The Characterization of Rapid Spontaneous Adenosine with Fast-scan Cyclic Voltammetry

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Abstract

Adenosine is a complex signaling molecule with a wide array of functions that include regulation of sleep, breathing, and neuromodulation. Recently adenosine was recognized as a fast acting modulator that can signal on a rapid time scale. Monitoring adenosine on a sub-second time scale with fast-scan cyclic voltammetry (FSCV) allowed for many advancements in understanding adenosine function on a rapid time scale. Our laboratory pioneered the field of adenosine measurements *in vivo* and in brain slices with FSCV. Here, I discovered and characterized spontaneous, transient adenosine release *in vivo*.

Chapter 1 introduces adenosine, FSCV, and other methods of measuring adenosine. The chapter focuses on understanding adenosine modulation on a rapid time scale through micro-dialysis, biosensors, electrophysiology, and FSCV. The strengths and weaknesses of these techniques were examined in some seminal studies on fast acting adenosine. Studies on electrically evoked, mechanically stimulated, and spontaneously released adenosine were reviewed here. I examine recent characterizations of rapid adenosine modulation as well as outline future studies of adenosine.

Chapter 2 examines a new mode of signaling: spontaneous, transient adenosine release. For the first time, naturally occurring adenosine release was observed in the caudate-putamen and prefrontal cortex of anesthetized rats. I characterized the concentration, duration, and frequency of spontaneous adenosine release in both brain regions. The prefrontal cortex has a higher frequency of release than the striatum, while

the concentration and duration remain constant in both regions. The frequency of transient adenosine release is modulated through the A_1 receptors, demonstrating an auto-inhibition feedback loop of adenosine.

Chapter 3 describes the clearance of spontaneous, transient adenosine in the caudate-putamen of the *in vivo* rat model as well as in brain slices. Naturally occurring adenosine release is cleared from the extracellular space in 3-4 seconds, suggesting a rapid form of signaling, as most studies have focused on adenosine modulation on a minute time scale. In this chapter, I also examine uptake through transporters and metabolism. The equilibrative nucleoside transporter, ENT1, and the enzymes responsible for metabolizing adenosine, adenosine deaminase and adenosine kinase, all play a partial role in clearing adenosine. The rapid regulation in the extracellular space suggests there is a mode of adenosine signaling that is fast and takes place on the second time scale.

The **fourth chapter** examines how transient adenosine is regulated. I found that the concentration of spontaneous, transient adenosine is independent of all of the examined receptors: glutamate (ionotropic and metabotropic), GABA, and adenosine. However, the frequency of adenosine release is modulated by the glutamate receptor NMDA and the adenosine receptors A_1 and A_{2a}. The results demonstrate there is a feedback loop between released adenosine and adenosine receptors, as well as through the NMDA receptor.

The **5th chapter** describes a new phenomenon that I accidentally discovered in the laboratory. While inserting our carbon-fiber microelectrodes, significant release of adenosine was observed during implantation. In this chapter, mechanically stimulated adenosine release is characterized in the prefrontal cortex. The release was examined in anesthetized rats and brain slices and the average release lasted around 42 seconds. Using cell staining and multiple movements of our electrodes the results demonstrate that

the release of adenosine is not from cell death or damage. This new mode of signaling further suggests that adenosine is neuroprotective and might be involved in protection during mechanical perturbation in the brain.

Overall, the dissertation describes rapid detection of adenosine in the brain using FSCV. While the purinergic signaling field has been researching adenosine for over 80 years, the ability to directly detect adenosine on a sub-second time scale was only recently implemented using FSCV. Two new modes of rapid adenosine signaling were discovered: spontaneous, transient adenosine and mechanically stimulated adenosine. The discovery of two new modes of rapid signaling demonstrate that adenosine modulates neurotransmission in the brain as well as having the ability to be a therapeutic agent during brain injury.

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"Trust in the Lord with all your heart and lean not on your own understanding; in all your ways acknowledge him, and he will make your paths straight"

Proverbs 3:5-6

Chapter 1 Introduction: Fast-scan Cyclic Voltammetry for the Characterization of Rapid Adenosine Release

"If at first you don't succeed, try, try, try again"

William Edward Hickson

Chapter 1: Introduction

1.1 Overview of adenosine

Adenosine is a signaling molecule and downstream product of ATP that acts as a neuromodulator. Adenosine regulates physiological processes, such as neurotransmission and blood flow, on a time scale from minutes to hours. Recent developments in electrochemical techniques, including fast-scan cyclic voltammetry (FSCV), have allowed direct detection of adenosine with sub-second temporal resolution. FSCV studies have revealed a novel mode of rapid signaling that lasts only a few seconds. This rapid release of adenosine can be evoked by electrical or mechanical stimulations or it can be observed spontaneously without stimulation. Adenosine signaling on this time scale is activity dependent; however, the mode of release is not fully understood. Rapid adenosine release modulates oxygen levels and evoked dopamine release, indicating adenosine may have a rapid modulatory role. In this overview, we outline how FSCV can be used to detect adenosine release, compare FSCV with other techniques used to measure adenosine, and present an overview of adenosine signaling that has been characterized using FSCV. These studies point to a rapid mode of adenosine modulation, whose mechanism and function will continue to be characterized in the future. This chapter was modified from a publication in **Computational and Structural Biotechnology Journal**, 2015, **13**, 47-54.

1.2 Adenosine function

1.2.1 Adenosine and sleep

Adenosine regulates sleep (Huang *et al.* 2011) and one of the most common adenosine antagonists is caffeine, which causes a shift in rapid eye movement and increases wakefulness (Karacan *et al.* 1976). In the brain, adenosine increases sleep patterns and decreased wakefulness after two hours of perfusing adenosine into the basal forebrain (Portas *et al.* 1997). There are four different types of adenosine receptors: A_1, A_{2a}, A_{2b}, and A_3 (Cunha 2001). For sleep regulation, adenosine modulates the A_1 and A_{2a} receptors (Marks *et al.* 2003) through different mechanisms. A_1 receptors modify sleep patterns by inhibiting cholingeric neurons (Rainnie *et al.* 1994), while A_{2a} receptors inhibit histaminergic system by increasing GABA release (Hong *et al.* 2005). Adenosine promotes rapid eye movement and sleep cycles on a gradual time scale.

1.2.2 Adenosine and Breathing

Adenosine also regulates breathing and adenosine analogs are used to treat asthma (Vass and Horvath 2008). In the mouse brain, endogenous adenosine decreases breathing over a five minute period as measured with HPLC (Gettys *et al.* 2013). Adenosine depressed breathing through activation of the A_1 receptor, although in another study, A_{2a} receptor antagonists were shown to recover breathing rates following hypoxia (Koos *et al.* 2002). Although adenosine modulates breathing on a faster time scale, than sleep, the regulation of breathing is still on the minute time scale.

1.2.3 Adenosine and heart rate

In 1929, Drury found that adenosine lowers both heart rate and blood pressure (Drury and Szent-Gyorgyi 1929). In the brain, adenosine increases cerebral blood flow,

however in the kidneys, adenosine has a more dynamic effect (Hansen and Schnermann 2003). Initially, adenosine acts as a vasoconstrictor decreasing blood flow but after 2-3 minutes adenosine then exerts a vasodilator effect and increasing blood flow (Tagawa and Vander 1970). Similarly another study found the same effect of adenosine going from blood vessel constriction to dilation in 40 seconds (Hall *et al.* 1985). Adenosine is a dynamic regulatory molecule that can have different effects over time.

1.2.4 Adenosine and neuroprotection

One of adenosine's primary roles is that of a neuroprotective agent. During ischemic events, when oxygen and glucose levels to the brain are reduced, adenosine is released to decrease brain activity (Rudolphi *et al.* 1992). Adenosine neuroprotection is mediated through the inhibitory A_1 receptor, which decreases cAMP levels, hyperpolarizing neurons, and decreasing neuronal activity. The brain has the ability to pre-condition against stroke like events, where prior non-lethal ischemic events are beneficial in protecting against future larger ischemic events. The pre-conditioning in hippocampal slices was found to be modulated by A_1 receptors after 30 minutes of exposure to A_1 agonists (Perez-Pinzon *et al.* 1996). Prolonged increases in basal adenosine levels exert neuroprotective effects in the brain.

1.2.5 Adenosine and neurotransmission

Glutamate is most prevalent excitatory neurotransmitter in the brain and can modulate adenosine levels. Together, adenosine and glutamate regulate nociceptive pain receptors, traumatic brain injury, and neuroinflammation. Following the formalin pain test (Hunskaar *et al.* 1985;Hunskaar and Hole 1987), adenosine levels increased over 10 minute intervals (Liu *et al.* 2000). Adenosine was also released as a result of directly

injecting glutamate into the rat hind paw (Liu *et al.* 2002) and the downstream release of adenosine exerted an inhibitory influence on A_1 adenosine receptors (Aumeerally *et al.* 2004). Twenty-four hours following traumatic brain injury and neuroinflammation, high levels of glutamate cause A_{2a} adenosine receptors to be anti-inflammatory, while low levels produce inflammation from A_{2a} adenosine receptors. Glutamate and adenosine exert downstream effects on each other that are modulated over a long periods of time.

The two primary types of ionotropic glutamate receptors, NMDA and AMPA, are characterized by the specific antagonists which functionally block their receptors. NMDA modulates endogenous adenosine levels, while adenosine has a downstream effect on AMPA activation. NMDA evokes adenosine release in the rat striatum (Delaney and Geiger 1995) and inhibiting adenosine deaminase and adenosine transport caused increases in adenosine levels (Delaney and Geiger 1998). AMPA receptor induced currents were enhanced after thirty minutes of A_{2a} receptor activation (Dias *et al.* 2012). NMDA and AMPA receptor effects on adenosine and vice versa show that adenosine and glutamate modulation are paired and that regulation takes place over long periods of time.

1.3 Adenosine formation and clearance

1.3.1 Adenosine Formation

Adenosine is formed both extra- and intra-cellularly and through multiple pathways. The primary source of adenosine is from metabolism of ATP and AMP. Both outside and inside the cell, adenosine is dephosphorylated from AMP and the level of dephosphorylation varies in different brain regions (Nagata *et al.* 1984). Additionally, ATP activates P_2 receptors which causes a release of adenosine. Adenosine is produced from the SAM (S-adenosyl methionine) pathway, which is a common biological pathway for methylation (Beluzic *et al.* 2008). SAH hydrolase breaks SAH down to adenosine

intracellularly, however the pathway only produces a small fraction of adenosine during hypoxic events and none during regular physiological conditions (Lloyd *et al.* 1988). In hippocampal slices, adenosine kinase increased basal levels of adenosine during ischemic events, while SAH hydrolase did not affect adenosine levels (Pak *et al.* 1994). Thus the primary source of adenosine is not from the SAM pathway.

1.3.2 Adenosine Clearance

Adenosine is cleared from the extracellular space through two primary mechanisms: metabolism and transport. The most common enzymes for breaking down adenosine are adenosine kinase and adenosine deaminase. Adenosine kinase, phosphorylates adenosine to form AMP and adenosine deaminase breaks adenosine down to inosine. Both enzymes reduce basal adenosine levels over long periods of time. Adenosine kinase inhibitors are more effective than adenosine deaminase inhibitors at increasing basal levels of adenosine in the brain at least 20 minutes after administration (Sciotti and van Wylen 1993). For NMDA induced adenosine release, adenosine kinase has a higher specificity (K_m = 0.2-2.0 µM) than adenosine deaminase (K_m = 20-100 µM) in the rat striatum (Delaney and Geiger 1998). Adenosine deaminase is found in discrete brain regions(Nagy *et al.* 1984) and inhibition increases basal levels of adenosine up to 24 hours later (Pazzagli *et al.* 1993).

The second mode of clearance is through equilibrative nucleoside transporters which moves adenosine across a cell following the gradient (Thorn and Jarvis 1996). Blocking adenosine transporters in the pig brain increased extracellular adenosine levels ten minutes after ischemia (Park and Gidday 1990). This demonstrates that ischemic adenosine is cleared but not released through nucleoside transporters. Similarly, adenosine released in hippocampal slices through electrical stimulation and

hypoxia/hypoglycemia was increased after inhibition of nucleoside transporters (Fredholm *et al.* 1994c). Inhibition of adenosine transporters can also decrease the amount of adenosine in the extracellular space when other mechanisms of release are blocked. Adenosine released by depolarizing neurons with K^+ was blocked with the removal of Ca^{2+} and blocking nucleoside transporters after ten minutes (Sweeney 1996).

1.4 Techniques to measure adenosine

Techniques that directly measure adenosine release can be used to understand the amount of adenosine in the extracellular space. Early studies used radiometric labeling of adenosine coupled with HPLC analysis to examine the breakdown of ATP to adenosine (Cunha *et al.* 1996). Microdialysis coupled to HPLC was also used to measure adenosine increases (Park and Gidday 1990). These methods measured adenosine on the minutes time scale but recently, electrochemical techniques have been developed that allow direct measurements on the second and even sub-second time scale. Fast, discrete release of adenosine has been characterized, which shows that adenosine exhibits characteristics of a neurotransmitter, as it is tightly regulated and cleared on a fast time scale. However, while adenosine has recently been reported in vesicles (Corti *et al.* 2013), there is currently no direct evidence that adenosine is released through exocytosis (Dale and Frenguelli 2009). Enzyme biosensors specific for adenosine (Dale 1998) have a response time of two seconds and were used to show that increases in adenosine occur within two minutes following ischemic events (Dale *et al.* 2000). Fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes directly measures adenosine on a sub-second time scale (Brajter-Toth *et al.* 2000;Swamy and Venton 2007), with a sampling rate of 10 times a second. FSCV has been used to study stimulated release *in vivo* (Cechova and Venton 2008;Cechova *et al.* 2010) and in brain slices (Xie *et al.* 2006;Pajski

and Venton 2010a;Pajski and Venton 2013). These studies revealed that adenosine can be released and cleared in only a few seconds. However, the function of rapid adenosine release is still being elucidated. FSCV is the fastest method currently available for measuring adenosine changes and combined with pharmacology and electrophysiology, it has the capability of revealing how adenosine signals on a rapid time scale. Here, we examine the fundamental principles of adenosine detection by FSCV, compare it to other measurement techniques, and highlight the biological applications and future studies that rapid measurements with FSCV may enable.

1.4.1 Adenosine detection with Fast-scan Cyclic Voltammetry (FSCV)

FSCV is an electrochemical technique that was developed to measure changes in electroactive neurotransmitters, especially dopamine (Ewing and Wightman 1984). To measure adenosine with FSCV, a triangular potential is applied scanning from -0.40 V to 1.45 or 1.50 V and back versus a Ag/AgCl reference electrode at 400 V/s (Figure 1.1A). The scan takes less than 10 ms and scans are repeated at 100 ms, which is the temporal resolution of the technique. The working electrode is a carbon-fiber microelectrode with a 7 µm diameter, which allows measurements in discrete brain regions. The fast scan rates cause a large background charging current (Figure 1.1B) due to double layer charging at the electrode. The background current is stable over time and can be subtracted out from the signal (Hermans *et al.* 2008;Jacobs *et al.* 2014). The result of subtracting the background current of the dashed line (buffer only) and the red line (buffer and adenosine) in Figure 1.1B is a characteristic cyclic voltammogram for 1 µM adenosine (Figure 1.1C).

Adenosine is an electroactive molecule that can undergo a series of three, two-electron oxidations (Figure 1.2) (Dryhurst 1977). The initial oxidation of adenosine from product I to product II in Scheme 1 is observed at 1.4 V with FSCV. A secondary oxidation, from product II to product III, is detected at 1.0 V. The first two oxidation steps are irreversible and reduction peaks are not observed. The third oxidation in the scheme is seldom observed with FSCV at our carbon-fiber microelectrodes. Thus, the characteristic

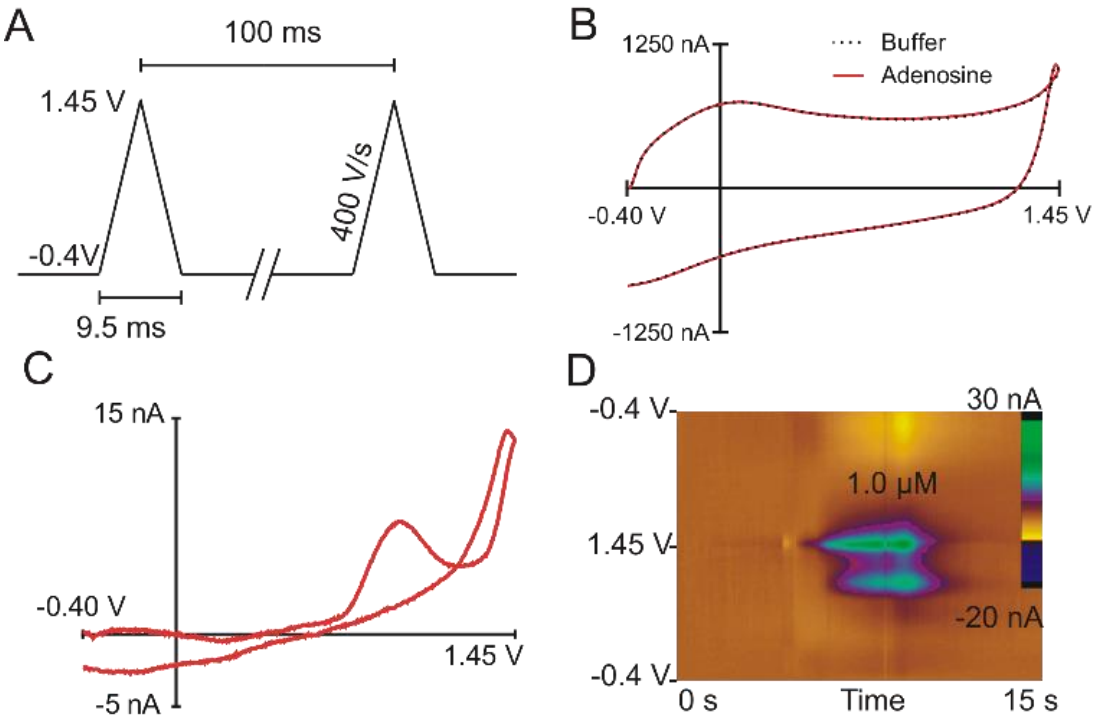

Figure 1.1 Detection of adenosine with FSCV

A) Applied potential waveform. The electrode is held at -0.40 V, ramped up to a switching potential of 1.45 V and back at 400 V/s. The scan is repeated every 100 ms. B) The cyclic voltammogram (CV) is large due to the background charging current in buffer (black dashed line) and the addition of 1.0 µM adenosine (red line). C) Subtracting out the background yields a background-subtracted CV of adenosine oxidation. The primary oxidation is observed at 1.4 V and the secondary oxidation at 1.0 V. The primary peak current is proportional to the concentration of adenosine detected at the electrode. D) False color plot of multiple background subtracted CVs. The *x*-axis is time, the *y*-axis is applied potential, and the color is current. This plot depicts an *in vitro* calibration experiment where buffer is flowed by the electrode for 5 seconds, then 1.0 µM adenosine is flowed by for 5 seconds and finally buffer is flowed again. The large green oval in the center of the plot is the primary oxidation peak and the smaller green below is the secondary oxidation peak. Data adapted from Nguyen et al. (Nguyen *et al.* 2014).

cyclic voltammogram (CV) for adenosine has two oxidation peaks, with the largest peak being **x**near the switching potential at 1.4 V (Figure 1.1C) (Swamy and Venton 2007).

Since many CVs are collected over time, it is useful to visualize multiple voltammograms simultaneously in false color plots (Figure 1.1D). A vertical slice through

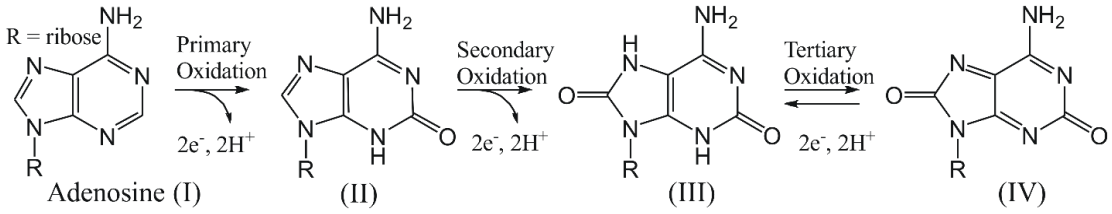

Figure 1.2 Oxidation mechanism of adenosine

Adenosine undergoes a three sequential, 2-electron oxidations. The first two oxidations are observed using FSCV and are irreversible. R is a ribose unit.

the color plot at 7.5 seconds gives a CV of adenosine (Figure 1.1C). The primary peak appears about a half second before the secondary peak on the color plot (Nguyen *et al.* 2014). A horizontal slice through the color plot at 1.4 V shows how the oxidation current of adenosine changes against potential. With an appropriate calibration value, that current can be converted to concentration.

Traditionally, FSCV has been used to detect catecholamines such as dopamine (Robinson *et al.* 2003) and norepinephrine (Palij and Stamford 1994) but it can also detect serotonin (Borue *et al.* 2009), histamine (Pihel *et al.* 1995a), and hydrogen peroxide (Sanford *et al.* 2010a). Dopamine, serotonin, and norepinephrine have oxidation peaks around 0.6 V and reductions peaks between 0.2 V to -0.2 V (Ewing *et al.* 1982;Jackson *et al.* 1995) and the peaks do not interfere with adenosine detection. Hydrogen peroxide has a similar oxidation peak as adenosine at 1.2 V, but has no secondary peak, which distinguishes it from adenosine (Sanford *et al.* 2010a). Histamine also has a similar oxidation potential as adenosine, however, the secondary peak potential is lower than that of adenosine (Pihel *et al.* 1995a).

