Student $t$-test was performed to compare duration, decay rate, and concentration before and after drug application. Kolmogorov–Smirnov (KS) test was used to determine the underlying interevent time distributions. All data were considered significant at the 95% confidence level.

## Results

### Spontaneous, transient adenosine clearance

Adenosine is spontaneously released without stimulation and is cleared in about 3 sec in anesthetized rats (Nguyen et al. 2014). Figure 1A shows a concentration versus time

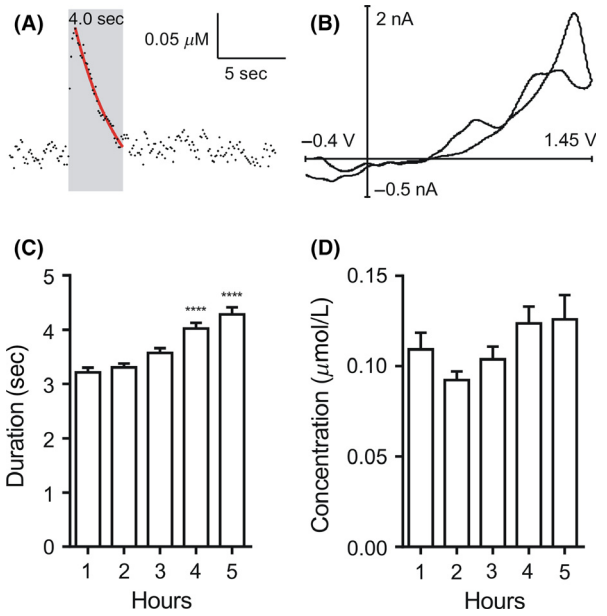


**Figure 1.** Stability of spontaneous, transient adenosine release in vivo using fast-scan cyclic voltammetry. (A) Concentration versus time trace of spontaneous adenosine release in vivo. The gray shading shows when adenosine levels are above 10% baseline and the clearance of adenosine is fit with a single exponential decay (red). (B) A characteristic CV of adenosine detected with fast-scan cyclic voltammetry. The primary oxidation is observed at 1.40 V and the secondary oxidation is observed at 1.0 V. (C) The duration of transient adenosine release was measured in the caudate-putamen of anesthetized rats over a 5 h period. The durations are placed into 1 h time bins. The fourth and fifth hour are significantly higher than the first 3 h (one-way ANOVA post-Bonferroni test, $n = 4$, $P < 0.05$). (D) Concentration of spontaneous adenosine release were placed into hour bins. There is a significant difference in concentration over time (one-way ANOVA, $n = 4$, $P < 0.05$), but not between the second and third hours (one-way ANOVA post-Bonferroni test, $n = 4$, $P > 0.05$). CV, cyclic voltammogram; ANOVA, analysis of variance. ****$P < 0.001$

plot of an example of spontaneously released adenosine. Adenosine is released and cleared within the extracellular space in 4.0 sec. The gray shaded area between the dotted vertical lines indicates when the adenosine concentration is 10% higher than baseline and shows how the peak duration was calculated in vivo. An exponential decay rate was fit from the apex to when the signal decayed to 90% of peak amplitude and is marked with a red line. The decay rate, $k$ ($sec^{-1}$), was calculated with a single exponential decay. A characteristic CV of transient adenosine is shown in Figure 1B. The working electrode is scanned from −0.40 to 1.45 V and back at 400 V/sec. The resulting current versus applied potential is a CV. Adenosine is oxidized at 1.4 V (primary oxidation) and the oxidation product undergoes a secondary oxidation at 1.0 V. The CV verifies that adenosine is detected.

## Stability of transient adenosine release

First, we examined spontaneous, transient adenosine release over a 5 h period to determine stability over time. The measured durations were placed into 1 h bins. The duration of spontaneous adenosine release significantly increases over time ($n = 4$, one way ANOVA, $P < 0.0001$); however the first 3 h are not significantly different from each other ($n = 4$, Bonferroni test, $P > 0.05$, Fig. 1C). Similarly, the concentrations of transient adenosine events were binned in 1 h increments (Fig. 1D) and there was a significant change over the 5 h period ($n = 4$, one way ANOVA, $P = 0.0279$); however, there was no significant difference between any individual hour periods ($n = 4$, Bonferroni test, $P > 0.05$). A possible explanation for the increase in duration after 3 h is surface fouling of the electrode by lipids and proteins onto the electrode (Park et al. 2005; Chandra et al. 2014). Fouling may restrict diffusion to and from the surface of the electrode, causing an apparent increase in duration of spontaneous adenosine release. Therefore, for drug experiments, we analyzed data from the second hour (predrug) and third hour (first hour postdrug) as the duration and decay rate ($n = 4$, unpaired $t$-test, $P = 0.8781$) were constant for this time period.

## Inhibiting ENTs

NBTI, a specific inhibitor of ENT1, was administered intraperitoneally (1 mg/kg). NBTI crosses the blood–brain barrier (Anderson et al. 1996) and at this dose, NBTI increased the concentration of adenosine (Salcedo et al. 1997). In Figure 2A, concentration versus time plots before (top) and after (bottom) NBTI administration demonstrate the increase in duration. The CVs are characteristic of adenosine and verify adenosine was detected pre and

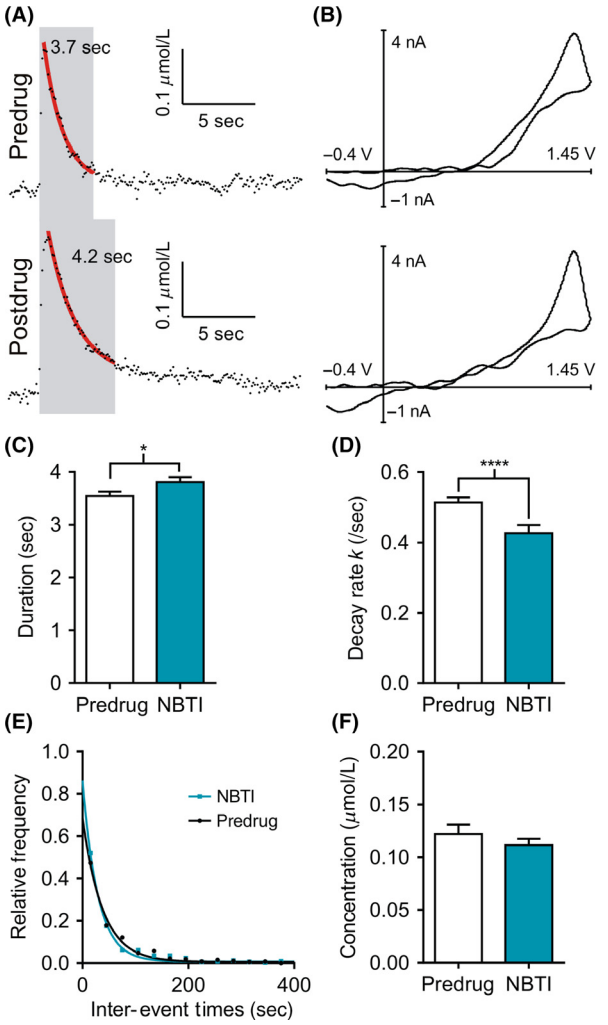


**Figure 2.** Inhibition of clearance of spontaneous, transient adenosine release by NBTI. (A) Concentration versus time trace of spontaneous adenosine release pre- (top) and post-NBTI (bottom, 1 mg/kg i.p.) which inhibits ENT1. Gray shading indicates when adenosine is above 90% of the baseline with exponential decay fit (red trace). (B) CVs of adenosine for predrug (top) and postdrug (bottom) transients. (C) The duration of adenosine significantly increased (unpaired $t$-test, $n = 6$, $P = 0.0327$). (D) The decay rate ($k$, $sec^{-1}$) following NBTI administration (blue) is significantly smaller than predrug values (white) (unpaired $t$-test, $n = 8$, $P = 0.0024$). (E) A histogram of relative frequency versus interevent time (time between consecutive transients) with 30 sec bins. Exponential fits are displayed for predrug (black) and post-NBTI (blue) with no significant difference in the underlying distributions (Kolmogorov–Smirnov test, $n = 8$ rats, $P = 0.2882$). (F) Spontaneous transient adenosine concentration predrug (white bars) and post-NBTI (blue bars) did not significantly change (unpaired $t$-test, $n = 8$, $P = 0.3585$). NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; ENT1, equilibrative nucleoside transport 1; CV, cyclic voltammogram. $^*P < 0.05$, $^{****}P < 0.001$

postdrug (Fig. 2B). Figure 2C shows the duration of transient adenosine significantly increased after NBTI from 3.5 ± 0.1 to 3.8 ± 0.1 sec ($n = 8$ animals, unpaired $t$-test,

$P = 0.0327$). NBTI significantly decreased the exponential decay value for clearance (Fig. 2D) from $0.51 \pm 0.01$ to $0.43 \pm 0.02$ $sec^{-1}$ ($n = 8$ animals, unpaired *t*-test, $P = 0.0024$). To make sure the dose was not too low, an increased NBTI dose (i.p. 40 mg/kg) was administered and the duration increased from $4.1 \pm 0.2$ to $4.6 \pm 0.2$ sec ($n = 3$ animals, unpaired *t*-test, $P = 0.0386$, Fig. S1A). The increased NBTI dose also significantly decreased the exponential decay value from $0.37 \pm 0.04$ to $0.26 \pm 0.03$ $sec^{-1}$ ($n = 3$ animals, unpaired *t*-test, $P = 0.0319$, Fig. S1B). The increase in duration and decrease in exponential decay were similar for the two doses of NBTI. The increase in duration for adenosine in the extracellular space demonstrates that spontaneous release is cleared by ENT1.

In order to further characterize the effects of NBTI on spontaneous, transient adenosine release, the interevent times and concentrations were examined. The interevent times, that is, the time between consecutive transients, were placed into 30 sec bins. The frequency distribution was examined for predrug (black line) and NBTI (blue line) (Fig. 2E). The underlying distribution of the frequency of transient adenosine release did not change following NBTI ($n = 8$ animals, KS test, $P = 0.2882$). The concentration of each transient did not significantly change from $0.12 \pm 0.01$ to $0.11 \pm 0.01$ $\mu$mol/L (Fig. 2F, $n = 8$ animals, unpaired *t*-test, $P = 0.3585$). The results demonstrate spontaneous adenosine is not released through equilibrative transport, since the frequency and concentration of adenosine did not decrease after ENT1 inhibition.