The same electroactive adenine moiety is present in ATP and adenosine, so the electrochemical signatures of adenosine and ATP are similar. However, interferences can be minimized and adenosine distinguished from ATP with FSCV. Regular carbon-fiber microelectrodes were more sensitive for adenosine than ATP when the applied waveform has a negative holding potential of -0.4 V (Swamy and Venton 2007). Electrodes coated with Nafion and carbon nanotubes were six-fold more sensitive for adenosine than ATP (Ross and Venton 2012). Recently, our laboratory developed a sawhorse waveform that helped distinguish adenosine and ATP (Ross and Venton 2014b). The altered waveform was more sensitive for adenosine and gave distinct signals for adenosine over ATP; however there was still some overlap between the two molecules. To positively identify adenosine release *in vivo*, pharmacological manipulations were used (Cechova and Venton 2008) and the identity of the analyte was verified by independent techniques, such as biosensors (Ross *et al.* 2014). These approaches were similar to those used to distinguish dopamine from other catecholamines (Phillips and Wightman 2003) and verified that adenosine was monitored by FSCV.

1.4.2 Comparison of adenosine detection with FSCV and other methods

1.4.2.1 Comparison of microdialysis and FSCV

Microdialysis is one of the most general techniques for monitoring neurochemical changes. A diagram of microdialysis is shown in Figure 1.3. A microdialysis probe is inserted into the brain and artificial cerebral spinal fluid is pumped through the probe. The probe is semi-permeable allowing molecules to diffuse and be collected in the dialysate.

The aliquot is usually analyzed with HPLC and one advantage is that many species can be measured from the same sample (Coolen *et al.* 2008). For example, adenosine was

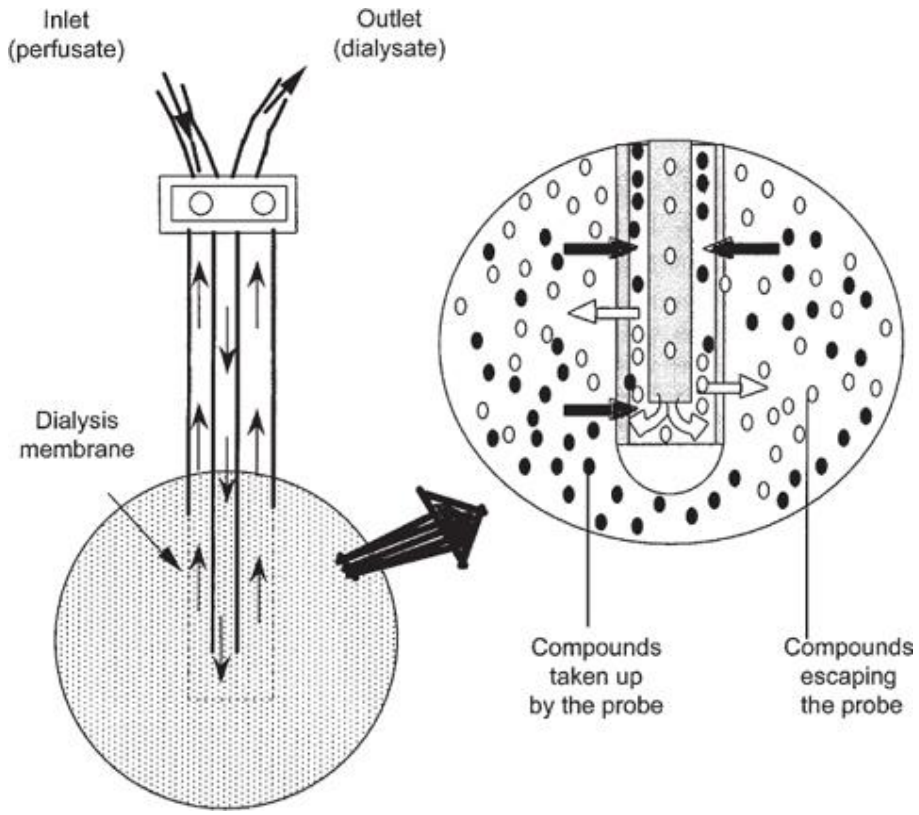

Figure 1.3 Microdialysis diagram

Perfusate is flown through the double layer microdialysis probe. The inner membrane is impermeable while the tip of the outer membrane is semi-permeable. Molecules follow the concentration gradient across the outer membrane and the dialysate which now contains molecules unique to the sampling area are collected in a sample for further analysis. Figure reprinted from (Barbe *et al.* 2001).

quantified and separated from hypoxanthine and uric acid (Fredholm and Sollevi 1981). The limit of detection for adenosine was as low as 5 nM using microdialysis(Haink and Deussen 2003). Microdialysis monitoring has revealed that adenosine is released during ischemia and built up in 15 minutes (Hagberg *et al.* 1987). A disadvantage of microdialysis, is the amount of sample and time required to draw a sufficient sample size. Adenosine A_1 and A_{2a} antagonists were administered with microdialysis to the lateral preoptic area for 90 minutes to test the effects on sleep (Methippara *et al.* 2005).

Microdialysis samples were collected for 5 to 10 minutes, so the time scale of the measurement is slower than FSCV. Microdialysis is good for studying slower changes in basal levels of adenosine while FSCV is better at detecting rapid fluctuations in extracellular adenosine.

1.4.2.2 Comparison of electrophysiology and FSCV

Electrophysiology studies monitor the firing of neurons and has been used to examine the effects of adenosine release on neurotransmission. Neurons have a resting membrane potential of -60 mV with more sodium ions outside the cell than potassium ions inside the cell. If a neuron reaches a sufficient potential of -55 mV, the neuron will depolarize to +40 mV with a flow of sodium ions into the cell causing an action potential resulting in neurotransmitter release. In cells, excitatory post-synaptic potentials (EPSPs) are caused by chemical signaling that excite the post-synaptic neurons. Action potentials and EPSPs occur rapidly, in less than a second. Adenosine has been applied to neuronal cells to examine the effect on EPSPs. For example, adenosine in the presence of neurons depressed EPSPs and thus decreased post-synaptic neurotransmission (Dunwiddie and Hoffer 1980). The depressions of neurotransmission in electrophysiology studies found that endogenous adenosine release in hippocampal slices was activity dependent and acted via A_1 receptors (Mitchell *et al.* 1993a).

Electrophysiology studies look at downstream effects of adenosine on cell firing, while electrochemical methods directly measure adenosine release. Thus, the two methods are complementary. Electrophysiology measurements are on the millisecond time scale and were used to demonstrate that adenosine acted at A_1 receptors on a 1-2 second time scale (Mitchell *et al.* 1993a). FSCV has confirmed adenosine release can last only a few seconds (Cechova and Venton 2008) and showed rapid signaling of

adenosine in the brain. FSCV and electrophysiology studies have been combined at the same microelectrode to measure dopamine (Belle *et al.* 2013) and similar studies could be performed in the future to monitor the amount of adenosine release and its effect on neuronal firing.

1.4.2.3 Comparison of FSCV and adenosine biosensors

Another method for detecting adenosine is using amperometric biosensors, which were developed by the Dale group (Dale 1998). Adenosine biosensors directly measure adenosine at platinum electrodes coated with enzymes that metabolize adenosine to hydrogen peroxide, which is detected amperometrically at +0.5 V (Llaudet *et al.* 2003). Adenosine is broken down to inosine, then to hypoxanthine, then to xanthine, urate, and hydrogen peroxide via adenosine deaminase, purine nucleoside phosphorylase, and xanthine oxidase, respectively (Figure 1.4A) (Dale and Frenguelli 2012). Although the biosensors are held at +0.5 V, the multiple polymer layers may act as a barrier to prevent electroactive species from oxidizing (Llaudet *et al.* 2003), while still allowing for detection of H_2O_2 without slowing response time (Dale and Frenguelli 2012). An identical null sensor which contains no adenosine deaminase is placed next to the biosensors to distinguish adenosine from any interferents, particularly downstream metabolites. Subtracting out the null sensor signal from the adenosine biosensor signal gives a specific response for adenosine. The limit of detection for adenosine biosensors is 12 nM and the rise time is about 2 seconds, which allows measurements of adenosine release on the seconds time scale (Llaudet *et al.* 2003). Figure 1.4 shows evoked adenosine release measured by biosensors. Adenosine release was decreased by EHNA (*erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride, which inhibits adenosine deaminase (Figure 1.4B);

tetrodotoxin, a blocker of action potentials (Figure 1.4C); and removal of Ca^{2+} (Figure 1.4D), which blocks activity dependent release (Klyuch *et al.* 2012).

Biosensors have been used to examine the mechanism and function of adenosine release, particularly in brain slices. In hippocampal slices, 5-10 minutes of hypoxia gradually increased adenosine over the ischemic event (Dale *et al.* 2000). Similarly, adenosine released during hypoxia was activity dependent and not a downstream breakdown product of ATP (Gourine *et al.* 2002;Klyuch *et al.* 2012). Enzyme biosensors were also used to measure electrically stimulated (Begg *et al.* 2002) and glutamate-induced adenosine release (Sims *et al.* 2013). These studies revealed that adenosine was released on a time scale of seconds to minutes following oxygen deprivation, electrical stimulation, or glutamate application.

FSCV and amperometric biosensors are both electrochemical techniques that measure adenosine directly. The limit of detection for adenosine at carbon-fiber

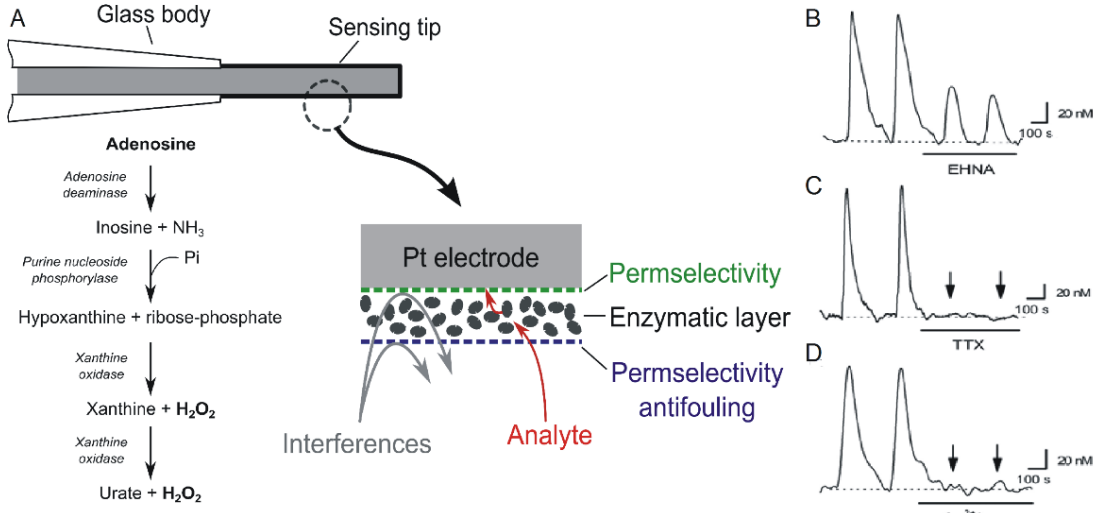

Figure 1.4 Biosensor detection of adenosine

Left: A) Schematic of adenosine biosensor. The platinum electrode is coated with a permselective layer to prevent fouling and an enzymatic layer that breaks down adenosine to hydrogen peroxide. Right: Evoked adenosine release in brain slices. B) EHNA, an adenosine deaminase inhibitor, lowers evoked adenosine release. C) Tetrodotoxin (TTX), which inhibits action potentials, completely eliminates stimulated adenosine release. D) Ca^{2+} free buffer abolishes evoked adenosine release. Data adapted with permission from Dale (Dale and Frenguelli 2012;Klyuch *et al.* 2012) and Springer Science and Business Media.

microelectrodes is comparable to biosensors (Llaudet *et al.* 2003), in the 15 nM range. FSCV at carbon-fiber microelectrodes has better temporal resolution than biosensors, with rise times for FSCV being only a few hundred milliseconds. Typically, adenosine biosensors are platinum electrodes with diameters of 25 or 50 µm while carbon-fiber microelectrodes are only 7 µm. Smaller electrodes cause less tissue damage (Nesbitt *et al.* 2013). Adenosine release measured with FSCV lasts only a couple of seconds while release measured with biosensors is usually longer, on the order of a minute. Biosensors require a null sensor to screen out metabolites, while metabolites such as inosine are electrochemically inactive at carbon-fiber electrodes using FSCV (Swamy and Venton 2007). However, biosensors are able to measure basal changes in adenosine over a minute to hour time scale, while FSCV cannot observe basal shifts because the signal is background subtracted. With both techniques, evoked adenosine release is activity dependent on the rapid time scale (Pajski and Venton 2010a;Klyuch *et al.* 2012). Biosensors for ATP have also been developed and can be used to distinguish adenosine and ATP signals (Llaudet *et al.* 2005).

1.4.3 Advantages and disadvantages of FSCV

There are several advantages of FSCV for detection of adenosine. The high sensitivity for adenosine allows measurements in the physiologically relevant range of receptor affinities (Fredholm *et al.* 1994a). Adenosine is directly detected, as opposed to measuring downstream effects, which is beneficial for determining mechanisms of release that may account for adenosine signaling (Wall and Dale 2008). Another benefit of FSCV with carbon-fiber microelectrodes is the small size of the electrodes (7µm), which are less invasive than microdialysis probes of 250-500 µm (Clapp-Lilly *et al.* 1999). Carbon-fiber

microelectrodes are also cheap and easy to make, as opposed to biosensors which are usually purchased from a commercial source and are more expensive.

The biggest advantage of FSCV is the time resolution of the measurements, 100 ms, which is the fastest method for measuring adenosine. Adenine nucleotides undergo conversion to adenosine within 200 milliseconds, suggesting a need to detect adenosine on a fast time scale (Dunwiddie *et al.* 1997a). Biosensors take around 2 seconds to respond to a change in adenosine and thus are not fast enough to characterize many of the spontaneous adenosine transient signals that last only 3 seconds on average (Nguyen *et al.* 2014). The consequence of fast scanning is a large background current, so local changes in concentration are typically only measured over a 90 second window due to background subtraction (Heien *et al.* 2005). Using analog background subtraction with principal component analysis, changes in dopamine concentrations have been quantified over a 30 minute period (Hermans *et al.* 2008); however this method has not been tested with adenosine. A disadvantage of FSCV is that it cannot measure basal concentrations of adenosine because it requires background subtraction. However, FSCV is the best method for measuring rapid changes and when combined with pharmacology can be a powerful tool to understand transient adenosine signaling.

1.5 Biological studies of adenosine with FSCV

1.5.1 Electrically-stimulated adenosine release

Using FSCV, stimulated adenosine was characterized in anesthetized animals (Cechova and Venton 2008) and brain slices (Xie *et al.* 2006). Electrically-stimulated release was first characterized *in vivo* in the caudate-putamen after dopamine neurons in the medial forebrain bundle were stimulated (Cechova and Venton 2008). Both the evoked dopamine and adenosine release occurred immediately. Figure 1.5 shows a false

color plot in brain slices of stimulated dopamine and adenosine release being cleared in 2 and 5 seconds, respectively (Pajski and Venton 2010a). On average, the peak evoked adenosine was 0.94 µM and lasted 15 seconds, *in vivo* (Cechova and Venton 2008). Pharmacological experiments demonstrated that propentofylline, an inhibitor of adenosine transport, decreased stimulated release while ABT 702, an adenosine kinase inhibitor,

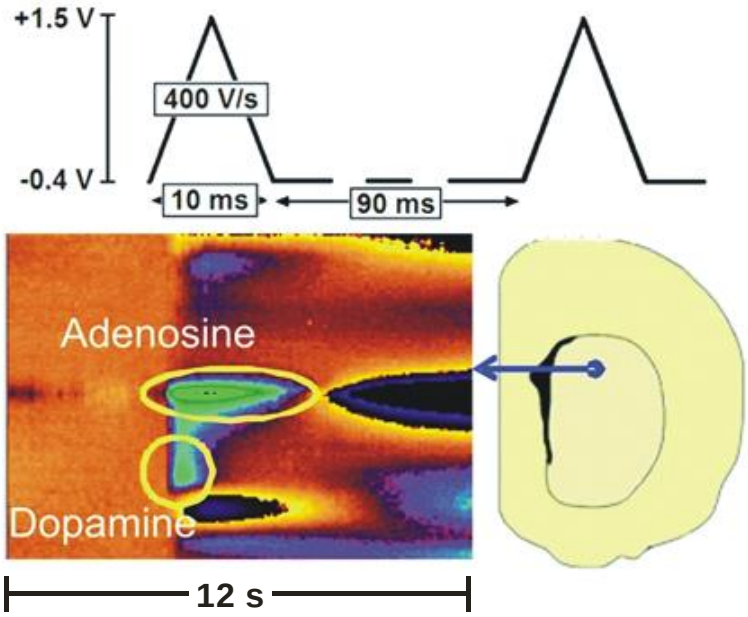

Figure 1.5 Electrically stimulated adenosine in slices using FSCV.

The applied waveform is shown above the color plot. Electrically stimulated adenosine and dopamine are shown on the false color plot in yellow circles. The dopamine and adenosine are cleared within 2 and 5 seconds of stimulation, respectively. A diagram of the location of electrode implantation in the caudate-putamen is shown to the right. Figure reprinted from (Pajski and Venton 2010a) Copyright 2010 American Chemical Society.

increased evoked release. Stimulated adenosine release increased oxygen flow within 5 seconds at the electrode, demonstrating that adenosine can control cerebral blood flow on a rapid time scale. A_1 receptors self-regulated adenosine release (Cechova *et al.* 2010); the A_1 agonist, CPA, decreased evoked adenosine while the A_1 antagonist, DPCPX, increased adenosine release rapidly.

FSCV was also used to characterize stimulated adenosine release in brain slices. Two different pulse trains, low and high frequency, were compared in rat striatal slices

(Pajski and Venton 2010a). The high frequency stimulations (5 pulses, 60 Hz) evoked more adenosine than the low frequency stimulations (5 pulses, 10 Hz). Blocking nucleoside transporters did not change evoked adenosine concentration so stimulated adenosine was not released through equilibrative transporters. Stimulated adenosine release varied in different brain regions (Pajski and Venton 2013), with higher levels evoked in the caudate-putamen and nucleus accumbens compared to the cortex and hippocampus. While basal levels also differ in distinctive regions (Kovacs *et al.* 2010a), there was little trend between evoked levels of adenosine (Pajski and Venton 2013) and previously measured basal levels (Kobayashi *et al.* 1998;Akula *et al.* 2008).

The mechanism of stimulated adenosine release was examined on a rapid time scale. Evoked adenosine release was activity dependent because Ca^{2+} chelation or application of tetrodotoxin (TTX) almost completely reduced the signal (Pajski and Venton 2013). Thus, rapid adenosine release was likely due to exocytosis of adenosine, exocytosis of its precursor ATP, or the downstream result of activity-dependent neurotransmitter release (Wall and Dale 2008). NMDA and AMPA antagonists decreased the release of adenosine in the caudate-putamen and nucleus accumbens, showing the dependence of adenosine release on ionotropic glutamate receptors (Pajski and Venton 2013). The effect of blocking ATP metabolism varied by brain region. AOPCP and ARL 67156, inhibitors of ATP metabolism, had no effect in the caudate-putamen, but decreased adenosine in the nucleus accumbens, hippocampus, and cortex. Thus, there are least two mechanisms of stimulated adenosine release: breakdown of extracellular nucleotides and downstream effects of ionotropic glutamate receptors, but these mechanisms differ by brain region.

1.5.2 Adenosine release during deep brain stimulation probe implantation

FSCV is being pioneered for human clinical trials in order to monitor neurotransmitters during deep brain stimulation (DBS) (Shon *et al.* 2010). DBS is used to treat tremors but a phenomenon known as the microthalamotomy effect has been observed where simply implanting a probe in the thalamus reduces tremors without applying an electrical stimulation (Tasker 1998). Release of adenosine during probe implantation was hypothesized to be important for this effect. Using FSCV, the implantation of a carbon-fiber electrode into the thalamus was shown to release adenosine within seconds (Figure 1.6) (Chang *et al.* 2009). Adenosine release was also observed concurrently with tremor arrest (Chang *et al.* 2012). Thus, FSCV in clinical trials is a valuable technique for understanding the release of adenosine on a rapid time scale during microelectrode implantation and DBS treatment.

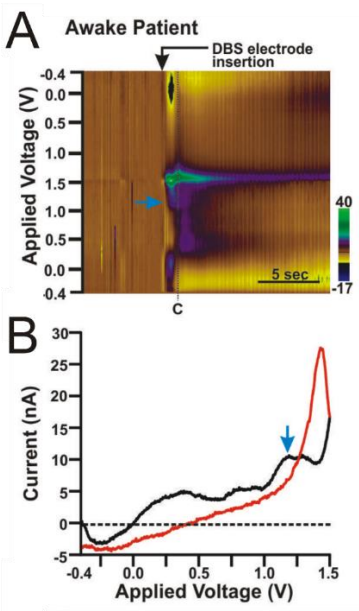

Figure 1.6 Adenosine release by DBS probe implantation

A) False color plot of DBS electrode implantation in human patient. The black arrow denotes the insertion of the electrode and the blue electrode shows the immediate release of adenosine. B) A cyclic voltammogram of adenosine shows the characteristic primary and secondary oxidation of adenosine at 1.4 V and 1.0 V, respectively. Figure adapted from Lee's Group (Chang *et al.* 2012) Copyright Elsevier (2012).

1.5.3 Mechanically stimulated adenosine release

Our laboratory characterized the effect of small mechanical stimulations by moving an electrode, or a pipette near an electrode, small distances. Adenosine transiently increased after mechanical stimulation and was cleared within 20 seconds (Ross *et al.* 2014). Mechanical release of adenosine was observed *in vivo* and in brain slices and was not due to cell death or tissue damage (Ross *et al.* 2014). Mechanically-stimulated adenosine decreased following application of EDTA (to complex Ca^{2+}) and TTX, showing the release was activity dependent. Blocking the nucleoside transporter with NBTI did not diminish the signal, proving the release was not through transporters. Mechanically-stimulated release was partially blocked by POM-1, which inhibits the breakdown of ATP, showing some of the release was from ATP metabolism. The rapid release of adenosine following electrode implantation or brain damage could be neuroprotective.