Dipyridamole inhibits both ENT1 and ENT2, although it has a higher affinity for ENT1 (Ward et al. 2000). At 10 mg/kg i.p., dipyridamole decreased aggressive behaviors in mice by increasing levels of adenosine (Ushijima et al. 1984). Dipyridamole (10 mg/kg, i.p.) did not increase the duration of transient adenosine in the extracellular space as shown in the concentration versus time traces (Fig. 3A). The CVs of adenosine remain unchanged in the presence of dipyridamole, with the secondary peak on the anodic scan at 1.0 V and the primary peak on the cathodic scan at 1.40 V (Fig. 3B). Dipyridamole did not significantly change the duration from $3.2 \pm 0.1$ predrug to $3.2 \pm 0.1$ sec (Fig. 3C, $n = 6$ animals, unpaired *t*-test, $P = 0.6921$). However, the exponential decay rate for clearance significantly decreased from $0.39 \pm 0.02$ to $0.31 \pm 0.02$ $sec^{-1}$ (Fig. 3D, $n = 6$ animals, unpaired *t*-test, $P = 0.0141$).

Nonselective inhibition of ENT1 and ENT2 with dipyridamole did not affect the magnitude or frequency of spontaneous adenosine release. The interevent time distribution did not significantly change (Fig. 3E, $n = 6$ animals, KS test, $P = 0.3141$). The concentration of transient adenosine following ENT1/2 inhibition remained unchanged from $0.12 \pm 0.01$ to $0.13 \pm 0.02$ $\mu$mol/L (Fig. 3F, $n = 6$ animals, unpaired *t*-test, $P = 0.4893$). The results show that spontaneous transient adenosine is not affected by inhibition of ENT1/2 with dipyridamole.

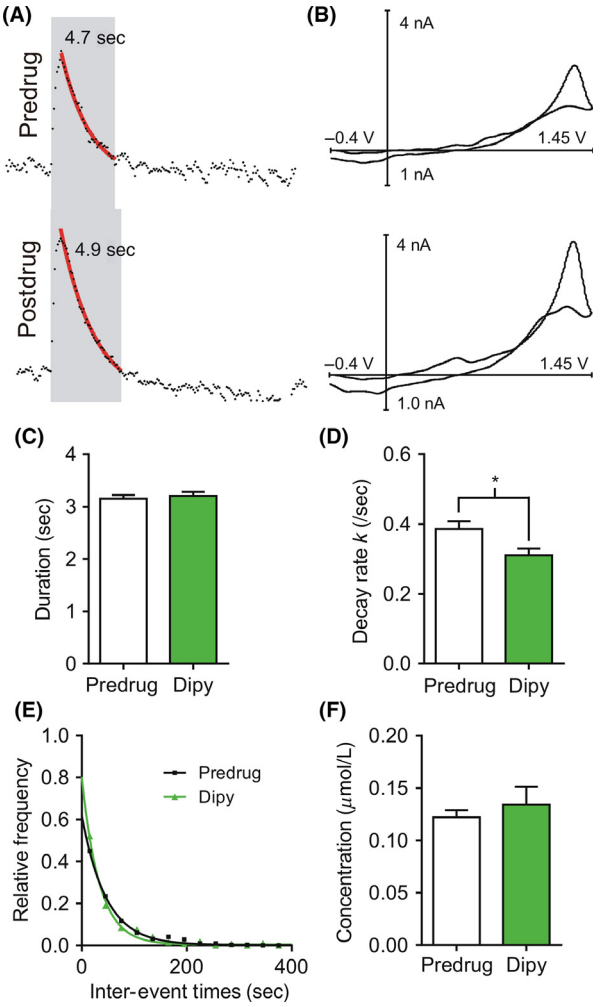


**Figure 3.** Inhibition of clearance of spontaneous, transient adenosine release through ENT1/2. (A) A predrug transient (top) and post-dipyridamole (ENT1/2 inhibitor, 10 mg/kg i.p., bottom) concentration versus time graph with duration shaded gray and exponential decay fit in red. (B) CVs of adenosine before and after dipyridamole. (C) Duration before (white) and after dipyridamole (green) did not significantly change (unpaired *t*-test, $n = 6$ animals, $P = 0.6921$). (D) The decay rate following dipyridamole significantly decreased (unpaired *t*-test, $n = 6$ animals, $P = 0.0141$). (E) Interevent time histogram before (black) and after (green) dipyridamole. The underlying distributions are not significantly different (KS test, $n = 6$ animals, $P = 0.3141$). (F) Concentration of spontaneous adenosine did not significantly change postdrug (unpaired *t*-test, $n = 6$ animals, $P = 0.4893$). ENT, equilibrative nucleoside transport; CV, cyclic voltammogram; KS, Kolmogorov–Smirnov. $^*P < 0.05$

## Inhibiting metabolism

There are two primary enzymes that remove adenosine from the extracellular space: adenosine kinase and ade-

nosine deaminase. ABT-702 inhibits adenosine kinase and increased extracellular adenosine concentrations (Kowaluk et al. 2000) and at a dose of 5 mg/kg i.p. increased stimulated adenosine release (Cechova and Venton 2008). Figure 4A shows concentration versus time traces of spontaneous adenosine release before and after adenosine kinase inhibition with the exponential fit of the decay portion in red. The CVs of adenosine pre- and post-ABT-702 do not change (Fig. 4B). Inhibition of adenosine kinase with ABT-702 (5 mg/kg, i.p.) significantly increased the duration of spontaneous adenosine release from $2.9 \pm 0.1$ to $3.3 \pm 0.1$ sec (Fig. 4C, $n = 5$ animals, unpaired $t$-test, $P = 0.0155$). The decay rate significantly decreased from $0.42 \pm 0.03$ to $0.31 \pm 0.02$ $sec^{-1}$ (Fig. 4D, $n = 5$ animals, unpaired $t$-test, $P = 0.0127$).

After administration of ABT-702, the interevent time distribution is significantly different (Fig. 4E, $n = 5$ animals, KS test, $P = 0.0414$) with frequency decreasing. The concentration of spontaneous transients significantly increased from $0.14 \pm 0.01$ to $0.20 \pm 0.03$ $\mu$mol/L (Fig. 4F, $n = 5$ animals, unpaired $t$-test, $P = 0.0483$). The results are in agreement with previous studies that showed inhibition of adenosine kinase increased stimulated adenosine release (Cechova and Venton 2008).

Adenosine deaminase, an enzyme that metabolizes adenosine into inosine was inhibited with EHNA. EHNA increased adenosine levels and decreased inosine levels during cerebral ischemia at 10 mg/kg i.p. in vivo (Kobayashi et al. 1998). Concentration versus time plots (Fig. 5A) and CVs (Fig. 5B) show the effect of adenosine deaminase inhibition. After EHNA (10 mg/kg, i.p.), the duration of spontaneous transients significantly increased from $3.5 \pm 0.1$ to $3.9 \pm 0.1$ sec (Fig. 5C, $n = 6$ animals, unpaired $t$-test, $P = 0.0049$). In Figure 5D, the exponential decay rate of clearance significantly decreased from $0.60 \pm 0.02$ to $0.44 \pm 0.02$ $sec^{-1}$ ($n = 6$ animals, unpaired $t$-test, $P < 0.0001$).

Figure 5E shows the frequency distribution of interevent times for predrug (black line) and post-EHNA (red line). The underlying distributions of the time between transient events are not significantly different ($n = 6$ animals, KS test, $P = 0.8506$). The concentration significantly decreased from $0.18 \pm 0.01$ to $0.15 \pm 0.01$ $\mu$mol/L (Fig. 5F, $n = 6$ animals, unpaired $t$-test, $P = 0.0404$). Concentration decreased after adenosine deaminase inhibition, showing the opposite effect of adenosine kinase inhibition.

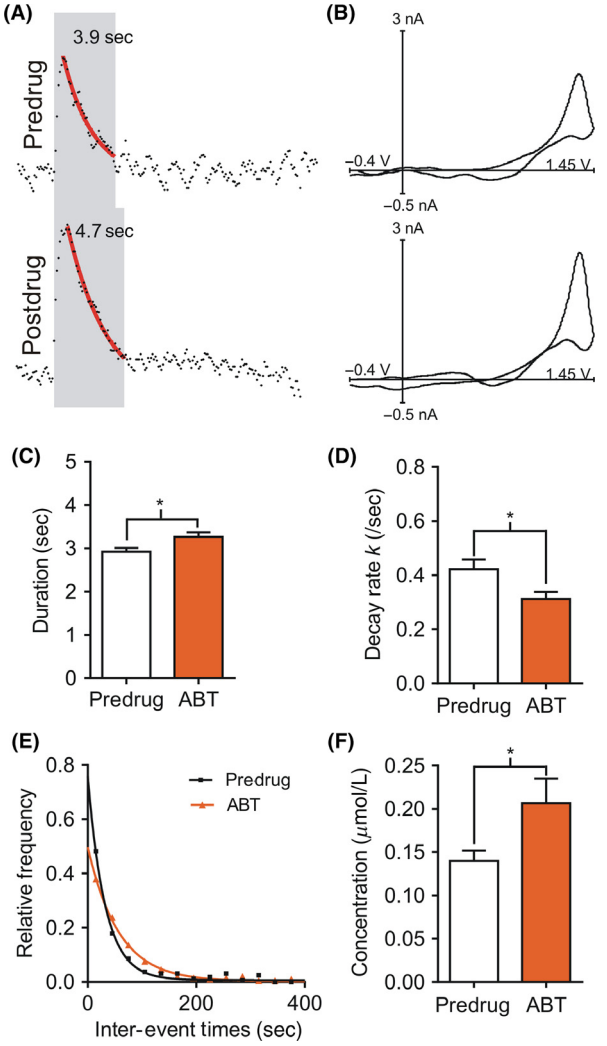


**Figure 4.** The effect of adenosine kinase on clearance of transient adenosine release. (A) Concentration versus time of spontaneous, transient adenosine release before (top) and after (bottom) ABT-702 (5 mg/kg, i.p.). (B) CVs of adenosine pre and postdrug. (C) The duration significantly increased (unpaired $t$-test, $n = 5$ animals, $P = 0.0155$) post-ABT-702 (orange). (D) The decay rate significantly decreased after administration of ABT-702 (unpaired $t$-test, $n = 5$ animals, $P = 0.0127$). (E) Interevent time histogram of predrug (black) and postdrug (orange) with no significant difference between the underlying distributions (KS test, $n = 5$ animals, $P = 0.0414$). (F) The concentration of spontaneous adenosine release significantly increased (unpaired $t$-test, $n = 5$ animals, $P = 0.0483$) after ABT-702 administration. ABT-702, 5-(3-bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*]byrimidin-4-amine dihydrochloride; CV, cyclic voltammogram; KS, Kolmogorov–Smirnov. $*P < 0.05$

## Inhibition of ENTs and metabolism

Adenosine kinase, adenosine deaminase, and ENT1 were inhibited with a mixture of ABT-702 (5 mg/kg), EHNA (10 mg/kg), and NBTI (1 mg/kg) in order to test the effects of blocking the main adenosine clearance mechanisms simultaneously. The cocktail significantly increased the duration of spontaneous adenosine from $4.3 \pm 0.1$ to $5.0 \pm 0.1$ sec (Fig. 6A, $n = 5$ animals, unpaired $t$-test, $P < 0.0001$). In Figure 6B, the decay rate after the cocktail significantly