1.5.4 Spontaneous, transient adenosine release

Spontaneous, transient adenosine release was discovered in spinal cord slices of mice using FSCV (Street *et al.* 2011a). The duration of adenosine release was only 1.5 seconds and the average concentration was 0.53 µM. This rapid, transient release could not have been detected with microdialysis or biosensors, as only FSCV has sub-second temporal resolution. The frequency of transient release was low, with events occurring once every three minutes and was significantly reduced when Ca^{2+} was removed from the buffer, suggesting activity dependent release of adenosine. Knockout mice lacking NT5E and CD73, enzymes that convert AMP to adenosine, also had a lower frequency of transients. A third enzyme, tissue non-specific alkaline phosphatase (TNAP), was later found to inhibit the rapid conversion of applied AMP to adenosine in the double knockout mice with FSCV (Street *et al.* 2013). The results suggested that adenosine transients

were formed from rapidly hydrolyzed AMP; however the inhibition of TNAP has not been tested for spontaneous, transient adenosine release.

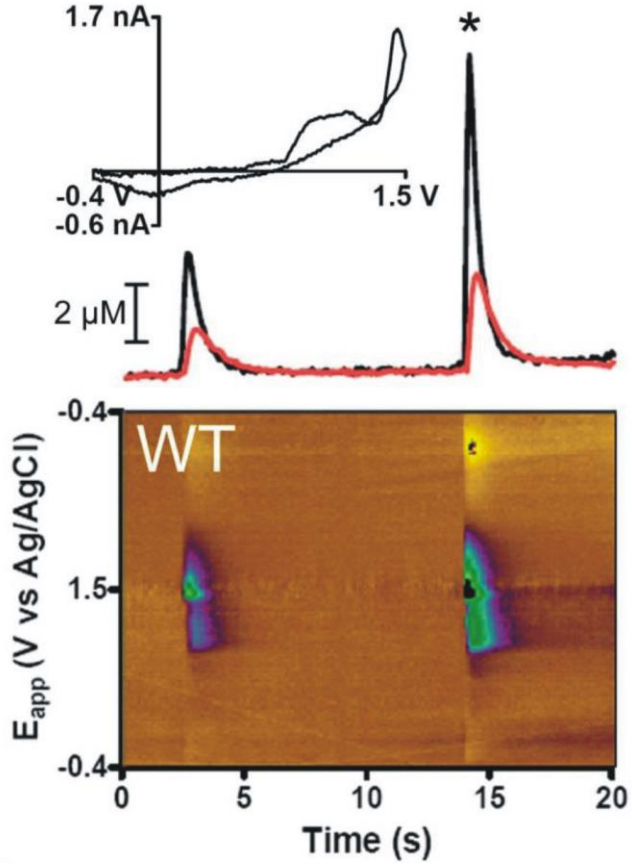

Figure 1.7 Spontaneous transient adenosine release with FSCV

A false color plot shows two spontaneous release events of adenosine in a 20 second window. The concentration vs time trace above the color plot shows the two discrete events of adenosine at the primary peak (black) and the secondary peak (red). Figure adapted from Street et al. (Street *et al.* 2011a)

Spontaneous, transient adenosine release was also characterized *in vivo* in spinal cord slices in the mouse model (Street *et al.* 2011a). No stimulation was applied and adenosine release was observed that lasted less than 2 seconds. Figure 1.7 shows a false color plot of two transients that occur within 20 seconds of each other. FSCV has the temporal resolution to detect three distinct adenosine events in this short time frame. The average adenosine concentration was 0.17 μM in the caudate-putamen and 0.19 μM in the prefrontal cortex, however transients as large as 2.5 μM were observed. The release was random and events occurred on average about once every three minutes. A_1 receptors modulated the frequency of transient adenosine release, but the mechanism of regulation has not been elucidated. Interestingly, comparing the mice and rat studies, the characteristics of spontaneous transient adenosine release were similar even though they were measured

in two different rodent species and in two different regions (Nguyen *et al.* 2014;Street *et al.* 2011a). This suggests that spontaneous, transient adenosine release is a conserved signaling pathway across species and regions.

1.6 Concluding remarks

The biggest finding from FSCV research on adenosine has been the discovery of a mode of signaling that lasts only a few seconds. The sub-second temporal resolution of FSCV was needed to understand the temporal dynamics of rapid release. Electrically-stimulated, mechanically-stimulated, and spontaneous, transient adenosine release all occur on this rapid time scale. Transient adenosine release can be regulated by A_1 receptors and partially by ionotropic glutamate receptors (Pajski and Venton 2013). FSCV has facilitated a better understanding of a rapid signaling role for adenosine but questions still persist.

1.6.1 Understanding the function of rapid adenosine release

When a new signaling mode of a molecule is identified, it is important to characterize the function of that signaling. In **Chapter 2**, the characteristics of spontaneous, transient adenosine were defined in two brain regions. The concentration, duration, and frequency of this new mode of adenosine signaling was described in the striatum and the prefrontal cortex. This was the first in-depth characterization of a spontaneous adenosine in the anesthetized rat. Transient adenosine likely acts locally, as the distance a molecule such as adenosine could diffuse in a couple of seconds is only 10-20 μm (Garris *et al.* 1997). In **Chapter 3**, I examined the clearance of fast acting adenosine by inhibiting various pathways of clearance. The results demonstrate that adenosine is cleared through both uptake and metabolism.

Adenosine is known to modulate neurotransmission in the brain and so an obvious hypothesis is that transient adenosine regulates neurotransmission on a rapid time scale. Recently, our laboratory identified that transient adenosine release rapidly modulated phasic dopamine release. When adenosine was transiently applied less than 10 seconds before dopamine was electrically evoked, the stimulated dopamine concentration was reduced by 50% (Figure 1.8) (Ross and Venton 2014a). This inhibitory effect was mediated by A_1 receptors and only occurred while adenosine was present in the extracellular space. Thus, rapid adenosine is able to modulate neurotransmission in discrete locations. Adenosine also modulated oxygen concentrations in the brain (Cechova and Venton 2008), suggesting that transient adenosine release can locally modulate oxygen and blood flow. In **Chapter 4**, modulation of different glutamate, GABA, and adenosine receptors effect on spontaneous, transient adenosine was examined. I found that A_1 and A_{2a} adenosine receptors and ionotropic glutamate NMDA receptors modulate the frequency of release but not the concentration.

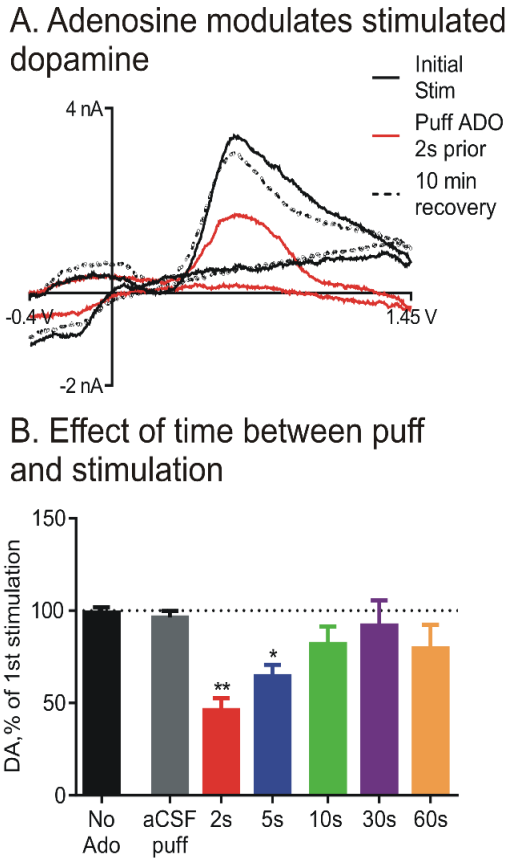

Figure 1.8 Transient modulation of stimulated dopamine release with FSCV

(A) Cyclic voltammograms for stimulated dopamine release initially (black line), 2 seconds after adenosine application (red line), and 10 minutes after adenosine application (dashed line). (B) Stimulated dopamine after application of adenosine over different times. Evoked dopamine significantly decreased if adenosine was applied 2 or 5 seconds before stimulation. Figure adapted from Ross et al. (Ross and Venton 2014a) with permission from Wiley Company.

In the **final data chapter**, I discovered another mode of adenosine signaling, mechanically stimulated adenosine. We characterized mechanosensitive adenosine release in anesthetized rats and in brain slices. The release was constant over time and multiple stimulations demonstrating release of adenosine was not from cell death.

In conclusion, this dissertation examines two new modes of adenosine signaling, spontaneous and mechanical stimulation with FSCV in rat brains and how they function on a rapid time scale.

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Chapter 2 Characterization of Spontaneous, Transient Adenosine Release in the Caudate-Putamen and Prefrontal Cortex

"The true sign of intelligence is not knowledge but imagination"
Albert Einstein

2 *Abstract*

Adenosine is a neuroprotective agent that inhibits neuronal activity and modulates neurotransmission. Previous research has shown adenosine gradually accumulates during pathologies such as stroke and regulates neurotransmission on the minute-to-hour time scale. Our lab developed a method using carbon-fiber microelectrodes to directly measure adenosine changes on a sub-second time scale with fast-scan cyclic voltammetry (FSCV). Recently, adenosine release lasting a couple of seconds has been found in murine spinal cord slices. In this study, we characterized spontaneous, transient adenosine release *in vivo,* in the caudate-putamen and prefrontal cortex of anesthetized rats. The average concentration of adenosine release was 0.17 ± 0.01 μM in the caudate and 0.19 ± 0.01 μM in the prefrontal cortex, although the range was large, from 0.04 to 3.2 μM. The average duration of spontaneous adenosine release was 2.9 ± 0.1 seconds and 2.8 ± 0.1 seconds in the caudate and prefrontal cortex, respectively. The concentration and number of transients detected do not change over a four hour period, suggesting spontaneous events are not caused by electrode implantation. The frequency of adenosine transients was higher in the prefrontal cortex than the caudate-putamen and was modulated by A_1 receptors. The A_1 antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine, 6 mg/kg i.p.) increased the frequency of spontaneous adenosine release, while the A_1 agonist CPA (N^6-cyclopentyladenosine, 1 mg/kg i.p.) decreased the frequency. These findings are a paradigm shift for understanding the time course of adenosine signaling, demonstrating that there is a rapid mode of adenosine signaling that could cause transient, local neuromodulation. The chapter was published in the Public Library of Open Science ONE (**PLOS ONE**, *2014*, DOI: 10.1371)

2.1 Introduction

Adenosine is an important neuroprotective modulator in the brain that regulates neurotransmission and blood flow. Adenosine increases in the brain during pathological events, such as ischemia (Van Wylen *et al.* 1986) and seizures (During and Spencer 1992), where it can act as a retaliatory metabolite. The increases in adenosine during these pathologies typically last for minutes to hours (Zetterstrom *et al.* 1982). For example, the concentration of adenosine doubled one minute following ischemia and was ten-fold larger twenty minutes afterwards (Berne *et al.* 1974). During hypoxia, adenosine activates inhibitory A_1 adenosine receptors a couple of minutes after onset, which decreases cAMP concentrations, hyperpolarizes neurons, and prevents excitatory firing (Cunha 2001;Cunha 2005). While these studies demonstrate adenosine signaling on a longer time scale, there is growing evidence that adenosine also signals on a much shorter time scale.

Using electrophysiological techniques, Dunwiddie's group explored a rapid modulatory role for adenosine in the brain. During very short electrical stimulations (1-5 pulses at 100 Hz), adenosine regulated glutamate receptor-mediated excitatory postsynaptic potentials (EPSPs) in the hippocampus in an activity dependent manner (Mitchell *et al.* 1993a). The duration of change in EPSPs was only 2 seconds, implying fast adenosine changes were responsible. However, the experiment did not directly measure adenosine concentration on a rapid time scale.

Recently, electrochemical sensors were developed for rapid measurements of changes in local adenosine concentration. Enzyme biosensors can quantitate adenosine with a time resolution of 2 seconds (Llaudet *et al.* 2003). Using these biosensors, electrically-stimulated adenosine release in the cerebellum was measured that lasted 100 seconds (Klyuch *et al.* 2012). Our lab developed fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes (Swamy and Venton 2007) to directly detect electrically-

stimulated adenosine release in the caudate-putamen that lasts only 10-40 seconds (Cechova and Venton 2008). The concentration of stimulated release was regulated by A_1 receptors (Cechova *et al.* 2010). Using FSCV, the Zylka group recently discovered spontaneous adenosine transients. The adenosine lasted less than 2 seconds in the extracellular space and was a result of extracellular ATP metabolism (Street *et al.* 2011b;Street *et al.* 2013). These studies establish that adenosine can be released on a more rapid time scale; however, the characteristics and regulation of rapid adenosine signaling *in vivo* are not well understood.

In this study, we measured spontaneous, transient adenosine release *in vivo* for the first time. Spontaneous adenosine release, not evoked by electrical stimulation, was measured in the caudate-putamen and the prefrontal cortex of the anesthetized rat. The duration of direct adenosine release *in vivo* was only about 3 seconds, a time scale that matches the previous electrophysiological study (Mitchell *et al.* 1993b). The concentration of adenosine release was on average 0.18 μM but a large range of concentrations was detected. The frequency of spontaneous adenosine events was one transient every 2-3 minutes and was modulated by A_1 receptors; however, the events follow a random, not a regular firing pattern. The spontaneous adenosine transients observed *in vivo* were similar in concentration, duration, and frequency to what others have found in murine spinal cord slices (Street *et al.* 2011b), suggesting transient adenosine release is common in multiple regions in the nervous system and across different species. This study demonstrates that adenosine is rapidly released and cleared in the brain and may have a rapid neuromodulatory role in addition to the previously characterized function as a long-term modulator.

2.1.1 **Experimental Methods**

2.1.2 Ethics

All animal experiments were carried out under strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Virginia (Protocol Number 3517). All surgery was performed under urethane anesthesia and all efforts were made to minimize suffering.

2.1.3 Chemicals

All reagents were purchased from Fisher Scientific (Fair Lawn, NJ, USA) unless otherwise stated. Phosphate buffered saline (PBS) was used to calibrate electrodes containing (in mM): 131.25 NaCl, 3.0 KCl, 10.0 NaH_2PO_4, 1.2 $MgCl_2$, 2.0 Na_2SO_4, and 1.2 $CaCl_2$ with the pH adjusted to 7.4. Sodium phosphate was purchased from RICCA Chemical Company (Arlington, TX, USA). All aqueous solutions were prepared with deionized water (Milli-Q Biocel; Millipore, Billerica, MA, USA). Adenosine was prepared as a 10 mM stock solution in 0.1 M $HClO_4$ and stored in the refrigerator.

DPCPX (6 mg/kg, i.p., 8-cyclopentyl-1,3-dipropylxanthine, Sigma Aldrich) was dissolved in dimethylsulfoxide (DMSO). CPA (N^6-cyclopentyladenosine) was purchased from Tocris Bioscience (Ellisville, MO, USA), dissolved in saline, and administered at 1 mg/kg. These doses were chosen as large doses that were previously used in the literature (Karcz-Kubicha *et al.* 2003;Prediger *et al.* 2004;Cechova *et al.* 2010).

2.1.4 Electrodes and FSCV

Carbon-fiber microelectrodes were prepared as previously described (Huffman and Venton 2008). Briefly, cylindrical microelectrodes were prepared with 7 µm diameter T-650 carbon-fibers (Cytec Engineering Materials, West Patterson, NJ, USA). Fibers were aspirated into glass capillaries (1.2 mm x 0.68 mm; A-M Systems, Inc., Seqium, WA, USA),

pulled by a vertical pipette puller (model PE-21; Narishige, Tokyo, Japan) into two microelectrodes, and the extended fiber cut with a scalpel to about 50 µm. The fiber/glass interface was sealed with epoxy [Epon resin 828] (Miller-Stephenson Chemical Co. Inc.; Danbury, CT, USA) and 14% wt m-phenylenediamine heated to 80°C. Excess epoxy was rinsed with acetone and electrodes were dried overnight at room temperature, then cured at 100°C for two hours, and then at 150°C overnight. The Nafion-CNT coated electrodes were prepared as previously described (Ross and Venton 2012). High pressure carbon monoxide conversion single-walled CNTs (Carbon Nanotechnologies, Houston, TX, USA) were functionalized as described here (Wei *et al.* 2006). The electrodes were dipped in 0.05 mg/mL CNTs suspended 5% wt Nafion in methanol (Ion Power, New Castle, DE, USA) for 5 minutes, air dried for 10 seconds, placed in the oven for 10 minutes at 70 ºC, and stored at room temperature overnight. Electrical connections were created by backfilling the electrodes with 1 M KCl. Bare electrodes were soaked in 2-propanol for at least 10 minutes before use.

Fast-scan cyclic voltammetry (FSCV) was used to monitor adenosine with sub-second temporal resolution (Swamy and Venton 2007). Waveform generation and data collection was computer controlled by Tar Heel CV (gift of Mark Wightman, UNC at Chapel Hill) (Heien *et al.* 2003). A Dagan ChemClamp potentiostat (Dagan Corporation; Minneapolis, MN, USA) was used to collect data. Electrical stimulations were applied with a BSI-950 Bi-Phasic Stimulus Isolator (Dagan).

Electrodes were continuously held at -0.40 V and scanned to 1.45 V and back every 100 milliseconds against a Ag/AgCl reference electrode, at a rate of 400 V/s. All data was background subtracted to remove any non-Faradic currents by averaging 10 CVs from no more than 20 seconds before the analysis point. Electrodes were calibrated using flow injection analysis (Strand and Venton 2008) with 1.0 µM adenosine in PBS made fresh daily from the 10 mM stock solution. Pre- and post-calibrations were

performed and no significant difference in adenosine oxidation current was found after electrode implantation (data not shown).

2.1.5 Animals and Surgery

Male Sprague-Dawley Rats (250-350 g; Charles-River, Wilmington, MA, USA) were housed in a vivarium with 12-h light/dark cycles and provided food and water *ad libitum*. The rats were anesthetized with 50% wt urethane (Sigma Aldrich) solution in saline (Baxter; Deerfield, IL, USA) (1.5 g/kg, i.p). The surgical site was shaved and 0.25 mL of bupivicaine (Sensorcaine® MPF, APP Pharmaceuticals, LLC; Schaumburg, IL, USA) was administered subcutaneously for local analgesia. After exposing the skull, holes were drilled for placement of electrodes using a stereotaxic drill (Paxinos and Watson 2007). Adenosine transients were measured in either the caudate or the prefrontal cortex in each rat. The coordinates for the caudate-putamen are (in mm from bregma): anterior-posterior (AP): +1.2, mediolateral (ML): +2.0, and dorsoventral (DV): -4.5. Coordinates for the prefrontal cortex are: AP: +2.7, ML: +0.8, and DV: -3.0. The Ag/AgCl reference electrode was inserted on the contralateral side of the brain. The rat's body temperature was maintained at 37°C using a heating pad with a thermistor probe (FHC, Bowdoin, ME, USA).

2.1.6 Data Collection and Analysis

Electrodes were implanted into the tissue and allowed to equilibrate with the waveform being applied for at least 30 minutes before any data collection. After equilibration, data was continuously collected and if no transients were found after 30 minutes or if the electrode baseline was unstable, a new electrode was inserted until transients were observed. Up to three electrodes per animal were inserted to search for transient adenosine release. Any animals with fewer than four transients in the hour long pre-drug time period were excluded. The overall success rate for finding transients was

80% in both the caudate-putamen and in the prefrontal cortex. After electrode placement was deemed optimal, one hour of pre-drug data was collected and then drug was injected. Nafion-CNT coated electrodes were placed *in vivo* and data collected for one hour.

2.1.7 Principal Component Analysis

All adenosine transients were qualitatively and quantitatively analyzed using High Definition Cyclic Voltammetry (HDCV) Analysis software (from Mark Wightman, UNC at Chapel Hill). A training set was compiled for each rat of the five largest, most definitive adenosine transients with a clear secondary peak. The largest transients were chosen because they were easily identifiable, whereas smaller transients created poor principal component correlations. Principal components were extracted from the training set and all data was analyzed using principal component regression (Jolliffe 1986). This produced an adenosine concentration vs. time trace that was used to identify and determine the amount and duration of each transient. Every training set has residuals which account for currents from unknown signals, such as noise (Keithley *et al.* 2009). The sum of squares of the residuals for each variable, or the Q score, was calculated and any signal above Q failed and was not counted as an adenosine transient. With a limit of quantitation at ten times the noise for the secondary peak, the smallest transients that could be quantified were 40 nM. Any transient signal without a secondary peak or any sustained signal greater than 10 seconds, where the background could not be accurately subtracted out, was not counted.

2.1.8 Statistics

Statistics performed using GraphPad PRISM 6 (GraphPad Software Inc., San Diego, CA, USA), MatLAB® (The MathWorks, Inc., Natick, MA, USA) and OriginPro 7.5 (OriginLab Corporation, Northampton, MA, USA). Data presented as mean ± SEM with *n* number of animals. A Kolmogorov-Smirnov (KS) test was used to determine underlying

distributions between inter-event times (time between consecutive transients). All data was considered significant at the 95% confidence level.

2.2 Results

2.2.1 Adenosine detection using FSCV

Spontaneous adenosine transients were monitored using FSCV, which has sub-second temporal resolution, allowing real time measurements of adenosine changes in the brain. No information about basal levels is obtained with FSCV because all data are background subtracted. The carbon-fiber microelectrode was scanned from -0.40 V to 1.45 V and back at 10 Hz. Adenosine undergoes two sequential, two electron oxidations (Dryhurst 1977) that produce two peaks in the background-subtracted cyclic voltammogram (CV) (Swamy and Venton 2007) (Figure 2.1A top). Both oxidation peaks are evident in the color plot of an electrode calibration with 1.0 µM adenosine (Figure 2.1A). The primary oxidation occurs at 1.4 V and the secondary oxidation at 1.0 V is slightly delayed in time from the main oxidation peak because the secondary product forms after the primary product is produced.

Spontaneous adenosine release was identified by its two oxidation peaks in the CVs and color plots, which help distinguish adenosine from other compounds with similar oxidation potentials including hydrogen peroxide (Sanford *et al.* 2010b), histamine (Pihel *et al.* 1995b), and hypoxanthine (Ross and Venton 2012). In addition, the slight delay in the formation of the second peak helps identify the analyte as adenosine (Xu and Venton 2010). While adenosine and ATP have similar cyclic voltammograms, carbon-fiber electrodes are more sensitive for adenosine than ATP (Swamy and Venton 2007) and ATP breaks down to adenosine in the extracellular space within 200 milliseconds (Dunwiddie *et al.* 1997b). As an additional test to confirm adenosine was detected and

not ATP, we used Nafion-CNT coated electrodes, which are three times more sensitive for adenosine than ATP compared to bare carbon-fiber microelectrodes. Spontaneous adenosine transients were found with Nafion-CNT electrodes implanted in the caudate-putamen with similar concentrations (Nafion-CNT average = 0.13 ± 0.01 µM, n = 3, unpaired t-test, p = 0.1751). The duration of the Nafion-CNT transients were longer than those detected with bare electrodes (average = 3.8 ± 0.2 seconds, n = 3, unpaired t-test, p = 0.0002). Nafion is known to slow the temporal response of electrodes so longer durations are due to a slower electrode response (Peairs *et al.* 2011). The similar magnitude of transient release suggests that the signal is from spontaneous adenosine

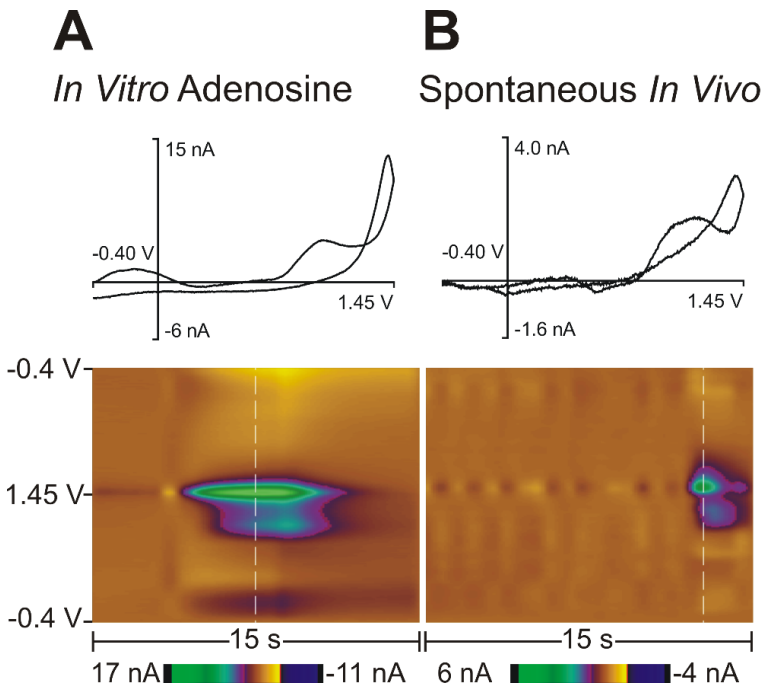

Figure 2.1 Detection of adenosine *in vitro* and *in vivo* using fast-scan cyclic voltammetry at the same carbon-fiber microelectrode

(A) *In vitro* calibration of adenosine. A 3-D color plot (bottom) depicts time on the x-axis, potential on the y-axis, and current in false color. The primary oxidation occurs at 1.4 V (large green oval in center of color plot) and the secondary oxidation occurs at 1.0 V (green/purple oval below center oval). The dashed white line on the color plot denotes where the background subtracted cyclic voltammogram (CV, top) is obtained. (B) *In vivo* spontaneous, transient adenosine event. The 3-D color plot and CV shows primary and secondary oxidation peaks that match the *in vitro* calibration.

release and not ATP release. Due to the labor intensive process of making Nafion-coated electrodes and the loss of temporal resolution, bare electrodes were used in all the other experiments.

Representative color plots and matching CVs from the same electrode are shown for an *in vitro* calibration of adenosine (Figure 2.1A) and a spontaneous adenosine transient (Figure 2.1B) in the caudate-putamen. The cyclic voltammograms of *in vitro* adenosine and spontaneous adenosine release *in vivo* show a clear primary oxidation peak at 1.4 V and a definitive secondary peak at 1.0 V. The CV for the calibration was similar to the *in vivo* transient and there was an R^2 correlation value of 0.81 between the two CVs. The color plots illustrate that both have a minor delay in the secondary peak formation. Stimulated adenosine release has been previously characterized in our laboratory (Cechova and Venton 2008), but the secondary peak was not consistently observed due to the overall small signals and other chemical changes. The CVs for stimulated adenosine can be convoluted with residual dopamine, pH, and oxygen changes (Venton *et al.* 2003). Thus, stimulated adenosine release was not a good comparison for identifying spontaneous adenosine release.

2.2.2 Automated identification of spontaneous adenosine transients

An automated system was needed to identify transient adenosine release events without bias. Principal components analysis (PCA) (Heien *et al.* 2004) has been previously used to identify spontaneous dopamine transients (Wightman *et al.* 2007); therefore we adapted PCA for use with adenosine. Unlike dopamine release (Garris *et al.* 1997), the CV of adenosine is not constant over time, due to the secondary product having a delayed increase in current. Figure 2.2A shows a color plot of a spontaneous adenosine transient with a current vs. time trace (top) through the primary (orange) and secondary

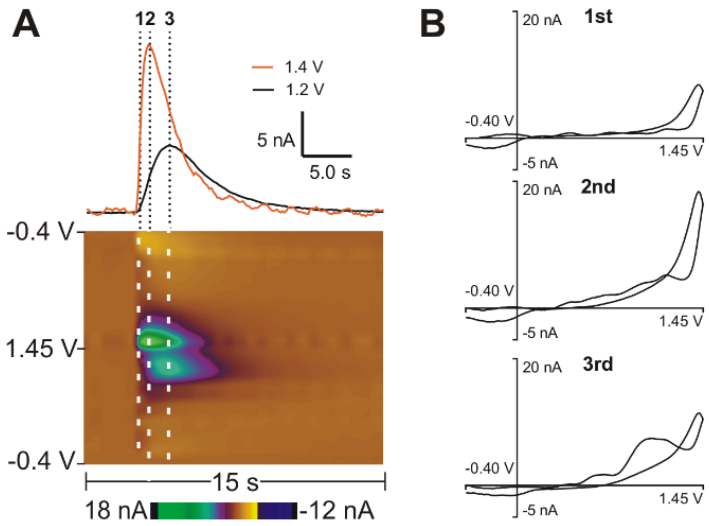

Figure 2.2 Detection of spontaneous, transient adenosine release *in vivo*

(A) *In vivo* spontaneous, transient adenosine release. The current vs. time plot has two traces, an orange line at 1.4 V for the primary oxidation and a black line at 1.2 V for the secondary oxidation. The dashed lines on the color plot and current vs. time plots indicate where CVs were taken. (B) Cyclic voltammograms of adenosine over time. The top (1st) is when adenosine first appears, the middle CV (2nd) is half a second later when the primary peak is at a maximum and the bottom (3rd) is half a second later when the secondary peak is at its maximum. The ratio of the primary and secondary oxidation peaks can change over time.

(black) oxidation peaks overlaid. The maximum signal at the second peak is 1.0 second after the first peak, demonstrating the lag time between the primary and secondary oxidation peaks. Cyclic voltammograms (Figure 2.2B) were taken at sequential times and show that the CV of adenosine release changes over time. The first CV has a primary oxidation peak and no secondary peak. Half a second later, the primary peak is more prominent while the secondary peak has grown, and in the third CV, the secondary and primary peak are almost equal in magnitude. The CVs for the training set of spontaneous adenosine release were taken at the apex of the primary oxidation peak, which gives the most accurate calibration of adenosine. Although the residuals were higher for adenosine than for dopamine because of the variable CVs, adenosine transients were still easily determined at a 95% confidence level.

A training set was created for each individual rat from five of the largest, easily identifiable spontaneous adenosine transients, each containing a characteristic secondary peak. From the training set, the eigenvalues were calculated for CVs of varying concentrations of adenosine. The highest eigenvalues correspond to the principal components with the highest variance, and thus the best correlation to the data (Malinowski 1977). A residual Q-score from the training set was used to reject any data that did not significantly match the principal components. After removing the residuals, the concentration and duration of spontaneous adenosine release was determined from a concentration vs. time plot. The limit of quantitation for our electrodes was 40 nM, so any transients with concentrations below this value were excluded. Below 40 nM, the secondary peak is difficult to identify and is obscured by noise. Thus, for a transient to be considered adenosine, it had to have a secondary peak, be below the residual Q-score (corresponding to 95% confidence level), and be above 40 nM, the quantitation limit.

2.2.3 Concentration of spontaneous adenosine transients

Transient adenosine release was examined in the caudate-putamen and prefrontal cortex. The color plots and concentration vs. time profiles of example adenosine transients show the magnitude of concentration released, as well as the duration of adenosine in the extracellular space in the caudate-putamen (Figure 2.3A) and the prefrontal cortex (Figure 2.3C). The average adenosine concentration was 0.17 ± 0.01 μM in the caudate-putamen and 0.19 ± 0.01 μM in the prefrontal cortex, which is significantly different (n = 30 and 29 rats, t-test, p = 0.0238). Table 1 gives the average, SEM, and range for the concentration in each brain region. The range of recorded adenosine transients was large, spanning almost two orders of magnitude. While the majority of adenosine release events were in the hundreds of nM range, 1% of transients

were greater than 1 µM, which demonstrates that large amounts of adenosine can be spontaneously released. To further investigate spontaneous adenosine release,

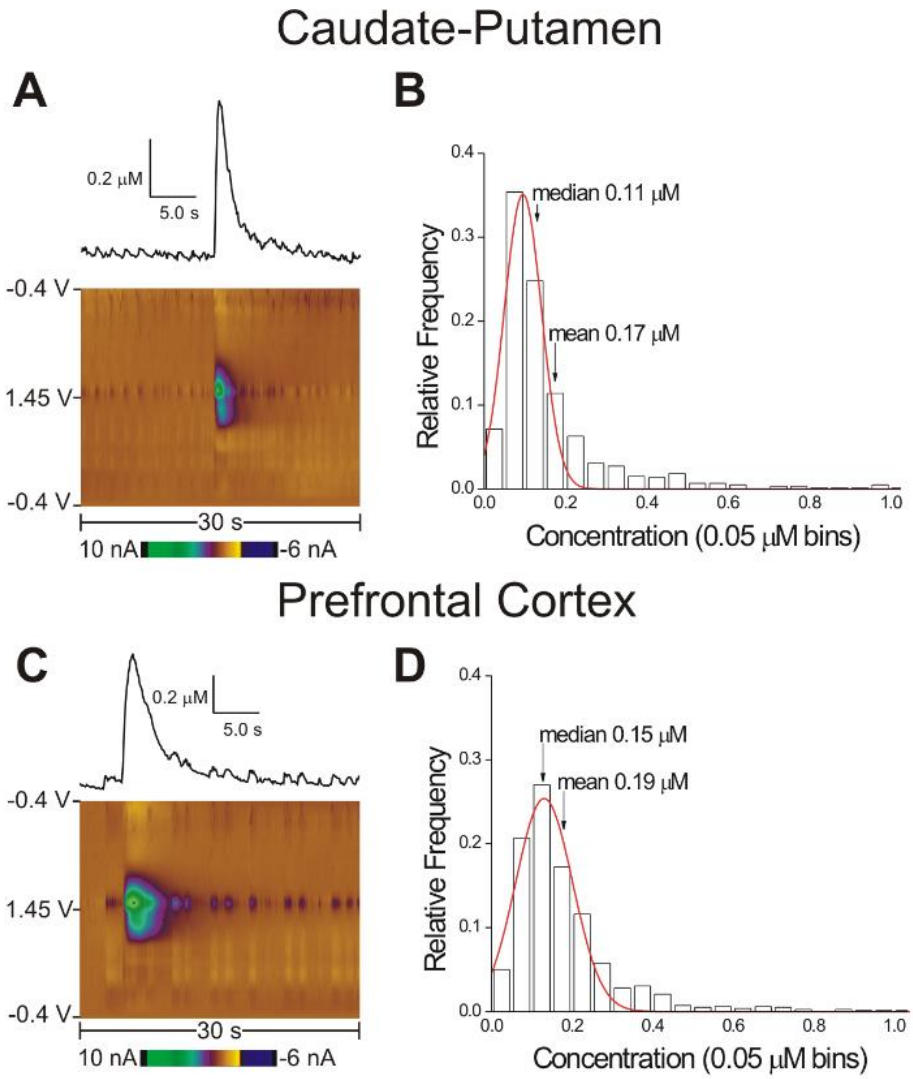

Figure 2.3 Spontaneous adenosine transient concentration

(A) A color plot of a spontaneous adenosine transient in the caudate putamen with a corresponding concentration vs. time plot above. (B) Caudate-putamen concentration histogram. The *y*-axis is relative frequency. All concentrations from the first hour of data collection in the caudate putamen were placed into 0.05 µM bins (*x*-axis) and fit with a Gaussian distribution (red line). The Gaussian fit equation is $y = 0.3512e^{-0.5\frac{(x-0.09549)^2}{0.4588}}$ (R^2 = 0.9327, *n* = 30 rats). The mean and median are marked. The majority of transients are in the 100-200 nM range. (C) An example color plot and concentration vs. time plot in the prefrontal cortex. (D) The histogram of concentrations in the prefrontal cortex fit a Gaussian distribution with the equation: $y = 0.2538e^{-0.5\frac{(x-0.1305)^2}{0.07079}}$ (R^2 = 0.9505, *n* = 29 rats).

concentrations were placed in 0.05 µM bins and histograms examined for both brain regions. The histograms have normal distributions and are overlaid with Gaussian fits with positive skews, which is expected when non-negative values are excluded. In the caudate-putamen (Figure 2.3B) over half of the values fall between 0.05 and 0.15 µM, although the average value was 0.17 µM, a reflection of the positive skew. Similarly, in the prefrontal cortex (Figure 2.3D), a little less than half of the measurements fall between 0.05 and 0.15 µM concentrations and the average is 0.19 µM. Both graphs show that although the bulk of transient adenosine events are in the 0.10 µM range, about 4% of release is 0.50 µM and higher.

2.2.4 Duration of transient adenosine release

The duration of adenosine release provides information about how long adenosine is available for signaling in the extracellular space. Color plots of typical adenosine transients with corresponding concentration vs. time plots from PCA (above) show adenosine is rapidly cleared in the caudate-putamen (Figure 2.4A) and the prefrontal cortex (Figure 2.4C). From the top plots the duration was calculated as the amount of time adenosine was over 10% (horizontal dashed line) of the peak concentration for each transient to eliminate any effects from noise in the baseline. The average duration of an adenosine transient was 2.9 ± 0.1 seconds in the caudate-putamen and 2.8 ± 0.1 seconds in the prefrontal cortex, which is not significantly different (n = 30 and 29 rats, t-test, p = 0.0826). The duration ranged from less than a second to ten seconds (Table 1). Histograms of the duration of spontaneous adenosine transients were plotted using 0.5 second bins for the caudate-putamen (Figure 2.4B) and the prefrontal cortex (Figure 2.4D). The plots are overlaid with a Gaussian distribution fit with positive skews. The

majority of transients lasted only 2-4 seconds, but outliers were present in both brain regions. Thus, adenosine is only available for signaling for a few seconds.

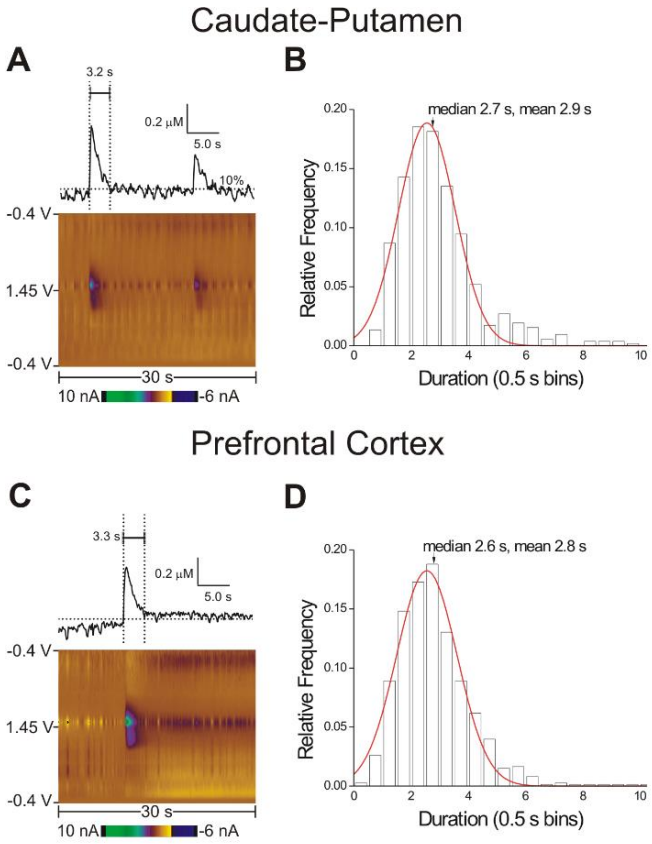

Figure 2.4 Spontaneous adenosine duration

(A) A color plot and concentration vs. time plot of adenosine transients in the caudate-putamen. The horizontal dashed line is 10% of the concentration of the first peak and the vertical lines are when the baseline value crosses this value, showing the duration. (B) Caudate-putamen duration histogram. The *y*-axis is relative frequency and the *x*-axis shows 0.5 second bins. The Gaussian distribution equation is $y = 0.1886e^{-0.5\frac{(x-2.554)^2}{0.9908}}$ (red line, $R^2 = 0.9712$, $n = 30$ rats). (C) Color plot and concentration vs. time plot of an example spontaneous adenosine transient in the prefrontal cortex. The duration is marked with vertical lines. (D) Adenosine duration histogram for the prefrontal cortex is plotted with a Gaussian distribution equation $y = 0.1823e^{-0.5\frac{(x-2.556)^2}{0.1060}}$ (red line, $R^2 = 0.9765$, $n = 29$ rats).

2.2.5 Frequency of adenosine transients

On average, spontaneous, transient adenosine release occurs once every several minutes (Table 2.1). The inter-event times, or the time between consecutive transients,

were calculated to examine if the adenosine transients were regularly spaced. Histograms of inter-event times are plotted for the caudate (Figure 2.5A) and prefrontal cortex (Figure

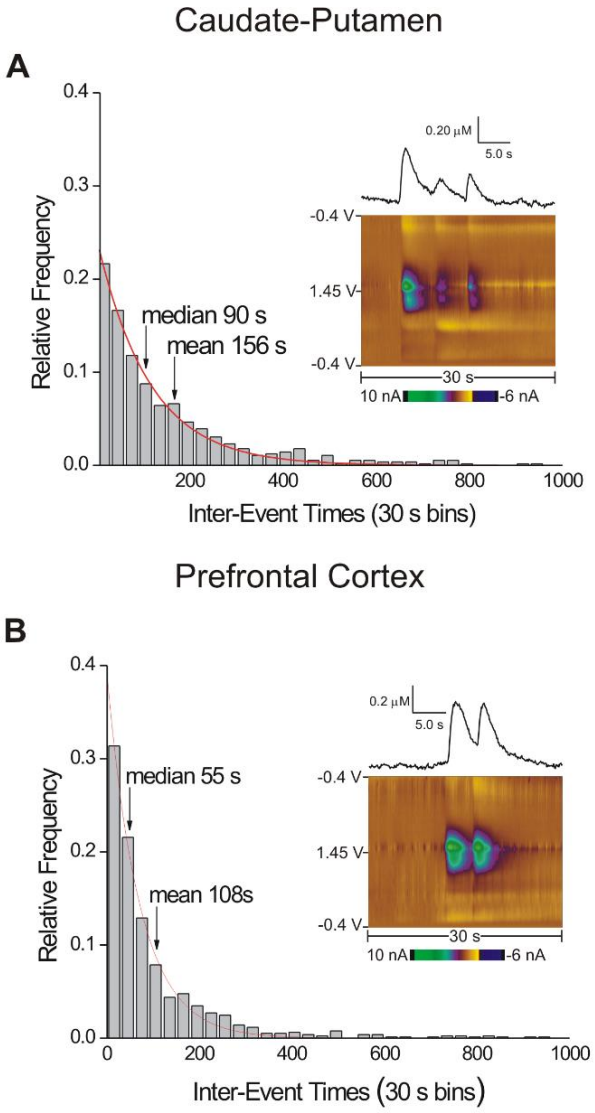

Figure 2.5 Histograms of inter-event times

(A) Inter-event histograms in the caudate-putamen. The time between consecutive transients (termed the inter-event time) was calculated and plotted for the first hour of data collection. The *x*-axis shows 30 second time bins and the *y*-axis is relative frequency of inter-event times. Median, mean and exponential fit ($y = 0.242\ e^{-0.00891x}$ ($R^2 = 0.9926$)) are plotted on the histogram. The inset plots show an example of three consecutive transients. (B) Inter-event histograms for the prefrontal cortex. The exponential fit is $y = 0.388\ e^{-0.0141x}$ ($R^2 = 0.9933$). The inset color plot shows an example of two transients that occurred close together. The underlying distribution of inter-event times was significantly different between the caudate-putamen and prefrontal cortex ($n = 30$ and 29 animals, 588 and 804 inter-event times respectively, Kolmogorov-Smirnov test, $p = 0.0338$). The time between transients is shorter in the prefrontal cortex.

2.5B), with the median and mean values marked. Relative frequency is plotted on the *y*-axis and 30 second time bins are on the *x*-axis. The distribution was not Gaussian and shows that adenosine transients occur closer together than expected from mean inter-event values. The average time between transients for the caudate putamen was 156 seconds, however, the majority of transients occurred within 2 minutes of each other. Similarly, in the prefrontal cortex, the mean inter-event time was 108 seconds, but a majority of transients happened less than a minute apart. Thus, adenosine release occurs randomly and is not a result of pacemaker firing. Color plots show that occasionally spontaneous release events occur within a couple of seconds of each other (Figure 2.5A

Caudate-Putamen

	Avg.	*SEM*	*Range*
Concentration (μM)	0.17	0.01	0.04-2.5
Duration (s)	2.9	0.1	0.8-9.9
Inter-event Time (s)	*156	8	1.4-1405

Prefrontal Cortex

	Avg.	*SEM*	*Range*
Concentration (μM)	0.19	0.01	0.04-3.2
Duration (s)	2.8	0.1	0.3-10
Inter-event Time (s)	*108	6	0.9-2069

Table 2.1 Averages for spontaneous adenosine release

Data from the caudate-putamen and prefrontal cortex. Concentration was significantly different between the two regions (n = 588 transients in 30 rats and 804 transients in 29 rats, t-test, p = 0.0238, respectively). The duration of transient adenosine was not different between the caudate and prefrontal cortex (n = 30 and 29 rats, t-test, p = 0.0826). * Inter-event times, or the time between consecutive transients have significantly different underlying distributions between the two regions (n = 30 and 29 rats, KS test, p = 0.0338).

inset) and the amount of adenosine release does not always decrease with the sequential release (Figure 2.5B inset).