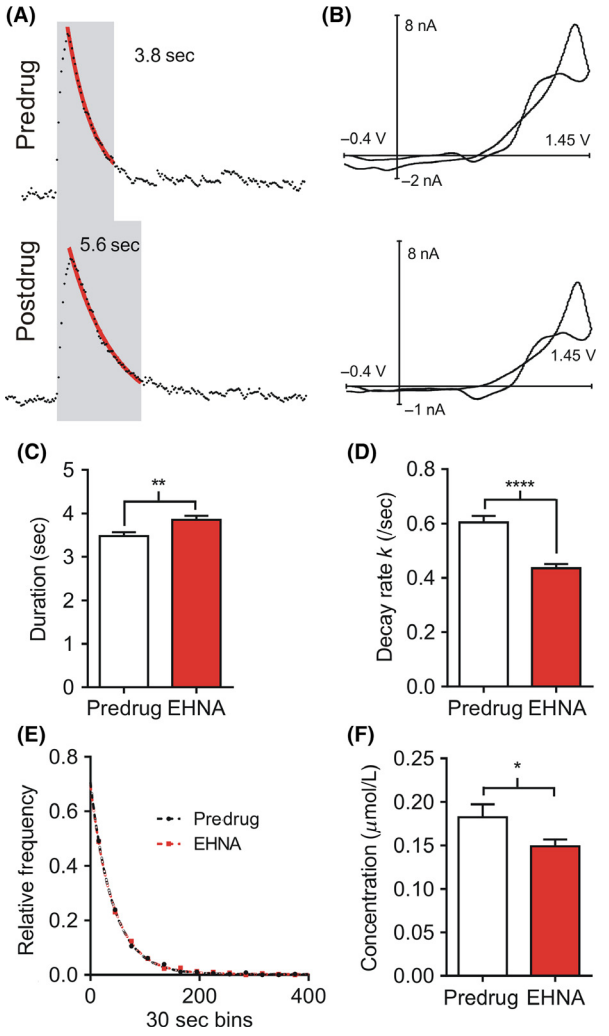


**Figure 5.** The effect of adenosine deaminase on clearance of transient adenosine release. (A) Effect of adenosine deaminase inhibition with EHNA (10 mg/kg i.p.) on spontaneous adenosine release. Concentration versus time plots before and after inhibition with gray indicating the duration of release and red showing the exponential decay fit. (B) CVs for adenosine pre- and post-EHNA. (C) The duration of transient adenosine significantly increased pre and postdrug (unpaired *t*-test, $n = 6$ animals, $P = 0.0049$). (D) Decay rates were significantly different after EHNA administration (unpaired *t*-test, $n = 6$ animals, $P < 0.0001$). (E) Interevent time histogram with predrug trace (black) and post-EHNA (red). EHNA had no significant effect on the underlying distribution ($n = 6$ animals, KS test, $P = 0.8506$). (F) The concentration of transient adenosine decreased following adenosine deaminase (red) inhibition (unpaired *t*-test, $n = 6$ animals, $P = 0.0404$). EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; CV, cyclic voltammogram; KS, Kolmogorov–Smirnov. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$

decreased from $0.23 \pm 0.02$ to $0.16 \pm 0.02$ sec$^{-1}$ ($n = 5$ animals, unpaired *t*-test, $P = 0.0315$).

The combined inhibition of adenosine kinase, adenosine deaminase, and ENT1 did not significantly alter the underlying interevent distribution of spontaneously released adenosine ($n = 5$ animals, KS test, $P = 0.3485$, Fig. 6C). Similarly, the concentration of transient adenosine release did not significantly change from $0.16 \pm 0.01$ to $0.18 \pm 0.02$ $\mu$mol/L (Fig. 6D, $n = 5$ animals, unpaired *t*-test, $P = 0.1951$).