The underlying distributions between the two brain regions were compared and the patterns are significantly different (n = 30 and 29 rats, Kolmogorov-Smirnov test, p = 0.0338). Thus, the time between adenosine transient events was shorter in the prefrontal cortex than in the caudate-putamen. An exponential decay was fit to the data and plotted on the histogram. The exponential fit in the caudate-putamen has a rate constant of 0.00891 s^{-1} or a firing rate of every 112 seconds. The rate constant in the prefrontal cortex is 0.0141 s^{-1} or a firing rate of every 71 seconds.

2.2.6 Adenosine transients continue over time

To examine adenosine transients over time, data were collected continuously over three hours. Figure 2.6A and 6D display the average concentration of adenosine transients in 30 minute bins in the caudate and prefrontal cortex, respectively. The average concentration in both brain regions was around 0.13 μM and did not significantly differ with time (one-way ANOVA, caudate: n = 6, p = 0.2013; prefrontal cortex: n = 6, p = 0.6268). The duration of adenosine release was also binned into 30 minute epochs for the caudate-putamen (Figure 2.6B) and the prefrontal cortex (Figure 2.6E). There was a significant effect of time on event duration between the 30 minute bins in the caudate-putamen (n = 6, one-way ANOVA, p = 0.0005) but not the prefrontal cortex (n = 6, one-way ANOVA, p = 0.8449). The duration of the fifth bin was significantly longer than the durations of the first and second bins in the caudate-putamen (Bonferroni post-test, one-way ANOVA p > 0.05); however, since the sixth bin was lower, we conclude there is no trend of increasing duration over time. Figure 2.6C and 6F show the average number of transient release events normalized in 30 minute time bins. The large error bars were due to the large variability in the number of transients from animal to animal. There was no

significant effect of time on the average number of transients, indicating time after implantation had no effect in the caudate-putamen (n = 6, one-way ANOVA, p = 0.4782) or the prefrontal cortex (n = 6, one-way ANOVA, p = 0.9299). These results indicate that the number, concentration, or duration of adenosine transients does not change for at least three hours following electrode implantation.

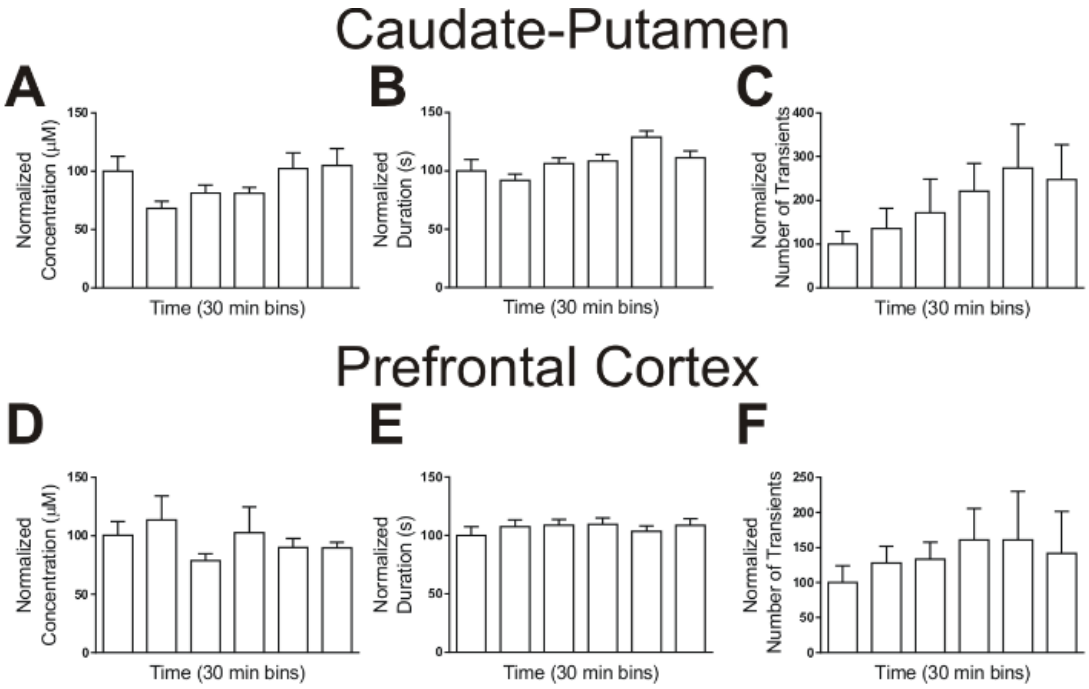

Figure 2.6 Spontaneous transient adenosine over time

All x-axes are time in 30 minute bins and all y-axes were normalized to the first bin. (A) Normalized concentrations of adenosine transients in the caudate putamen. There was no significant effect of time on the concentration (n = 6 animals, one-way ANOVA, p = 0.2013). (B) Duration of adenosine release from the caudate-putamen. There was a main effect of time on durations (n = 6 animals, one-way ANOVA, p = 0.0005). (C) Number of transients in the caudate-putamen. There was no significant effect of time on the number of transients (n = 6 animals, one-way ANOVA, p = 0.4782). (D) Concentrations in the prefrontal cortex. There was no significant effect of time on the concentration (n = 6 animals, one-way ANOVA, p = 0.6268). (E) Durations of transient adenosine in the prefrontal cortex. There was no significant effect of time on the duration (n = 6 animals, one-way ANOVA, p = 0.8449). (F) Number of transients from the prefrontal cortex. There was no significant effect of time on the number of transients (n = 6 animals, one-way ANOVA, p = 0.9299).

2.2.7 A_1 receptor modulation

Previous studies found A_1 receptors modulate stimulated adenosine release (Cechova *et al.* 2010), so we tested the effects of A_1 receptor drugs on spontaneous

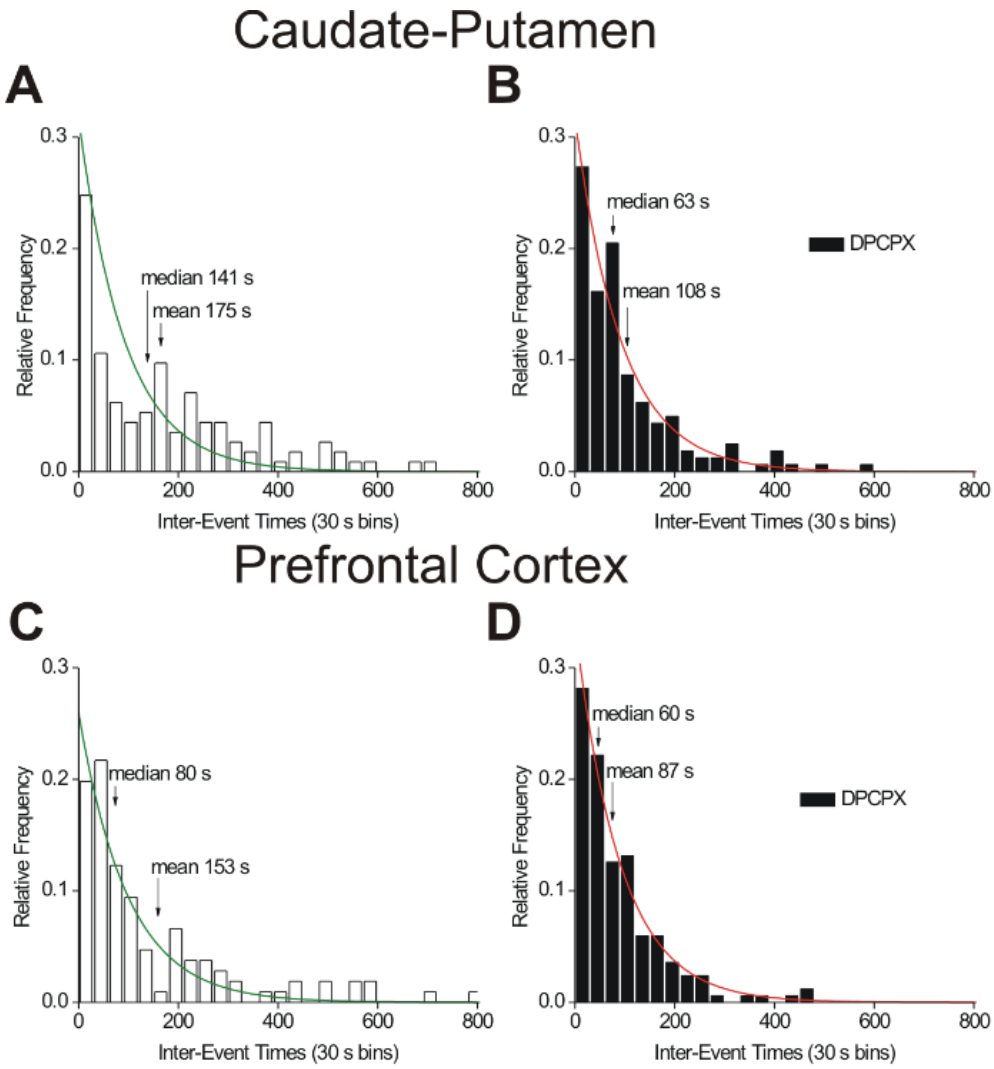

Figure 2.7 Effect of the A_1 antagonist, DPCPX (6 mg/kg, i.p.), on adenosine transients

(A) Inter-event time histograms of first hour of pre-drug in caudate. Median, mean and exponential fit (green line) ($y = 0.295\ e^{-0.0186}$ ($R^2 = 0.7889$)) are plotted on the frequency distribution. (B) Inter-event time histogram for transients in the first hour post-DPCPX in caudate. The exponential fit (red line) is $y = 0.318\ e^{-0.0101x}$ ($R^2 = 0.9490$). In the caudate, there was a significant difference between the underlying distributions before and after DPCPX ($n = 6$ animals, KS-test, $p < 0.0001$). (C) Inter-event histograms pre- and (D) post-DPCPX in the prefrontal cortex. The exponential fit equations are $y = 0.260\ e^{-0.0101x}$ ($R^2 = 0.9124$) pre-drug and $y = 0.340\ e^{-0.0111x}$ ($R^2 = 0.9851$) after DPCPX. In the prefrontal cortex, there was a significant difference between underlying distributions pre- and post-DPCPX ($n = 5$ animals, KS-test, $p = 0.0287$).

adenosine release. For all drug experiments, after the electrode was equilibrated, one hour of baseline data was collected and then the drug administered. DPCPX, an A_1 receptor antagonist, was administered at 6 mg/kg, i.p. The CVs of adenosine transients observed after DPCPX were similar to those observed before drug, indicating that DPCPX did not interfere with the detection of adenosine using FSCV. The concentration and duration of adenosine release were compared before and after DPCPX using paired *t*-tests to examine release in the same animal. DPCPX did not significantly affect the concentration of release in the caudate ($n = 6$, paired *t*-test, $p = 0.1186$) or prefrontal cortex ($n = 5$, paired *t*-test, $p = 0.8547$). However, in the caudate-putamen, DPCPX significantly increased the duration from 2.6 to 3.0 seconds ($n = 6$, paired *t*-test, $p = 0.0092$). The duration of adenosine release in the prefrontal cortex increased from 2.4 to 2.9 seconds after DPCPX, but the increase was not significant ($n = 5$, paired *t*-test, $p = 0.1300$). Following DPCPX administration, there was a decrease in the mean and median inter-event times and a significant difference in the underlying frequency distribution in the caudate-putamen ($n = 6$ rats, KS test, $p > 0.0001$) (Figure 2.7A and 7B) and the prefrontal cortex ($n = 5$ rats, KS test, $p = 0.0286$) (Figure 2.7C and 7D). The time between events decreased after DPCPX administration from a median of 141 seconds pre-drug to 63 seconds in the caudate and from 80 seconds to 60 seconds in the prefrontal cortex. Therefore, blocking A_1 receptors with DPCPX in both brain regions increases the frequency of spontaneous, transient adenosine release.

The A_1 agonist, N^6-cyclopentyladenosine (CPA), was administered to determine if activation of A_1 receptors affected spontaneous adenosine transients. There was no significant change in adenosine concentration in either the caudate or the prefrontal cortex following administration of CPA (paired *t*-test, $n = 6$, $p = 0.9818$; $n = 6$, $p = 0.4607$, respectively). However, CPA did increase the duration of adenosine transients in the caudate from 2.7 to 3.4 seconds ($n = 6$, paired *t*-test, $p = 0.0195$) and from 3.2 to 3.8

seconds in the prefrontal cortex (n = 6, paired t-test, p = 0.0259). The drugs may interfere with uptake or metabolism, thus increasing duration. In the caudate-putamen, the median

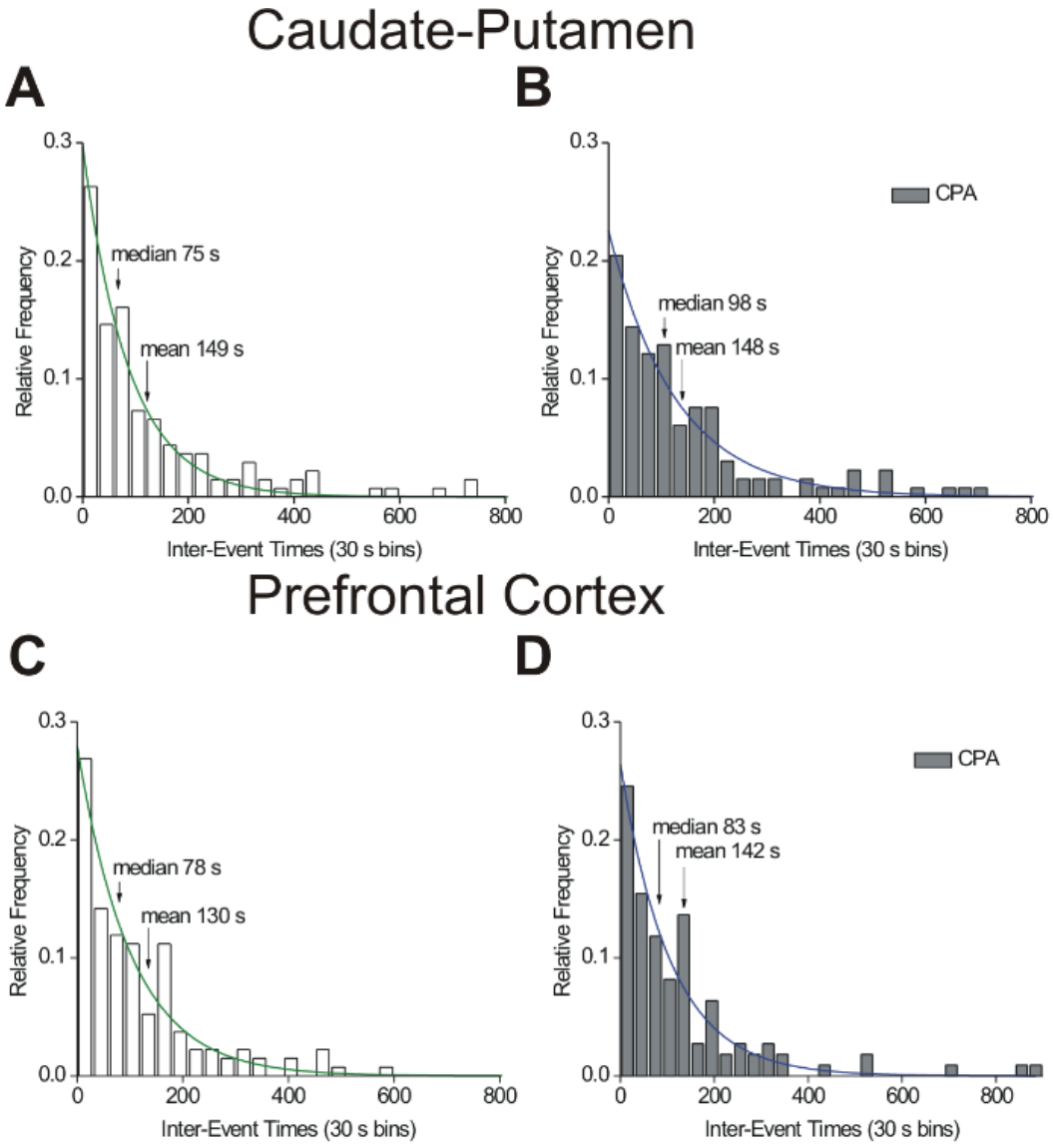

Figure 2.8 Effect of the A_1 agonist, CPA (1 mg/kg, i.p.), on adenosine transients

(A) Inter-event time histograms for the caudate. Median, mean and exponential fit (green line) ($y = 0.298\ e^{-0.0115x}$ ($R^2 = 0.9636$)) are plotted on the frequency distribution. (B) Inter-event time histograms for the first hour after CPA in caudate. The exponential fit (blue line) is $y = 0.226\ e^{-0.00787x}$ ($R^2 = 0.9528$). There was a significant difference between the underlying distributions before and after CPA (n = 6 animals, KS-test, p = 0.0308). (C) Inter-event histograms pre- and (D) post-CPA in the prefrontal cortex. Exponential equations are $y = 0.280\ e^{-0.00983x}$ ($R^2 = 0.9430$) pre-drug and $y = 0.264\ e^{-0.00938x}$ ($R^2 = 0.9337$) after CPA. In the prefrontal cortex, there was no significant difference in the underlying distributions before and after CPA (n = 6 animals, KS-test, p = 0.9299).

and mean inter-event times increased after CPA (Figure 2.8A and 8B) and the underlying distributions were significantly different than pre-drug (n = 6 rats, KS test, p = 0.0308). The time between events increased from a median of 75 seconds to 98 seconds following CPA in the caudate putamen, the opposite effect on inter-event time as DPCPX. However, in the prefrontal cortex (Figure 2.8C and 8D), CPA did not significantly change the underlying distribution and the inter-event times were not different (n = 6 rats, KS test, p = 0.9299). Thus, A_1 activation with CPA decreased the frequency of spontaneous transient adenosine release in the caudate-putamen but not the prefrontal cortex.

2.3 Discussion

Spontaneous, transient adenosine release occurs in both the caudate-putamen and prefrontal cortex. Transients were on average a couple hundred nM, which is sufficient to activate adenosine receptors (Fredholm *et al.* 2011). Adenosine was elevated for only a few seconds and these are the most rapid adenosine changes that have been measured *in vivo*. While adenosine transients occurred on average once every several minutes, at least 30% of transients were less than one minute apart and there was no regularity to adenosine release. A_1 receptors modulated the time between adenosine events but not the concentration. These studies reveal that large amounts of adenosine can be quickly released and cleared from the extracellular space, a contrast to previous studies which have documented a slower role for adenosine (Kaku *et al.* 1994;Lasley *et al.* 1995;Sharma *et al.* 2010). Thus, adenosine has a rapid signaling mode that may locally cause transient neuromodulation.

2.3.1 The concentration of transient release is sufficient for adenosine receptor activation

The average concentration of adenosine released was 180 nM, but the amount varied widely from 40 nM to 3.2 µM. Spontaneous adenosine release was the same order of magnitude as stimulated release in brain slices from the cerebellum (Klyuch *et al.* 2012), caudate-putamen (Pajski and Venton 2010b) and prefrontal cortex (Pajski and Venton 2013). However, evoked adenosine release in the caudate-putamen of anesthetized rats was typically 600-900 nM (Cechova and Venton 2008;Cechova *et al.* 2010), larger than the average spontaneous transient. Electrical stimulation likely activates all cells but different firing rates or number of cells activated during spontaneous adenosine release could lead to lower release.

In the prefrontal cortex, the concentration of adenosine release was significantly higher than in the caudate, although the difference was not large enough to suggest that different types of adenosine receptors are being activated in the two brain regions. Inhibitory A_1 receptors and excitatory A_{2a} receptors both have affinities in the low nanomolar range (Latini and Pedata 2001) and have high to intermediate distributions in the caudate and prefrontal cortex (Fastbom *et al.* 1987). About one percent of transients had concentrations greater than 1 µM, which could be sufficient to activate A_{2b} and A_3 receptors (Fredholm *et al.* 1994b). These large transients demonstrate the potential for transient, micromolar adenosine signaling and high amounts of receptor activation.

2.3.2 Spontaneous adenosine release is random and the frequency is regulated by A_1 receptors

Adenosine release occurred on average once every 3-4 minutes; however, about half of the transients occurred within two minutes of each other in the caudate and one

minute of each other in the prefrontal cortex. Spontaneous adenosine release is not periodic and consequently is unlikely to be caused by pacemaker firing or to directly modulate a rhythmic process such as breathing or tonic cell firing (Watt and Routledge 1985;Nayebpour *et al.* 1993). Instead, release was random and the inter-event times fit an exponential decay, which arises from a discrete Poisson process (Milton and Arnold 2003) where each transient is not dependent on the prior adenosine event. The shortest time between transients was less than one second, indicating a long time is not required to reset adenosine release (Fig. 5A and 5B insets). Some of the larger transients may be two or more release events that occur simultaneously and cannot be temporally resolved. The frequency and concentration of release did not change over three hours, demonstrating that transient adenosine continues for long periods of time and that adenosine release is not just a response to immediate damage after electrode implantation (Street *et al.* 2011b;Klyuch *et al.* 2012;Chang *et al.* 2012).

A_1 receptors are inhibitory adenosine receptors with low nM affinities that downregulate cAMP, hyperpolarize neurons, and can be neuroprotective during ischemia (Fredholm *et al.* 2011). The cortex and striatum have intermediate to high levels of A_1 receptor expression (Fastbom *et al.* 1987). Previously, A_1 receptors were shown to have autoreceptor characteristics and regulate the amount of stimulated adenosine release in the caudate *in vivo* (Cechova *et al.* 2010). Here, A_1 receptors modulated the frequency of spontaneous adenosine release but not the concentration. DPCPX, an A_1 antagonist, decreased the inter-event times in both brain regions. CPA, an A_1 agonist, had the opposite effect and increased the inter-event times in the caudate-putamen but did not have a significant effect in the prefrontal cortex. A_1 receptors are part of a feedback loop controlling transient adenosine release in the brain, where activation of A_1 receptors decreases transient adenosine events. A_1 receptor modulation does not affect concentration suggesting that the receptors do not control synthesis or the amount

packaged into vesicles. Instead, A_1 receptors modulate event frequency, such as regulating the number of docking or release events.

2.3.3 Spontaneous adenosine release occurs in multiple brain regions

Spontaneous, transient adenosine release was measured in both the caudate-putamen and prefrontal cortex. Basal adenosine levels are higher in the prefrontal cortex than the caudate-putamen (Kobayashi *et al.* 1998;Akula *et al.* 2008;Kovacs *et al.* 2010b). Similarly, the concentration of transient adenosine release is larger in the prefrontal cortex. However, transient adenosine release is unlikely to contribute to the basal levels of adenosine because of the rapid clearance. Spontaneous release was more frequent in the prefrontal cortex than in the caudate, which is similar to electrically-evoked release which was also more frequently observed in the prefrontal cortex (Pajski and Venton 2013). The higher frequency of adenosine release in the prefrontal cortex could be due to either additional release sites or more release events per site.

The spontaneous adenosine transients measured here in the rat caudate and cortex were remarkably similar to the spontaneous adenosine transients measured in murine slices from lamina II of the spinal cord by Zylka's group (Street *et al.* 2011b). Despite the differences in brain region, preparation (slices vs. *in vivo*), and species, the average concentrations were the same order of magnitude (180 nM in rats compared to 570 nM in mouse slices). Differences could be a result of different numbers of cells activated or the amount of adenosine available for release. The average frequencies of spontaneous adenosine transients were also similar, with events occurring every two to three minutes. The comparable adenosine transients in different regions of rats and mice, along with previous electrophysiology work in the hippocampus implicating fast adenosine release regulating glutamate receptor excitability (Mitchell *et al.* 1993a), show that

transient adenosine release is a common feature in the nervous system. These transients could be an important mechanism of adenosine signaling, facilitating rapid neuromodulation throughout the brain.

2.3.4 Spontaneous adenosine release provides local, transient modulation

Spontaneous adenosine signaling was fast, with the average transient lasting only about three seconds. This mode of transient signaling is a contrast to gradual adenosine buildup for minutes in the extracellular space during pathological events, such as ischemia (Phillis *et al.* 1987). Spontaneous adenosine transients were even more rapid than electrically-stimulated adenosine release, which elevated adenosine for up to 20 seconds in the caudate-putamen (Cechova and Venton 2008) or 100 seconds in the cerebellum (Klyuch *et al.* 2012). Our time course matched well with previous measurement of transient adenosine release in spinal cord slices (Street *et al.* 2011b) and electrophysiology studies that observed a transient, 2 second variation in glutamate-evoked EPSPs after stimulated adenosine release (Mitchell *et al.* 1993b). The short duration demonstrates that the adenosine clearance mechanism from the extracellular space is very rapid, likely due to uptake (Latini and Pedata 2001). Future studies will examine the effects of adenosine clearance mechanisms. Since the duration of adenosine release was the same in both brain regions, the mechanism of clearance is expected to be conserved between the caudate and prefrontal cortex.

The time course of spontaneous adenosine signaling is similar to that of transient, exocytotic release events of neurotransmitters. For example, dopamine signaling after phasic firing lasts only three to four seconds and dopamine is rapidly cleared from the extracellular space (Wightman and Robinson 2002). While our study did not address the pathway of extracellular adenosine formation, the mechanism is likely breakdown of

exocytotically released ATP or direct, activity-dependent release of adenosine. Recent evidence shows transient, electrically-stimulated adenosine is activity dependent and a portion is not dependent on extracellular breakdown of ATP (Pajski and Venton 2010b;Klyuch *et al.* 2012). Spontaneous adenosine release in slices of the spinal cord of mice is ATP-dependent (Street *et al.* 2011b;Street *et al.* 2013). While the mechanism of spontaneous transient release is difficult to elucidate *in vivo*, future experiments could be performed in brain slices to determine if transient adenosine release is exocytotic or a downstream product of ATP.

The rapid release and clearance of adenosine has two consequences: adenosine can only signal locally and receptors will be activated transiently. The distance a molecule can travel in three seconds in the extracellular space is only 10-20 micrometers (Wightman *et al.* 1988). Therefore, only receptors close to the release event would be activated and transient adenosine release would provide local neuromodulation, close to the site of release. In addition, there could be heterogeneity within a region for receptor activation if not all areas experienced adenosine transients at the same time. The local variation of release was not directly assessed in these studies, but moving the electrode did change the number of adenosine transients detected suggesting a spatial effect on release events.

The function of rapid adenosine release is likely transient neuromodulation in the brain. Although most studies of adenosine function have not been performed on the seconds time scale, adenosine is known to regulate cerebral blood flow and neurotransmission on a longer time scale. It is likely that faster regulation of these processes is caused by transient adenosine release. Adenosine increases cerebral vasodilation in less than 60 seconds (Winn *et al.* 1985) and also increases cerebral blood flow (Soricelli *et al.* 1995). Transient adenosine release could increase the flow of blood to localized areas in the brain that require an immediate boost in oxygen and nutrients. Adenosine regulation of neurotransmitter release can be inhibitory; for example,

adenosine inhibits acetylcholine, glutamate, serotonin, dopamine, noradrenaline signaling by activation of A_1 receptors (Sperlagh and Vizi 2011). Adenosine can also increase the release of acetylcholine and glutamate through A_{2a} receptor activation (Sperlagh and Vizi 2011). Although A_1 and A_{2a} adenosine receptor affinities are in the low nanomolar range, the effective *in vivo* EC_{50} is estimated to be 600 nM (Dunwiddie and Diao 1994), so changes on the order of hundreds of nanomolar concentrations could have significant physiological effects. This study shows that A_1 receptors control the frequency of adenosine transients, but the downstream modulatory effects of the transient adenosine activation of A_1 receptors are not known and should be explored in the future. A rapid, transient mode of adenosine signaling would provide discrete, local neuromodulation, which could facilitate fine control of neurotransmission.

2.4 Conclusions

Spontaneous transient adenosine release was characterized for the first time *in vivo*. The spontaneous release in the caudate and prefrontal cortex is fast, lasting only a few seconds, and large, in the hundred nM range. The frequency of release was random, higher in the prefrontal cortex, and modulated by A_1 receptors. These findings are a paradigm shift for understanding the time course of adenosine signaling. Previous studies have documented a long-term modulatory effect of adenosine during pathologies but here we demonstrate a new mode of transient adenosine signaling that could lead to rapid, local modulation. Future studies investigating the formation and function of this new type of adenosine signaling will reveal how adenosine modulates on the second time scale and regulates local brain function.

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Chapter 3: Rapid adenosine release is cleared by nucleoside transporters and metabolism

“If our brains were simple enough to understand, we wouldn’t be smart enough to understand them”

Anonymous

3 Abstract

Adenosine is a neuromodulator that regulates neurotransmission in the brain and central nervous system. Basal extracellular levels of adenosine are maintained by metabolism and nucleoside transporters. Recently, spontaneous adenosine release was discovered in mouse spinal cord slices and anesthetized rat brains. This release is transient and is cleared within 3-4 seconds. Here we examined the mechanism of clearance of transient adenosine release by measuring spontaneous adenosine release 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 µM/s. The equilibrative nucleoside transport 1 (ENT1) inhibitor, NBTI (1 mg/kg, i.p.), significantly increased the duration of adenosine *in vivo* while the ENT1/2 inhibitor dipyridamole (10 mg/kg, i.p.) did not affect duration. ABT-702, an adenosine kinase inhibitor (5 mg/kg, i.p.), increased the duration of spontaneous adenosine release. The adenosine deaminase inhibitor EHNA (10 mg/kg, i.p.) also increased the duration *in vivo*. The increase in duration for blocking ENT1, adenosine kinase, and adenosine deaminase were similar, about 0.4 s *in vivo*; thus, the removal of adenosine on a rapid time scale occurs through three mechanisms that have similar effects. Similarly, NBTI (10 µM), ABT-702 (100 nM) or EHNA (20 µM) also decreased the clearance rate of exogenously applied adenosine in brain slices. The presence of multiple mechanisms for adenosine clearance on a second time scale demonstrates that it is tightly regulated in the extracellular space.

3.1 Introduction

Adenosine modulates neurotransmission in the brain and is neuroprotective during stressful conditions, such as ischemia and hypoxia. Recently, adenosine was found to be rapidly released on a time scale of 3-30 seconds following electrical stimulation (Cechova and Venton, 2008;Cechova et al., 2010;Pajski and Venton, 2010;Pajski and Venton, 2013;Klyuch et al., 2012), mechanical stimulation (Chang et al., 2009;Ross et al., 2014), and ischemia (Dale et al., 2000;Dale and Frenguelli, 2009). In addition to stimulated release, spontaneously released adenosine was discovered in spinal cord slices (Street et al., 2011) and *in vivo* in the rat brain (Nguyen et al., 2014). This form of adenosine is released and removed from the extracellular space in less than four seconds, implying adenosine is cleared on a rapid time scale. Although the mechanism of clearance for adenosine has been examined on a longer time scale, the clearance for rapid adenosine clearance has not been thoroughly characterized.

Once released into the extracellular space, adenosine is typically cleared using 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 follow a concentrative gradient through ENTs, while CNTs move nucleosides against a gradient (Thorn and Jarvis, 1996). There are four types of equilibrative transporters (ENT1-4). The inhibitor nitrobenzylthioinosine (NBTI/NBMPR) is selective for ENT1 (Ward et al., 2000). There are only non-specific 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 thus is not involved in adenosine clearance. ENT4 has a low affinity for adenosine at physiological pH but could be involved in adenosine clearance during acidotic conditions from ischemia (Barnes et al., 2006). There are three types of CNT (1-3), however, there is no evidence that CNTs are regulating physiological levels of adenosine

(Parkinson et al., 2011;Young et al., 2013). While there are a few reports of specific inhibitors of human CNTs, they are not widely available (Damaraju et al., 2011). Thus ENT1 and 2 were the primary focus for studying the regulation of transient adenosine release.

Adenosine is also cleared through metabolism. Adenosine kinase phosphorylates AMP to adenosine and adenosine deaminase breaks adenosine down to inosine. Both adenosine kinase and adenosine deaminase are extracellular enzymes that modulate adenosine concentrations thus they are expected to have an effect on transient adenosine release. In the hippocampus, extracellular adenosine concentrations were increased two-fold with adenosine kinase inhibition (Pak et al., 1994). Studies of the effect of adenosine deaminase inhibition on basal adenosine levels are mixed as one study showed it increases basal concentrations (Sciotti and van Wylen, 1993), while another study found little change (Lloyd and Fredholm, 1995). The effects of metabolism to clear adenosine on a faster time scale are unknown.

Here, we investigated the mechanism of release and clearance of transient adenosine release. We examined rapid adenosine clearance of spontaneous adenosine release *in vivo* and with exogenously applied adenosine in brain slices. Equilibrative nucleoside transporters of adenosine were blocked with NBTI and dipyridamole, and we found that ENT1 was responsible for rapid clearance of adenosine. Similarly EHNA, an inhibitor of adenosine deaminase, or 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 and thus it is tightly regulated by multiple clearance mechanisms.

3.2 Results

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 is swept from -0.40 V to 1.45 V and back at 400 V/s at 10 Hz. Adenosine is 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 vs time plot was using principal component analysis and can be used to track how long adenosine is elevated in the extracellular space (Nguyen et al., 2014). We examined spontaneous, transient adenosine release *in vivo* as well as the clearance of exogenously applied adenosine in brain slices to determine the mechanism of rapid adenosine clearance.

3.2.1 Spontaneous, transient adenosine clearance

Adenosine is spontaneously released without stimulation and is cleared in about 3 seconds in anesthetized rats (Nguyen et al., 2014). Figure 3.1A shows a concentration versus time plot of an example transient adenosine event. Adenosine is released and cleared within the extracellular space in 4.0 seconds. The gray shaded area between the dotted vertical lines indicate when the adenosine concentration is 90% above background subtracted baseline levels and shows how the peak duration was calculated *in vivo*. An exponential decay rate is fit from the peak to when the signal decayed to 90% of peak the amplitude and is marked with a red line. The decay rate, k (s^{-1}), was calculated with a single exponential decay:

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

A characteristic CV of transient adenosine is shown in Figure 3.1B, where the primary oxidation is at 1.4 V and the secondary oxidation is at 1.0 V. The CV verifies that adenosine is detected. To examine clearance mechanisms, electrochemical data were collected for one hour pre-drug and then one hour after i.p. administration of various drugs

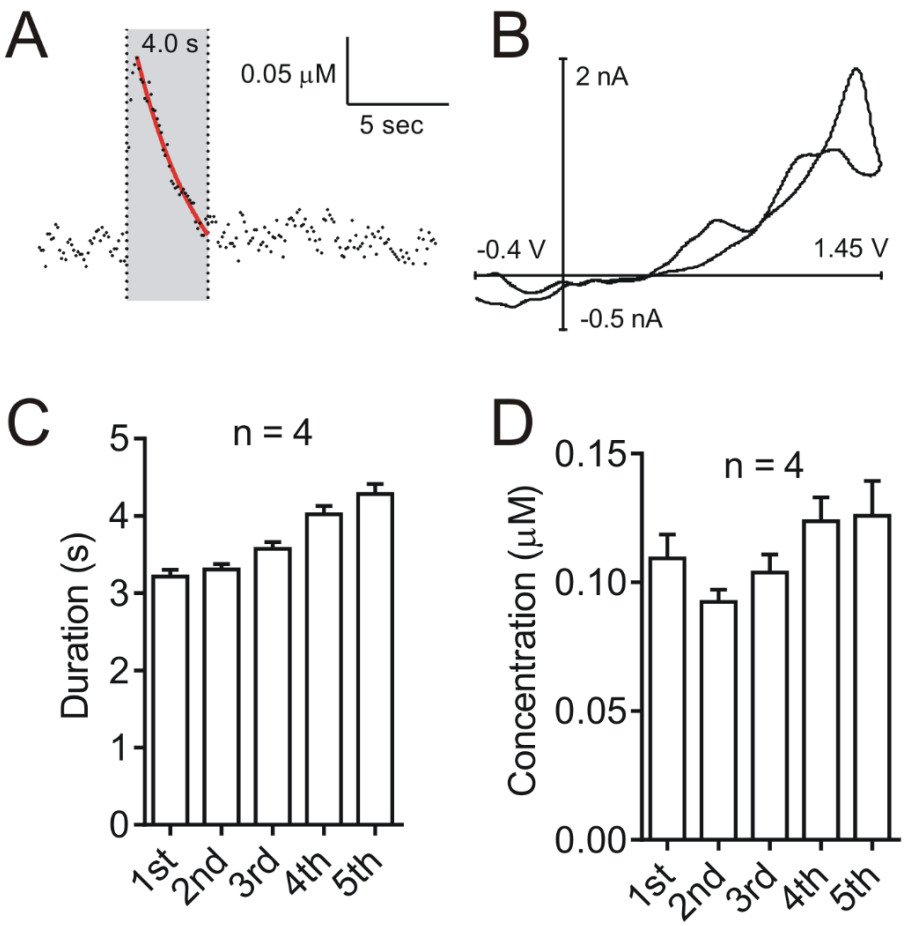

Figure 3.1 Stability of spontaneous, transient adenosine release *in vivo* using fast-scan cyclic voltammetry

(A) Concentration vs time trace of spontaneous adenosine release *in vivo*. The gray shading demonstrates when adenosine levels are above the 90% baseline and the clearance of adenosine is fit with a single exponential decay $[AD](t) = [AD]_{max}e^{-kt}$. (B) A characteristic cyclic voltammogram (CV) of adenosine from (A) 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 five hour period. The durations are placed into 1 hour time bins. The fourth and fifth hour are significantly higher than the first three hours (one-way ANOVA post-Bonferroni test, n = 4, $p < 0.05$). (D) Concentration of spontaneous adenosine release were placed into hour bins. There is no significant difference in concentration over time (One-way ANOVA, n = 4, $p > 0.05$).

to allow comparisons of transient frequency, duration, concentration, and clearance rates in the same animal.

3.2.2 *Stability of transient adenosine release*

First, we examined spontaneous, transient adenosine release over a five hour period to determine the extent to which adenosine release and clearance are stable over time. The measured durations were placed into one hour bins. The duration of spontaneous adenosine release significantly increases over time (One way ANOVA, $p < 0.0001$); however the first three hours are not significantly different from each other (One way ANOVA, Bonferroni test, $p > 0.05$, Figure 3.1C). Similarly, the concentration of transient adenosine was binned in one hour increments (Figure 3.1D) and there was a significant change over the 5 hour period (One way ANOVA, $p = 0.0279$); however, there was no significant difference between any individual hour periods (One way ANOVA, Bonferroni test, $p > 0.05$). A possible explanation for the increase in duration after three hours is surface fouling of the electrode by peptides, lipids, and proteins onto the electrode (Park et al., 2005;Chandra et al., 2014). This fouling may cause restricted 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 (pre-drug) and third hour (first hour post-drug) as the duration was constant for this time period.

3.2.3 *Inhibiting equilibrative nucleoside transporters*

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 has reduced NSAID gastric ulcer damage by increasing the concentration of adenosine

(Salcedo et al., 1997). Concentration vs time plots before (top) and after (bottom) NBTI administration demonstrate the increase in duration. The inset CV verifies adenosine was detected (Figure 3.2A). Figure 3.2 shows the duration of transient adenosine significantly increased after NBTI from 3.5 ± 0.1 to 3.8 ± 0.1 seconds (Figure 3.2B, n = 8 animals, unpaired *t*-test, $p = 0.0327$). NBTI significantly decreased the exponential decay value for clearance (Figure 3.2B) from 0.51 ± 0.01 to 0.43 ± 0.02 s^{-1} (n = 8 animals, unpaired *t*-test, $p = 0.0024$). The extended period of time for adenosine in the extracellular space demonstrates that spontaneous release is cleared by nucleoside transporters.

In order to further characterize the effects of NBTI on spontaneous, transient adenosine release, the inter-event times and concentrations were examined. The inter-event times, i.e. the time between consecutive transients, were placed into 30 second bins and the frequency distribution was examined for pre-drug (black dashed line) and NBTI (dashed blue line) (Figure 3.2D). 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 0.12 ± 0.01 to 0.11 ± 0.01 µM (Figure 3.2D, n = 8 animals, unpaired *t*-test, $p = 0.3585$). The results demonstrate spontaneous adenosine is not released through equilibrative transport, since the release 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 (Figure 3.2F) as the duration was 3.2 ± 0.1 pre-drug and 3.2 ± 0.1 after dipyridamole (Figure 3.2G, n = 6 animals, unpaired *t*-test, $p = 0.6921$). However, the exponential decay rate for clearance significantly decreased from 0.39 ± 0.02 to 0.31 ± 0.02 s^{-1} (Figure 3.2H, n = 6 animals, unpaired *t*-test, $p = 0.0141$).

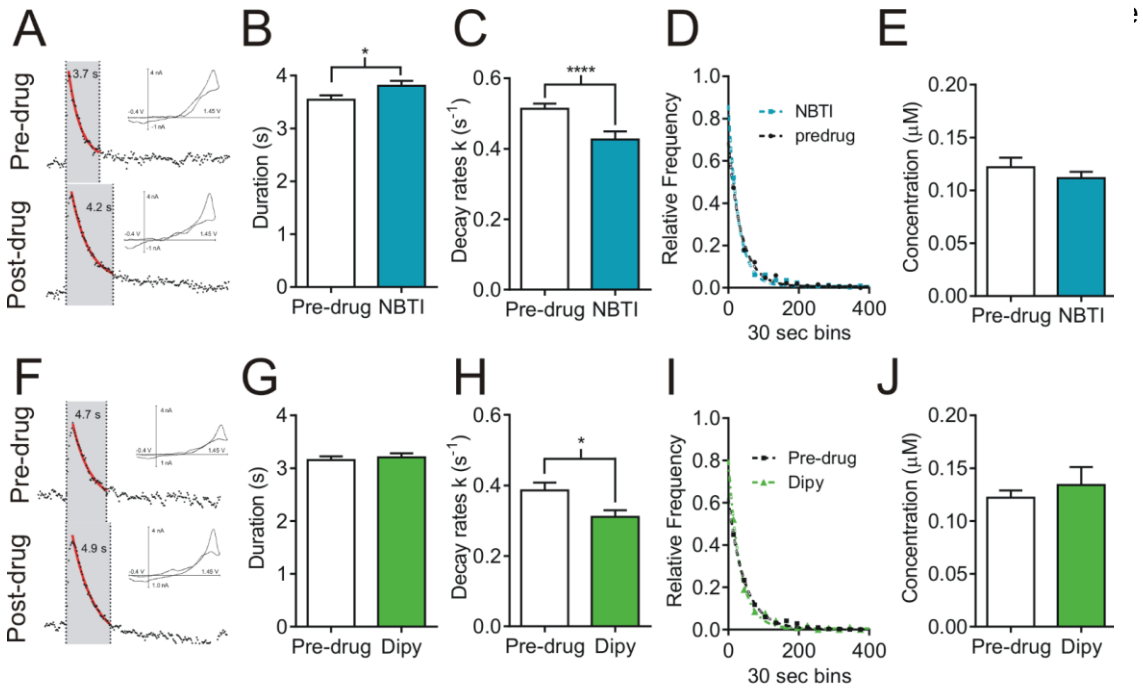

Figure 3.2 Inhibition of clearance of spontaneous, transient adenosine release through equilibrative nucleoside transporters

(A) Concentration vs time trace of spontaneous adenosine release pre- (top) and post-NBTI (bottom) which inhibits ENT1. Gray shading between dashed lines indicates when adenosine is above 90% of the baseline. Exponential fit of the clearance of adenosine is shown as red trace. CV of adenosine are inset for respective transients. (B) Pre-drug (white) and post-NBTI (blue) adenosine durations significantly increased (unpaired *t*-test, n = 6, $p = 0.0327$). The durations are significantly longer following inhibition of ENT1 with NBTI. (C) The decay rates (k, s^{-1}) following NBTI administration (blue) are significantly smaller than pre-drug values (white) (unpaired *t*-test, n = 8, $p = 0.0024$). (D) Inter-event times (time between consecutive transients) are plotted as histograms with *x*-axis in 30 second bins and the *y*-axis in relative frequency. The pre-drug values are in black dots and the NBTI values are in blue dots. Exponential fits are displayed for each set in their respective colors. There is no significant difference in the underlying distributions (Kolmogorov-Smirnov test, n = 8 rats, $p = 0.2882$). (E) Spontaneous transient adenosine concentration pre-drug (white bars) and post-NBTI (blue bars). The concentration did not significantly change (unpaired *t*-test, n = 8, $p = 0.3585$). (F) Dipyridamole (ENT1/2 inhibitor) is administered to see effects on spontaneous, transient adenosine release. A pre-drug transient (top) and post-dipyridamole (bottom) concentration vs time graph with duration shaded gray and exponential decay fit in red. CV of adenosine are inset. (G) Duration bar graph before (white) and after dipyridamole (green) did not significantly change (unpaired *t*-test, n = 6 animals, $p = 0.6921$). (H) The decay rate following dipyridamole significantly decreased (unpaired *t*-test, n = 6 animals, $p = 0.0141$). (I) Inter-event time histogram before (black) and after (green) dipyridamole. The underlying distribution are not significantly different (KS test, n = 6 animals, $p = 0.3141$). (J) Concentration of spontaneous adenosine release before and after drug. The concentration did not significantly change (unpaired *t*-test, n = 6 animals, $p = 0.4893$).

magnitude or frequency of spontaneous adenosine release. The inter-event time distribution did not significantly change (Figure 3.2I, n = 6 animals, KS test, p = 0.3141). The concentration of transient adenosine following ENT1/2 inhibition remained unchanged from 0.12 ± 0.01 to 0.13 ± 0.02 µM (Fig. 2J, n = 6 animals, unpaired t-test, p = 0.4893). The results show that spontaneous transient adenosine is not released or cleared through ENT2.