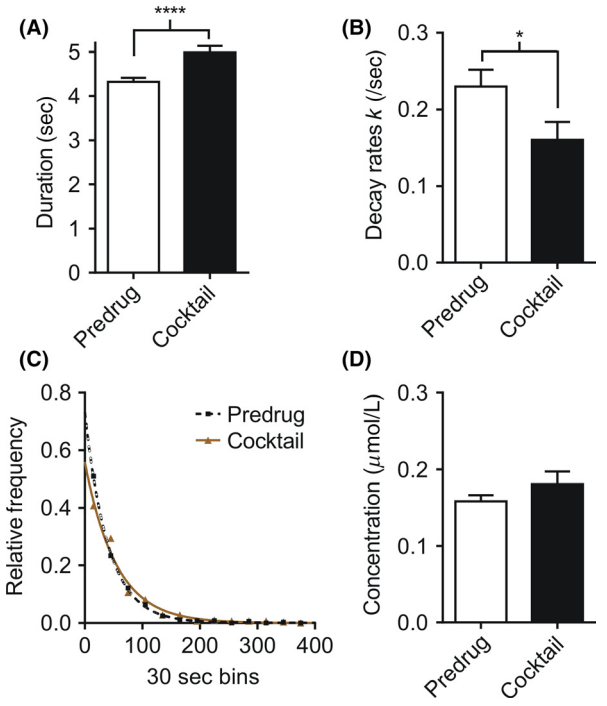


**Figure 6.** The effect of simultaneous inhibition of adenosine kinase, adenosine deaminase, and ENT1 on clearance of transient adenosine release. (A) Effect of ABT-702 (5 mg/kg), EHNA (10 mg/kg), and NBTI (1 mg/kg) on the duration of spontaneous, transient adenosine release. The inhibition of adenosine deaminase, adenosine kinase, and ENT1 significantly increased the duration of adenosine release (unpaired *t*-test, $n = 5$ animals, $P < 0.0001$). (B) The decay rates were significantly smaller following the cocktail administration (unpaired *t*-test, $n = 5$ animals, $P = 0.0315$). (C) The interevent distribution was not significantly different before and after inhibition ($n = 5$ animals, KS test, $P = 0.3485$). (D) The concentration of released adenosine did not change (unpaired *t*-test, $n = 5$ animals, $P = 0.1951$). ENT1, equilibrative nucleoside transport 1; ABT-702, 5-(3-bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*]byrimidin-4-amine dihydrochloride; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; KS, Kolmogorov–Smirnov. $^*P < 0.05$, $^{****}P < 0.0001$

## Exogenously applied adenosine

Different amounts of adenosine were pressure ejected onto brain slices using a pulled glass pipette that was calibrated for multiple ejection times at varying pressures (5–20 psi). Figure 7A shows a plot of raw data of concentration versus time with different amounts of exogenously applied adenosine in caudate-putamen brain slices. Increasing the amount of applied adenosine increased the

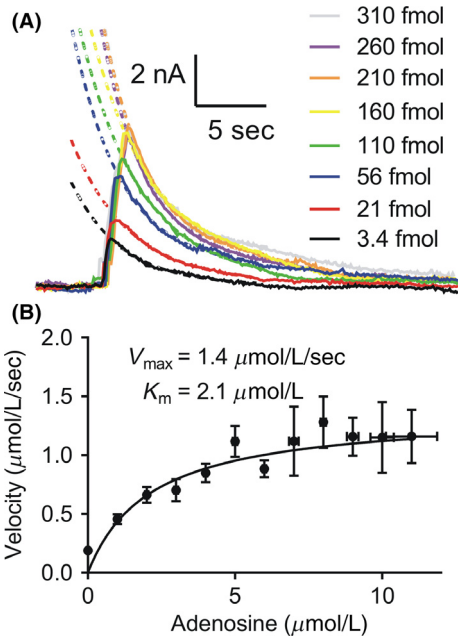


**Figure 7.** Application of exogenous adenosine in caudate-putamen brain slices. (A) Concentration versus time trace of increasing amounts of adenosine picospritzed onto brain slices. The concentrations range from 3.4 to 310 femtomol and are fit with single exponential decays (dashed lines). (B) Velocity of clearance from multiple electrodes plotted versus applied adenosine concentration. The concentrations were placed into 100 nmol/L bins. The Michaelis–Menten equation was fit to the curve in order to determine a $V_{max}$ of 1.4 μmol/L/sec for adenosine clearance.

rate of clearance until saturation. Each trace was fit with an exponential decay (dashed lines) to measure clearance. The initial velocity, $V$, was calculated from the rate constants using the equation:

$$V = k[\mathrm{AD}]_{\max}$$

A plot of velocity versus concentration (Fig. 7B) was fit with a nonlinear regression following Michaelis–Menten enzyme kinetics. The plot had a maximal clearance rate, $V_{max}$, of 1.4 ± 0.1 μmol/L/sec which includes all forms of adenosine clearance, including uptake and metabolism.

## Adenosine transporter clearance

Adenosine was pressure ejected before and after the brain slices were bathed in NBTI (10 μmol/L) to block ENT1 (Fig. 8A). At 10 μmol/L, NBTI inhibits adenosine transporters in brain slices (Bailey et al. 2004). The results show that ENT1 is partially responsible for the clearance of exogenously applied adenosine. The exponential decay rate after NBTI significantly decreased from 0.23 ± 0.01 to 0.16 ± 0.02 $\mathrm{sec}^{-1}$ ($n$ = 4, paired $t$-test, $P$ = 0.0002). The lowered rate of clearance shows blocking nucleoside transporters slowed the ability to remove adenosine from the extracellular space.

ENT1 and 2 were also inhibited with dipyridamole (10 μmol/L) (Fig. 8B). Dipyridamole reduced excitatory postsynaptic potentials in brain slices at a similar concentration (Narimatsu and Aoki 2000). The clearance rate of pressure evoked adenosine did not significantly change from 0.51 ± 0.04 to 0.46 ± 0.07 $\mathrm{sec}^{-1}$ ($n$ = 6, paired $t$-test, $P$ = 0.5209).

## Adenosine metabolism clearance

The clearance of exogenous transient adenosine in brain slices was examined following inhibition of adenosine

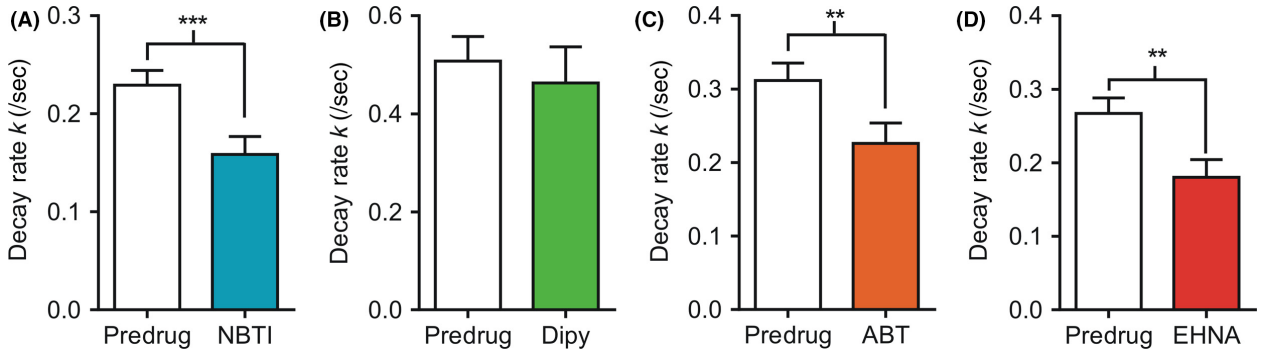


**Figure 8.** Clearance rate of exogenously applied adenosine during inhibition. Adenosine was picospritzed onto the brain slices and clearance rates were fit with a single exponential curve before and after drug application. (A) The ENT1 inhibitor, NBTI (10 μmol/L, blue), significantly decreased the clearance rate (paired $t$-test, $n$ = 4, $P$ = 0.0002). (B) The ENT1/2 inhibitor, dipyridamole (10 μmol/L, green), did not have an effect on clearance rate ($n$ = 6, paired $t$-test, $P$ = 0.5209). (C) The adenosine kinase inhibitor, ABT-702 (100 nmol/L, orange), significantly decreased the clearance rate ($n$ = 5, paired $t$-test, $P$ = 0.0053). (D) The adenosine deaminase inhibitor, EHNA (20 μmol/L, red), significantly decreased the clearance rate ($n$ = 4, paired $t$-test, $P$ = 0.0036). ENT1, equilibrative nucleoside transport 1; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; ABT-702, 5-(3-bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*]byrimidin-4-amine dihydrochloride; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine. $^{**}P < 0.01$, $^{***}P < 0.001$

kinase with ABT-702 (100 nmol/L) (Fig. 8C). The clearance rate of exogenously applied adenosine decreased after inhibition with ABT-702 from $0.32 \pm 0.02$ to $0.23 \pm 0.03$ $sec^{-1}$ ($n = 5$, paired $t$-test, $P = 0.0053$). Next, we examined the effect of adenosine deaminase inhibition with EHNA (20 $\mu$mol/L) (Barankiewicz et al. 1997) on adenosine clearance (Fig. 8D). The decay rate significantly decreased from $0.24 \pm 0.02$ to $0.18 \pm 0.03$ $sec^{-1}$ ($n = 4$, paired $t$-test, $P = 0.0036$). The results demonstrate that adenosine deaminase is responsible for clearing adenosine from the extracellular space in slices and agrees with the in vivo results.

## Discussion

Rapid adenosine release that only lasts for a few seconds has been recently discovered. Electrically stimulated and mechanically stimulated adenosine release lasts less than 20 sec (Cechova and Venton 2008), while spontaneous adenosine release is even faster, lasting less than 3 sec (Nguyen et al. 2014). The short duration of adenosine implies that adenosine is rapidly released and cleared from the extracellular space. Here, our studies in caudate brain slices found that adenosine is cleared from the extracellular space at a maximum velocity of 1.4 $\mu$mol/L/sec. The rate accounts for all forms of clearance and is comparable to maximal rates of clearance for volume neurotransmitters in vivo. For example, dopamine is cleared from the extracellular space at 0.2–5 $\mu$mol/L/sec, depending on the brain region (Wightman et al. 1988; Sabeti et al. 2002). The duration of adenosine transients, around 3–4 sec, is also similar to the duration of dopamine transients that underlie social behaviors (Robinson et al. 2002).

The main sources of adenosine clearance in vivo are transporters, metabolism, and diffusion. Diffusion is generally not fast enough on a 3 sec time scale to cause complete clearance, as tissue is tortuous and diffusion is restricted (Taylor et al. 2013). For volume transmitters such as dopamine, the main source of fast clearance is active transporters and metabolism only plays a minor role (Budygin et al. 1999). However, our studies show that clearance of rapid adenosine is more balanced, with contributions by both ENTs and metabolism.

### Transporters

There are two types of nucleoside transporters: ENTs and CNTs. ENTs carry molecules across a membrane either direction following a concentration gradient (Thorn and Jarvis 1996). Most studies of adenosine transport have focused on the ENTs as these transporters are ubiquitous in the body and have a wide variety of functions (Griffith and Jarvis 1996). ENT1 is sensitive to NBTI, distributed throughout the rat brain, and specifically present in the striatum (Anderson et al. 1999). ENT2 is found in the rat brain and is insensitive to NBTI (Lu et al. 2004).

NBTI is a potent inhibitor of ENT1 with a $K_i$ in the range of 0.1–1.0 nmol/L (Thorn and Jarvis 1996) but is less effective at binding ENT2, with a $K_i$ of 1 $\mu$mol/L (Belt and Noel 1985). Dipyridamole inhibits both ENT1 and ENT2, although dipyridamole is less sensitive for ENT2 than ENT1 (Hammond 1991). In humans, dipyridamole has a $K_i$ of 5.0 nmol/L for ENT1 and 360 nmol/L for ENT2 (Ward et al. 2000). NBTI had a significant effect on the duration and the exponential rate of clearance in vivo and the clearance rate in brain slices. NBTI did not change the concentration or frequency of transients demonstrating that release of rapid adenosine is not through ENT1. In contrast, dipyridamole did not change the duration of adenosine in vivo. The exponential decay rate did significantly decrease in vivo, but remained unchanged in brain slices. The different clearance rates for NBTI and dipyridamole might be due to the fact that adenosine clearance rates were only measured in a specified concentration range (150–250 nmol/L), which is outside the range for ENT2. Also, while the reported $K_i$ for dipyridamole is in the range of doses administered, it is possible dipyridamole is not as efficient at binding transporters and preventing uptake, even for ENT1. Thus, the NBTI data clearly show ENT1 is responsible for some clearance of transient adenosine and more potent drugs are needed to evaluate ENT2.