3.2.4 *Inhibiting metabolism*

There are two primary enzymes that remove adenosine from the extracellular space: adenosine kinase and adenosine deaminase. Adenosine kinase metabolizes adenosine to AMP. ABT-702 inhibits adenosine kinase and increases extracellular adenosine concentrations (Kowaluk et al., 2000). At a dose of 5 mg/kg i.p., ABT-702 increased stimulated adenosine release in the rat brain (Cechova and Venton, 2008). Figure 3.3A shows concentration vs time traces of spontaneous adenosine release before and after adenosine kinase inhibition with the exponential fit of the decay portion in red. Inhibition of adenosine kinase with ABT-702 (5 mg/kg, i.p.) increased the duration of spontaneous released adenosine from 2.9 ± 0.1 to 3.3 ± 0.1 seconds (Figure 3.3B, n = 5 animals, unpaired t-test, p = 0.0155). The decay rate significantly decreased from 0.42 ± 0.03 s^{-1} to 0.31 ± 0.02 s^{-1} (Figure 3.3C, n = 5 animals, unpaired t-test, p = 0.0127). This confirms that adenosine kinase is partially responsible for the clearance of spontaneously released adenosine.

After ABT-702, the inter-event time distribution is significantly different (n = 5 animals, KS test, p = 0.0414) demonstrating that inhibition of adenosine kinase affects the frequency of spontaneous adenosine release (Figure 3.3D). The median inter-event time increases and there are fewer transients occurring close together. The concentration of

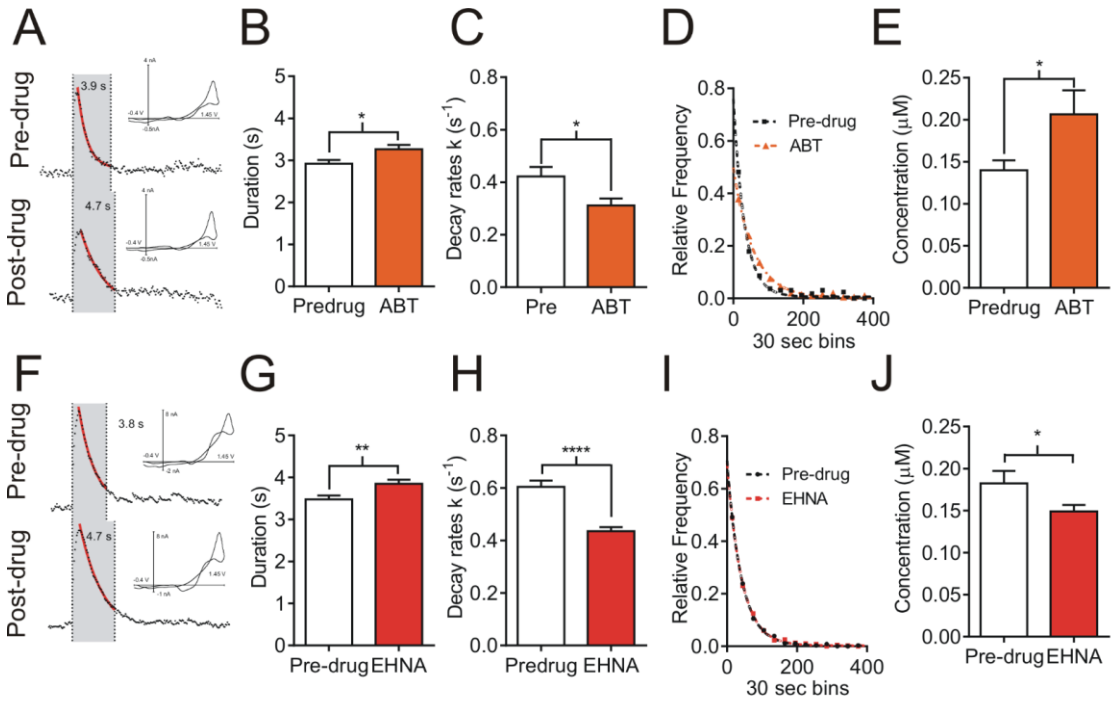

Figure 3.3 Metabolism effect on clearance of transient adenosine release

(A) Concentration vs time of spontaneous, transient adenosine release before (top) and after (bottom) ABT-702 with inset of CV of adenosine. (B) The effect of adenosine kinase inhibition on duration pre-drug (white) and post-ABT (orange). The duration significantly increased (unpaired *t*-test, n = 5 animals, $p = 0.0155$). (C) Decay rate before and after administration of ABT-702 significantly decreased (unpaired *t*-test, n = 5 animals, $p = 0.0127$). (D) Inter-event time histogram of pre-drug (black) and post-drug (orange) with 30 second bins. There was no significant difference between the underlying distributions (KS test, n = 5 animals, $p = 0.0414$). (E) The concentration of spontaneous adenosine release before and after ABT-707 administration. The concentration significantly increased (unpaired *t*-test, n = 5 animals, $p = 0.0483$). (F) Effect of adenosine deaminase inhibition with EHNA on spontaneous adenosine release. Concentration vs time plots before and after inhibition with gray indicating duration of release and exponential decay fit in red. Inset of CV for adenosine. (G) The duration of transient adenosine significantly increased pre- and post-drug (unpaired *t*-test, n = 6 animals, $p = 0.0049$). (H) Bar graph showing decay rates before and after EHNA administration show an increase (unpaired *t*-test, n = 6 animals, $p < 0.0001$). (I) Inter-event time histogram with pre-drug trace (black) and post-EHNA (red) with times placed into 30 second bins. There was no significant difference in the underlying distribution (n = 6 animals, KS test, $p = 0.8506$). (J) The concentration of transient adenosine decreased following adenosine deaminase (red) inhibition (unpaired *t*-test, n = 6 animals, $p = 0.0404$).

the spontaneous transients significantly increased from 0.14 ± 0.01 to 0.20 ± 0.03 µM

(Figure 3.3E, n = 5 animals, unpaired *t*-test, $p = 0.0483$). The results are in agreement

with previous studies that inhibition of adenosine kinase increases adenosine release (Cechova and Venton, 2008).

Adenosine deaminase, an enzyme that metabolizes adenosine to inosine, was inhibited with erthro-9-(2-hydroxy-3-nonyl) adenine (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 vs time plots with inset CV show the effect of adenosine deaminase inhibition (Figure 3.3F). After EHNA (10 mg/kg, i.p.), the duration of spontaneous transients significantly increased from 3.5 ± 0.1 to 3.9 ± 0.1 seconds (Figure 3.3G, n = 6 animals, unpaired *t*-test, $p = 0.0049$). In Figure 3.3H, the exponential decay rate of clearance significantly decreased from 0.60 ± 0.02 to 0.44 ± 0.02 s^{-1} (n = 6 animals, unpaired *t*-test, $p < 0.0001$). The results show that inhibition of adenosine deaminase also affects adenosine clearance.

Figure 3.3I shows the frequency distribution of inter-event times for pre-drug (black line) and EHNA (red line). The time between transient events are not significantly different (n = 6 animals, KS test, $p = 0.8506$). The concentration significantly decreased from 0.18 ± 0.01 µM to 0.15 ± 0.01 µM Figure 3.3J, n = 6 animals, unpaired *t*-test, $p = 0.0404$). Concentration decreases after adenosine deaminase inhibition, the opposite effect of adenosine kinase inhibition.

3.2.5 Exogenously-applied adenosine

Different amounts of adenosine were pressure ejected onto brain slices using a pulled glass pipette that was calibrated for different ejection times at varying pressures (5-20 psi). The picospritzing pipette was positioned 20-30 µm away from the carbon-fiber electrode and the rate of adenosine clearance and the concentration of adenosine that reached the electrode were measured. Figure 3.4A shows a plot of raw data of

concentration vs time with different amounts of exogenously-applied adenosine in caudate-putamen brain slices. Increasing the amount of applied adenosine led to an increased rate of clearance until saturation. Each trace was fit with an exponential decay (red) to measure clearance. The initial velocity, V, was calculated from the rate constants using the equation: $V = k[AD]_{max}$. A plot of velocity vs concentration (Figure 3.4B) was fit with a nonlinear regression following Michaelis-Menten enzyme kinetics with the equation:

$$V = \frac{V_{max}[AD]_{max}}{K_M + [AD]_{max}}$$

The plot had a maximal rate, V_{max}, of 1.4 ± 0.1 µM/s. This rate includes all forms of adenosine clearance, including uptake and metabolism.

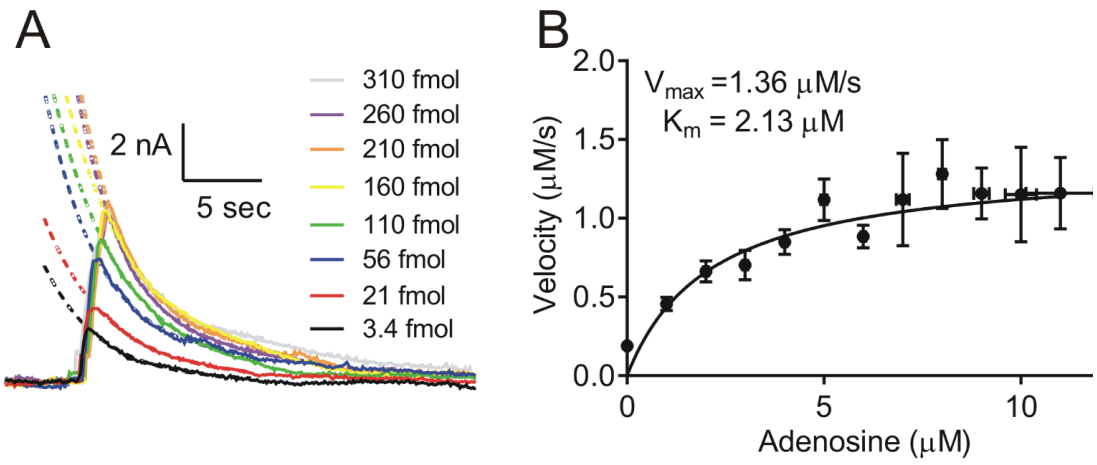

Figure 3.4 Application of exogenous adenosine in caudate-putamen brain slices

(A) Concentration vs time trace of increasing amounts of picospritzed adenosine onto brain slices. The concentrations ranges from 3.4 to 310 femptomol and are fit with single exponential decays (dashed lines) to the equation: $V = k[AD]_{max}$. (B) Rate of clearance (*k*) from multiple electrodes plotted vs applied adenosine concentration. The concentrations were placed into 100 nM bins. A Michaelis-Menten equation ($V = \frac{V_{max}[AD]_{max}}{K_M + [AD]_{max}}$) was fit to the curve in order to determine a V_{max} of 1.36 µM/s for adenosine clearance.

3.2.6 *Adenosine Transporter Clearance*

Adenosine was pressure ejected in brain slices before and after they were bathed in NBTI (10 µM) to block ENT1 (Figure 3.5A). At 10 µM, NBTI sufficiently binds adenosine transporters in brain slices (Bailey et al., 2004). The results show that ENT1 is partially responsible for the clearance of exogenously-applied adenosine. An exponential decay was fit for the current vs time trace to determine the clearance rates before and after drug application. The exponential decay rate significantly decreased from 0.23 ± 0.01 s^{-1} to 0.16 ± 0.02 s^{-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 µM) (Figure 3.5B). Dipyridamole reduced EPSPs 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 s^{-1} to 0.46 ± 0.07 s^{-1} (n = 6, paired *t*-test, $p = 0.5209$).

3.2.7 *Adenosine Metabolism Clearance*

The clearance of exogenous transient adenosine in brain slices was examined following inhibition of adenosine kinase with ABT-702 (Figure 3.5C). The clearance rate of exogenously applied adenosine decreased after inhibition with ABT-702 from 0.32 ± 0.02 s^{-1} to 0.23 ± 0.03 s^{-1} (n = 5, paired *t*-test, $p = 0.0053$). Thus adenosine kinase is responsible for clearing adenosine on a rapid time scale. Next, we examined the effect of adenosine deaminase inhibition with EHNA (20 µM) (Barankiewicz et al., 1997) on adenosine clearance (Figure 3.5D). The decay rate significantly decreased from 0.24 ± 0.02 s^{-1} to 0.18 ± 0.03 s^{-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 *in vivo* results.

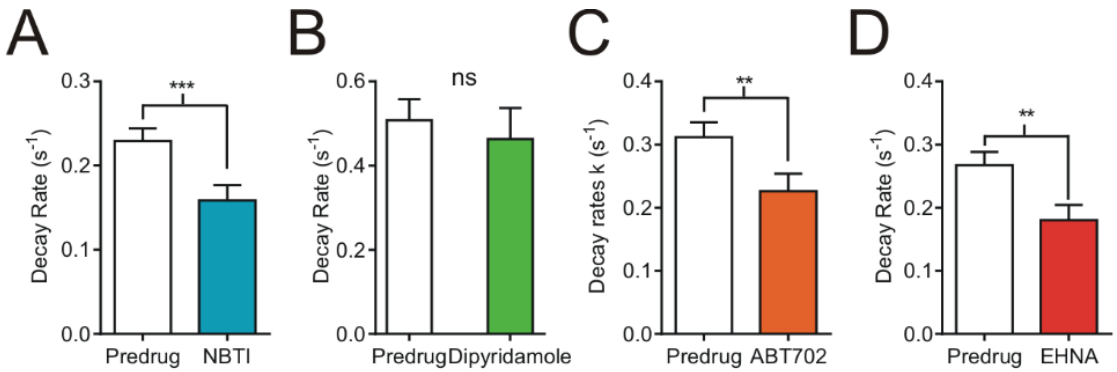

Figure 3.5 Clearance rate of exogenously applied adenosine during inhibition

(A) The clearance rate of applied adenosine is shown before (white) and after NBTI application (blue). Slices were bathed in normal aCSF and adenosine was picospritzed onto the brain slices. Clearance rates were fit from the peak maximum to 90% of baseline with a single exponential curve: $[AD](t) = [AD]_{max}e^{-kt}$. Slices were then bathed in aCSF with NBTI (10 µM) and duration and clearance was measured. The clearance rate decreased (paired *t*-test, n = 4, *p* = 0.0002). (B) Dipyridamole (10 µM), inhibitor of ENT1/2, effects on clearance rate. Dipyridamole (green) did not have an effect on clearance rate from (n = 6, paired *t*-test, *p* = 0.5209). (C) ABT-702, adenosine kinase inhibitor (100 nM) significantly decreased the clearance rate of exogenously applied adenosine (n = 5, paired *t*-test, *p* = 0.0053). (D) The adenosine deaminase inhibitor, EHNA (20 µM, red) significantly decreased the clearance rate from = 4, paired *t*-test, *p* = 0.0036).

3.3 Discussion

Recently, rapid adenosine release has been discovered that only lasts for a few seconds. Electrically stimulated adenosine release and mechanically stimulated release can last for 20 seconds (Cechova and Venton, 2008;Pajski and Venton, 2013;Ross and Venton, 2014) while spontaneous adenosine release *in vivo* and in brain slices is even faster, lasting less than 3 seconds (Street et al., 2011;Nguyen et al., 2014). The short duration of adenosine in these studies implies that adenosine can be both rapidly released and cleared from the extracellular space. Here, our studies in brain slices found that adenosine is cleared from the extracellular space at a maximum velocity of 1.4 µM/s in the striatum. Thus, adenosine is cleared rapidly and does not remain in the extracellular

space for a long time for signaling. This rate accounts for all forms of clearance and is comparable to maximal rates of clearance for some volume neurotransmitters *in vivo*. For example, dopamine is cleared from the extracellular space at 0.2-5 µM/s, depending on the brain region (Wightman et al., 1988;Zahniser et al., 1999;Sabeti et al., 2002) and serotonin clearance is about 0.5 µM/s in the hippocampus (Daws and Toney, 2007). The similar rates of clearance to other volume neurotransmitters is not surprising as the duration of adenosine transients, around 3-4 s, is similar to the duration of dopamine transients that underlie social behaviors (Robinson et al., 2002).

The main sources of clearance *in vivo* are transporters, metabolism, and diffusion. Diffusion is generally not fast enough on a 3 s time scale to cause complete clearance, as tissue is tortuous and thus diffusion is restricted (Sykova and Nicholson, 2008;Taylor et al., 2013). For volume transmitters such as dopamine, the main source of fast clearance is active transporters and metabolism plays an only minor role (Jones et al., 1998;Budygin et al., 1999). However, our studies show that clearance of rapid adenosine is much more balanced, with contributions by both equilibrative nucleoside transporters and metabolism.

3.3.1 Transporters

There are two types of nucleoside transporters: equilibrative nucleoside transporters (ENTs), which carry molecules across a membrane either direction following a concentration gradient, and concentrative nucleoside transporters (CNTs), which are sodium dependent and move nucleosides against a gradient (Thorn and Jarvis, 1996;Noji et al., 2004). Most studies of adenosine transport have been 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 and is distributed throughout the rat brain and present in the striatum (Anderson et al., 1999;Guillen-Gomez et al., 2004). ENT2 is

found in the rat brain and is insensitive to NBTI (Lu et al., 2004). ENT3, is located intracellularly (Baldwin et al., 2005) and is crucial for lysosomal recycling of nucleotides (Hsu et al., 2012) but is not relevant for extracellular clearance. ENT4 was found in human and mouse tissues (Hyde et al., 2001;Baldwin et al., 2004); however selective inhibitors are not widely available (Wang et al., 2013).

NBTI is a potent inhibitor of ENT1 with a K_m in the range of 0.1 to 1.0 nM (Thorn and Jarvis, 1996) but is less effective at binding ENT2, with a K_m of 1 μM (Belt and Noel, 1985). Dipyridamole inhibits both ENT1 and ENT2, although dipyridamole is less sensitive for ENT2 than ENT1 (Hammond, 1991;Belt et al., 1993). In humans, dipyridamole has a K_m of 5.0 nM for ENT1 and 360 nM for ENT2 (Ward et al., 2000). NBTI had a significant effect on the duration and the exponential rate of clearance *in vivo* (1 mg/kg, i.p.) and the clearance rate in brain slices (10 μM). The duration of spontaneous adenosine transients increased by about 0.3 sec after NBTI, showing that ENT1 can act quickly to clear adenosine from the extracellular space. However, NBTI did not change the concentration or frequency of transients which means that transient released adenosine is not being release through ENT1. In contrast, dipyridamole did not change the duration of adenosine *in vivo* (10 mg/kg, i.p.). The exponential clearance rates of adenosine did significantly decrease *in vivo*, but remained unchanged in brain slices. The different clearance rate might be due to the blockade of ENT1 and the fact that adenosine clearance rates were only measured in a specified concentration range (150-250 nM). Dipyridamole is less potent for ENT1 than NBTI and it is 2.8 fold more potent for ENT1 than ENT2 (Ward et al., 2000); however, at the given dose it likely does not block enough ENT1 or ENT2 to have an effect on duration. Thus, ENT1 is responsible for some clearance of transient adenosine and better, more potent drugs are needed to evaluate ENT2.

3.3.2 Adenosine metabolism

The two primary routes of adenosine metabolism are through adenosine deaminase and adenosine kinase. (Cristalli et al., 2001;Latini and Pedata, 2001). Adenosine deaminase expression in the brain is correlated with adenosine uptake sites, showing both mechanisms are responsible for adenosine clearance (Nagy et al., 1985). In the brain, adenosine deaminase is anchored to cell surfaces (Trams and Lauter, 1975;Franco et al., 1986;Franco et al., 1997), demonstrating extracellular activity of the enzyme. Inhibition of adenosine deaminase, with either EHNA or deoxycoformycin, increased basal concentrations of adenosine in the brain (Ballarin et al., 1991;Pazzagli et al., 1995). During ischemic events, adenosine deaminase inhibition increased adenosine levels in the brain (Phillis et al., 1991) and heart (Sandhu et al., 1993). Here, EHNA, a specific inhibitor of adenosine deaminase, increased the duration of adenosine *in vivo*. The decay rate also decreased which shows that adenosine deaminase is responsible for the 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 is known to modulate 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, we inhibited adenosine kinase with ABT-702 to determine its effect on the clearance of transient adenosine release and found the duration increased and the clearance rate decreased. The increase in duration and decrease in clearance rate was similar to that of adenosine deaminase, 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. This can be explained by a couple of hypotheses. First, adenosine kinase may increase release by preventing rapid breakdown of adenosine (Cechova and Venton, 2008). However, ENT1/2 inhibition had similar effects on duration but no effect on concentration, so this mechanism seems unlikely. Second, the difference in 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., 1986). Thus, inhibiting adenosine kinase would increase intracellular levels of adenosine which would allow more adenosine to be available for release.