### Adenosine metabolism

The two primary enzymes for adenosine metabolism are adenosine deaminase and adenosine kinase (Latini and Pedata 2001). In the brain, adenosine deaminase is anchored to cell surfaces (Franco et al. 1997), demonstrating extracellular activity of the enzyme. Inhibition of adenosine deaminase, with either EHNA or deoxycoformycin, increases basal concentrations of adenosine in the brain (Ballarin et al. 1991; Pazzagli et al. 1995). Here, EHNA, a specific inhibitor of adenosine deaminase, increased the duration of adenosine in vivo and decreased the decay rate, demonstrating that adenosine deaminase is responsible for some of the clearance of transient adenosine.

The other enzyme responsible for degrading adenosine, adenosine kinase, is expressed throughout the brain, with the striatum having a higher density than the cortex (Gouder et al. 2004). Adenosine kinase modulates adenosine levels as overexpression of adenosine kinase in epileptic mice increased seizure activity by decreasing the amount of adenosine available to reduce neuronal firing

(Gouder et al. 2004). Here, the adenosine kinase inhibitor ABT-702 increased the duration and decreased the clearance rate, similar to that of adenosine deaminase inhibition. Thus, adenosine kinase and adenosine deaminase play similar roles in the regulation of transient adenosine clearance.

Interestingly, inhibition of adenosine kinase or adenosine deaminase had opposite effects on the concentration of spontaneous adenosine release. ABT-702 increased the concentration of transient adenosine release while EHNA decreased the concentration of adenosine. The difference in the concentration of transients could be due to the location of the enzymes, as adenosine kinase is located intracellularly, either in the nucleus or the cytoplasm (Cui et al. 2009) while adenosine deaminase is located both intra and extracellularly (Franco et al. 1997). Thus, inhibiting adenosine kinase would increase intracellular levels of adenosine, which would allow more adenosine to be available for release. The inhibition of adenosine deaminase increases basal concentration of adenosine in the extracellular space over an extended period of time (Sciotti and van Wylen 1993). The increased levels of adenosine could activate additional $A_1$ adenosine receptors, which have auto-receptor characteristics and regulate transient, stimulated adenosine release (Cechova et al. 2010). Therefore, the decreased spontaneous adenosine release following adenosine deaminase inhibition could be from a feedback loop through basal levels of adenosine acting at adenosine receptors.

### Relative effects of transporters and metabolism on clearance

In this study, inhibition of ENT1, adenosine deaminase, or adenosine kinase individually increased the duration on a similar time scale, 0.3–0.4 sec. This shows that there is a balance of rapid adenosine clearance by both ENTs and metabolism. When all three pathways were inhibited, the duration increased by 0.7 sec, which is not fully additive of their individual effects. The fact that the duration is still short, even after inhibition of ENT1 and two metabolic enzymes implies that either the drugs do not fully block the mechanisms or that there are additional mechanisms of clearance. The doses we chose were from other studies which has significant effects and we tried a larger dose of NBTI without observing any further effect, but there is still a possibility that the clearance was only partially blocked. Other tightly regulated neurotransmitters are typically cleared by active transporters (Fuller and Wong 1977; Horn 1990), and CNTs are an analogous active transporter for adenosine. Unfortunately, good pharmacological tools are not available to probe these CNTs, but active transport should be examined in the future when possible. Diffusion is likely to play some role in clearance, but diffusion is too slow to completely clear adenosine in 3 sec. Other metabolic pathways exist, particularly intracellular pathways to *S*-adenosylhomocysteine (SAH) but previous studies show that adenosine release does not come from SAH hydrolysis under physiological or ischemic conditions in the brain (Pak et al. 1994), thus this pathway is unlikely to contribute to the rapid clearance.

For adenosine there are multiple competing mechanisms that are all important for clearance and blocking one single mechanism of clearance had no dramatic effect lengthening the duration of signaling. The presence of multiple mechanisms that all have similar effects on clearance implies that adenosine is important to tightly regulate and that clearance pathways are therefore redundant. Adenosine will act locally at its receptors and the rate of clearance also determines how quickly it can diffuse and act a neuromodulator in other areas. Evidence from our lab shows that adenosine transients can modulate dopamine transients (Ross and Venton 2014). The rapid clearance of transient adenosine in the striatum implies that adenosine acts locally and only diffuses on the scale of ~10 $\mu$m (Venton et al. 2003). Therefore, multiple clearance mechanisms for adenosine facilitate tight regulation and control of extracellular adenosine available for neuromodulation.

## Conclusions

Understanding the regulation of adenosine on a fast time scale is critical, as the clearance mechanism determines how long adenosine is available for signaling. These studies show three important pathways for adenosine clearance that can regulate the amount of adenosine present during transient signaling: ENT1, adenosine deaminase, and adenosine kinase. All three mechanisms had similar effects on duration although inhibiting all three mechanisms simultaneously did not have an additive effect. Thus, the extracellular concentration of adenosine is tightly regulated by multiple mechanisms which prevents transient adenosine from accumulating.

## Disclosures

None declared.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** The effect of ENT1 inhibition on spontaneous adenosine release with an increased dose of NBTI.