3.3.3 Relative effects of transporters and metabolism on clearance

In this study, inhibition of ENT1 increased the duration of spontaneous adenosine release by 0.3 s, while inhibition of adenosine deaminase increased it by 0.4 s and inhibition of adenosine kinase by 0.4 s. Thus, the duration increase by all three mechanisms was relatively similar. This shows that there is a balance of rapid adenosine clearance by both equilibrative nucleoside transporters and metabolism and that one mechanism does not appear to dominate. There could be additional mechanisms of clearance as well. Other neurotransmitters that are tightly regulated are typically cleared by active transporters and concentrative nucleoside transporters (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 when tools become available. Diffusion is likely to play some role in clearance, but diffusion is too slow to completely clear adenosine in 3 s. Other metabolic pathways exist, particularly intracellular pathways to S-adenosylhomocysteine (SAH), but these would require

clearance by transporters first. Previous studies show that adenosine release does not come from SAH hydrolysis under physiological or ischemic conditions in the brain (Pak et al., 1994;Latini et al., 1996), 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. Multiple mechanisms that all have similar effects on clearance might imply 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 likely only diffuses on the scale of ~10 µm (Venton et al., 2003). Therefore, multiple clearance mechanisms for adenosine facilitate tight regulation and control of extracellular adenosine available for neuromodulation.

3.4 Conclusions

These studies show three important pathways for adenosine clearance that can regulate the amount of adenosine present during transient signaling. There are at least three pathways of clearance for adenosine: ENT1, adenosine deaminase, and adenosine kinase. Understanding the regulation of adenosine on a fast time scale is critical, as the clearance mechanism and timeframe determine how long adenosine is available for signaling. These studies show that adenosine concentrations in the extracellular space are tightly regulated and adenosine is most likely regulating neurological processes on a rapid time scale.

3.5 Experimental Procedures

3.5.1 Chemicals and Drugs

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise noted. Phosphate buffered saline (PBS) was composed of (in mM): NaCl (Avantor Performance Materials, Center Valley, PA, USA) (131.25), NaH_2PO_4 (10.0), KCl (Fisher Scientific, Fair Lawn, NJ, USA) (3.0), Na_2SO_4 (Fisher) (2.0), $CaCl_2$ (1.2), $MgCl_2$ (Fisher) (1.2). PBS solution was pH adjusted to 7.4. All aqueous solutions were made with deionized water (Milli-Q Biocel, Millipore, Billerica, MA, USA). Adenosine was prepared daily in PBS from a 10 mM stock solution in 0.1M $HClO_4$.

S-(4-Nitrobenzyl)-6-thioinosine (NBTI) was dissolved in saline (Baxter; Deerfield, IL, USA) and DMSO (Amresco; Solon, OH, USA) injected i.p. at 1mg/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, USA) dissolved in DMSO and injected i.p. at 10 mg/kg. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was dissolved in saline and injected i.p. at 10 mg/kg. 5-(3-Bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*]byrimidin-4-amine dihydrochloride (ABT-702) was dissolved in DMSO and injected i.p. at 5 mg/kg.

3.5.2 Electrodes and Electrochemistry

Carbon-fiber cylinder microelectrodes were prepared as previously described (Huffman and Venton, 2009). T-650 carbon-fibers (7 um diameter, Cytec Engineering Materials, West Patterson, NJ, USA) were pulled in glass capillaries (A-M Systems Inc., Seqium, WA, USA) and cylinders were cut between 75-125 µm 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, USA). Electrodes were scanned from -0.40 V to 1.45 V and back at 400 V/s at 10 Hz against a Ag/AgCl reference electrode. Electrodes were post-calibrated with 1.0 µM adenosine after each experiment.

3.5.3 Animal Experiments

Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia. Male Sprague-Dawley Rats (250-350g) were anesthetized with a 50% wt urethane and saline solution (1.5 mg/kg i.p.) prepared daily. After shaving the surgical site and injecting 250 µL of bupivacaine (Sensorcaine, MPF, APP Pharmaceuticals, LLC; Schaumburg, IL, USA) subcutaneously, the skull was exposed. Adenosine transients were measured in the caudate-putamen, measured in mm from bregma: AP: 1.2, ML: 2.0, DV: 4.5 (Paxinos and Watson, 2007). The reference electrode was placed on the contralateral side of the brain and the rat's body temperature was maintained with a heating pad and thermistor probe (FHC; Bowdoin, ME, USA).

For brain slice experiments the protocol was used as described previously (Pajski and Venton, 2010;Ross et al., 2014). Briefly, rats were anesthetized with isoflurane (1 mL/100 g rat weight) and beheaded. The brain was extracted and placed in 0–5°C aCSF for 2 min for recovery. 400 µm slices of the caudate–putamen were prepared using a vibratome (LeicaVT1000S, Bannockburn, IL, USA) and transferred to oxygenated aCSF (95% oxygen, 5% CO_2), to recover for an hour. Artificial cerebral spinal fluid (maintained at 35–37°C) was pumped over the brain slices at 2 mL/min (Watson-Marlo 205U, Wilmington, MA, USA). The medial caudate–putamen coordinates are approximately (in mm from bregma): +1.2 AP, +2.0 ML, and −5.5 DV. The carbon-fiber electrode was inserted 50 µm into the tissue of the medial caudate–putamen and the picospritzing pipette

was placed 20–30 μm from the working electrode. Various amounts of adenosine were pressure ejected onto brain slices using a Parker Hannifin picospritzer (Picospritzer III, Cleveland, OH, USA). Short puff time at low pressuress were used to minimize tissue damage. The ejection parameters were 5-20 psi for 5-1800 ms with 0.7-11 μM adenosine detected at the electrode. High concentrations of exogenously-applied adenosine were needed to reach maximal clearance rates. The duration of exogenously applied adenosine was not measured as various puff times and pressures were used to create a Michaelis-Menten curve. Also, the distance from the pipette and slice was not always precisely the same, thus the amount of adenosine applied with the same parameters varied from slice to slice.

3.5.4 Data Collection and Analysis

Electrodes were allowed to equilibrate *in vivo* for at least an hour and a half in order to get a steady baseline. If transients were not seen within the first hour of equilibration, another electrode was inserted until transients were found. If the frequency of spontaneous transient adenosine was below five per hour, a new electrode was used. Two hours of pre-drug and two hours of post-drug were collected, however only the second hour of pre-drug and first hour of post-drug were used due to electrode stability. To calculate concentration and duration, principal component analysis was used in the High Definition Cyclic Voltammetry (HDCV) Analysis software (Nguyen et al., 2014). Using PCA, the smallest transients that can be detected are 40 nM.

We examined the exponential decay rates of transient adenosine release in the 150 to 250 nM range. Transients less than 150 nM have small peak signals, which can be obscured by baseline noise. The upper limit was set at 250 nM so the pre-drug and post-drug have similar numbers of transients for comparison. Curves were fit with a one-

phase exponential decay with the equation: $y = y_0\, e^{-kx}$ in GraphPad PRISM 6, (GraphPad Software Inc., San Diego, CA, USA). For the velocity vs concentration graph, the concentrations were placed into bins (Sabeti et al., 2002).

3.5.5 Statistics

Statistics were performed using MatLAB (The MathWorks, Inc., Natick, MA, USA) and GraphPad PRISM 6. Data shown as mean ± SEM with *n* number of animals. One-way 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 inter-event time distributions. All data was considered significant at the 95% confidence level.

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Chapter 4 Modulation of fast acting spontaneous adenosine release is NMDA and A_{2a} receptor dependent but not AMPA or GABA receptor dependent

"Scientific research is one of the most exciting and rewarding of occupations"

Frederick Sanger

"Science is a wonderful thing if one does not have to earn one's living at it"

Albert Einstein

4 Abstract

Adenosine is a neuroprotective agent that regulates neurotransmission with glutamate and other neurotransmitters. Spontaneous, transient adenosine is a mode of signaling where adenosine is cleared from the extracellular space in less than three seconds. Although fast acting adenosine has been characterized, the regulation of spontaneous adenosine release is not completely understood. Here, we examined the effect of NMDA, AMPA, metabotropic glutamate, $GABA_A$, $GABA_B$, and A_{2a} receptors on the concentration and frequency of spontaneous, transient adenosine release. None of the drugs administered affected the concentration of adenosine release. The NMDA antagonist, CPP, increased the frequency of adenosine release events but the agonist NMDA had no effect. In contrast, the AMPA antagonist, NBQX, and the $GABA_A$ antagonist, bicuculline, did not change the frequency. Thus, only one ionotropic receptor, NMDA, regulated adenosine release. The metabotropic mGlu2/3 glutamate receptor antagonist (LY 341495) and $GABA_B$ antagonist (CGP 52432) did not affect adenosine release. Thus, none of the metabotropic heteroreceptors regulated release. The A_{2a} antagonist, SCH 442416, increased the frequency of adenosine release but the A_{2a} agonist, CGS 21680, had no effect. A_{2a} receptors self-regulate adenosine release and have an opposite effect than previously characterized regulation by A_1 receptors. The interaction between spontaneous, transient adenosine and the NMDA receptor demonstrates a moderate feedback loop between adenosine and glutamate receptors.

4.1 Introduction

Adenosine is a well-known neuromodulator in the brain, regulating neurotransmission. Recently, our laboratory discovered a rapid mode of adenosine signaling that could have a rapid neuromodulatory effect in brain slices (Ross and Venton, 2014). Transient release of adenosine can be electrically-stimulated or mechanically stimulated and lasts just a few seconds (Cechova and Venton, 2008;Pajski and Venton, 2010;Ross et al., 2014). In addition, spontaneous transient adenosine release has been observed in both slices and *in vivo* (Street et al., 2011;Nguyen et al., 2014). The duration is only three seconds and blocking A_1 receptors increases the frequency of adenosine transients (Nguyen et al., 2014). However, the regulation of these spontaneous transients by other receptors is not well understood.

Adenosine regulates the release of neurotransmitters such as glutamate and GABA, but its release can be regulated by these same neurotransmitters. For example. NMDA evoked adenosine release in brain slices over a ten minute window, with the maximum release at 2.5 minutes (Hoehn et al., 1990). A decrease in neuromodulation following NMDA application is due to the release of adenosine acting specifically through the A_1 receptor (Manzoni et al., 1994). In preconditioned mice (24 hours), there is cross-talk between the glutamatergic and adenosinergic system during neuroprotection (Constantino et al., 2015). Similarly, GABA and glutamate application to brain slices evoked adenosine release (Hoehn and White, 1990). For rapid release, Dale's group has studied the effects of glutamate and GABA on both train-evoked and single pulse evoked release in the cerebellum (Wall and Dale, 2007). Train-evoked adenosine release was independent on NMDA and AMPA receptors. On the other hand single pulse evoked adenosine was dependent on NMDA and AMPA receptors. Single pulse adenosine release was also dependent on metabotropic receptors $GABA_B$ and mGlu4 in cerebellar brain slices (Klyuch et al., 2012). Thus there are different mechanisms of regulation for

single pulse and pulse trains of stimulated adenosine (Klyuch et al., 2011). Our group found that electrically-stimulated, rapid adenosine release was ionotropic glutamate dependent in the striatum (Pajski and Venton, 2013). These studies show that regulation of adenosine by ionotropic and metabotropic glutamate and GABA receptors differs by region and type of release.

Adenosine receptors self-regulate adenosine release. A_1 receptors are inhibitory and regulate both train-evoked and single pulse-evoked adenosine in the cerebellum (Klyuch et al., 2012). In the striatum, A_1 receptors have some auto-receptor characteristics and regulate electrically-stimulated adenosine release (Cechova et al., 2010). A_1 receptors also regulate the frequency of spontaneous, transient adenosine release *in vivo* (Nguyen et al., 2014). A_{2a} receptors are located on glutamatergic terminals (Corsi et al., 2000) and A_{2a} and metabotropic glutamate receptors are co-localized presynaptically (Tebano et al., 2005;Bogenpohl et al., 2012). The regulation of spontaneous, transient adenosine by A_{2a} receptors has not been characterized.

Here we examine the effects of glutamate, GABA, and A_{2a} receptors on spontaneous, transient adenosine release. We found that NMDA and A_{2a} receptors modulate the frequency of spontaneous adenosine release, while AMPA, $GABA_A$, $GABA_B$, and group II metabotropic glutamate receptors do not. None of the drugs administered had any effect on the concentration of adenosine release demonstrating that the amount of adenosine release is not regulated by any of these receptors. Understanding the receptor modulation of adenosine release is key to future studies and treatments that control spontaneous, transient release. Identifying receptors that regulate spontaneous, transient adenosine will facilitate the neuromodulatory properties of adenosine by controlling rapid adenosine release.

4.2 Methods

4.2.1 Chemicals and Drugs

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise noted. Phosphate buffered saline was adjusted to pH 7.4 and was made with sodium chloride (NaCl, 131.25 mM) (Avantor Performance Materials, Center Valley, PA, USA), sodium phosphate (NaH_2PO_4, monohydrate, 10.0 mM), potassium chloride (KCl, 3.0 mM) (Fisher Scientific, Fair Lawn, NJ, USA), Sodium sulfate (Na_2SO_4, anhydrous, 2.0 mM) (Fisher), calcium chloride, ($CaCl_2$, dihydrate, 1.2 mM), magnesium chloride ($MgCl_2$, hexahydrate, 1.2 mM) (Fisher).

All drugs administered to rats were purchased from Tocris Biosciences (Ellisville, MO, USA) unless otherwise noted. All drugs were injected i.p. The A_{2a} agonist CGS 21680 (4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl] benzenepropanoic acid hydrochloride) was dissolved in 1 mL of saline (Baxter; Deerfield, IL, USA) and injected at 3 mg/kg. The A_{2a} agonist SCH 442416 (2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine) was dissolved in 200 µL of DMSO (dimethyl sulfoxide) (Amresco; Solon, OH, USA) and injected at 3 mg/kg. NMDA (*N*-methyl-D-aspartate) was dissolved in 500 µL saline and injected at 50 mg/kg. The NMDA receptor antagonist, CPP (3-((*R*)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid) was dissolved in 200 µL saline and injected at 6.25 mg/kg. The AMPA antagonist NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide) was dissolved in saline and DMSO (500 µL each) at 15 mg/kg. The group II metabotropic glutamate receptor antagonist LY 341495 ((2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid) was dissolved in saline and DMSO (500 µL) each at 5 mg/kg. The $GABA_B$ receptor antagonist CGP 52432 (3-[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl)phosphinic acid) was dissolved in 2.5 mL DMSO and 1 mL saline and injected at 30 mg/kg. The $GABA_A$ receptor antagonist

bicuculline ([*R*-(*R**,*S**)]-5-(6,8-Dihydro-8-oxofuro[3,4-*e*]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-*g*]isoquinolinium iodide) was dissolved in 500 µL of saline and injected at 5 mg/kg.

4.2.2 Animal Experiments

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) between 250-350 grams were housed in 12/12 hour light/dark cycles and fed *ad libitum*. Rats were anesthetized with urethane (1.5 mg/kg, i.p.) and the fur around the surgical site was shaved. The rat was placed in a stereotaxic frame and 250 µL of bupivacaine (Sensorcaine, MPF, APP Pharmaceuticals, LLC; Schaumburg, IL, USA) was injected subcutaneously. Holes were drilled in the skull for the placement of electrodes (Paxinos and Watson, 2007). The working carbon-fiber microelectrode was placed in the caudate-putamen (in mm from bregma): AP: 1.2, ML: 2.0, DV: 4.5. The Ag/AgCl reference electrode was placed on the contralateral side. The rat's body temperature was regulated with a heating pad and thermistor probe (FHC; Bowdoin, ME, USA). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Virginia.

A working electrode was placed in the caudate-putamen until a steady background with optimal shape was observed. If transients were not seen in the first 30 minutes of equilibration or the optimal shape was not observed, a new electrode was inserted. The electrode was allowed to calibrate for a half hour. If the frequency of transient adenosine release was less than five transients per hour, a new electrode was used. An hour of pre-drug data was collected, then the drug was injected and an hour of post-drug was collected. Principal component analysis or regression was used to calculate concentration and frequency of transient adenosine release (Nguyen et al., 2014). A training set was

created for each animal using the five largest transients in the pre-drug data (Keithley et al., 2009). All transients were above 20 nM and had a delayed secondary peak.

4.2.3 Electrodes and Fast-scan Cyclic Voltammetry

Working carbon-fiber electrodes were prepared as previously described (Huffman and Venton, 2008). Carbon fibers (T-650, Cytec Engineering Materials, West Patterson, NJ, USA) 7 µm in diameter were aspirated into glass capillaries with an outer and inner diameter of 1.2 mm and 0.68 mm, respectively (A-M Systems Inc., Seqium, WA, USA). The capillary was pulled in a vertical pipette puller (model PE-21; Narishige, Tokyo, Japan) into two electrodes and the protruding carbon-fiber cut to 75-125 µm with a scalpel. Electrical connections made by backfilling the electrode with 1 M KCl. The carbon fiber electrodes were soaked in isopropanol for at least 10 minutes prior to use. The silver-silver chloride reference electrodes were made in house by electrodepositing chloride onto a silver wire (Acros Organics) at 2 V.

Fast-scan cyclic voltammetry was used to detect and quantitate adenosine on a sub-second time scale (Swamy and Venton, 2007). The waveform and data collection were computer controlled through High-Definition Cyclic Voltammetry software (from Mark Wightman, UNC Chapel Hill). Data collection was done through a LabView (National Instruments Corporations, Austin, TX, USA) DAQ card connected to a HDCV Breakout Box (UNC, Department of Chemistry Electronics Design Facility, Chapel Hill, NC, USA). The potentiostat used was a Dagan ChemClamp (Dagan Corporation, Minneapolis, MN, USA).

The applied waveform was from -0.40 V to 1.45 V and back at 400 V/s, every 100 milliseconds against a Ag/AgCl reference. Data was background subtracted (10 CVs

averaged) to removed non-Faradaic currents. Electrodes were post-calibrated for 1.0 µM adenosine in phosphate buffered saline immediately following animal experiments.

4.2.4 Statistics

All statistics were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA), MatLAB ® (The MathWorks, Inc., Natick, MA, USA). The data are presented as normalized mean ± SEM. A Kolmogorov-Smirnov (KS) test was used to determine underlying distributions between inter-event times (time between consecutive transients). All data are considered significant at the 95% confidence level.

4.3 Results

4.3.1 Adenosine detection with fast-scan cyclic voltammetry

Adenosine release was measured on a rapid time scale using fast-scan cyclic voltammetry (FSCV) and spontaneous events were monitored *in vivo* of the rat brain (Nguyen et al., 2014). Carbon-fiber microelectrodes were inserted into the striatum of the rat brain and a waveform from -0.40 V to 1.45 V and back to -0.40 V at 400 V/s at a frequency of 10 Hz was continuously applied. Applying the waveform causes a large background current, which is subtracted out, thus FSCV is a differential technique to measure rapid changes in concentration on a sub-second time scale. Adenosine undergoes a set of two electron oxidations (Dryhurst, 1977) at the microelectrode that results in a current that is proportional to the concentration of adenosine at the electrode (Swamy and Venton, 2007).

A cyclic voltammograms (CVs) for adenosine (Figure 4.1A) shows the characteristic electrochemical fingerprint for adenosine with a primary oxidation at 1.4 V and a secondary oxidation at 1.0 V. Three dimensional color plots are commonly plotted

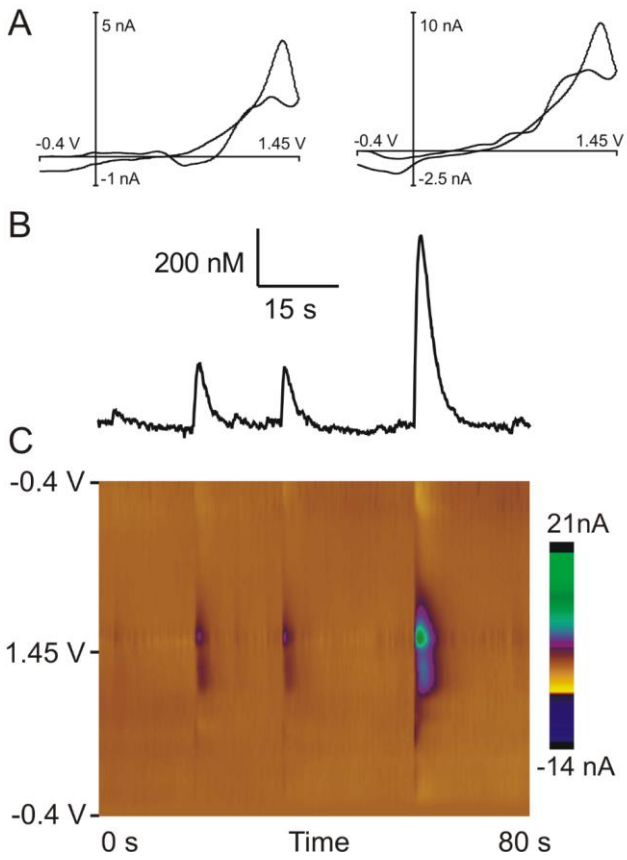

Figure 4.1 Spontaneous, transient adenosine release

A) Cyclic Voltammogram (CV) of spontaneously release adenosine *in vivo*. The primary oxidation of adenosine occurs at 1.4 V and the secondary peak is at 1.0 V. B) Concentration vs time trace collected *in vivo* over an 80 second window. The concentrations were calculated from calibrated *in vitro* values using principal component analysis. C) 3-D color plot of three example spontaneous events *in vivo*. The CV on the left (from A) is from the transient event around 15 seconds and the CV on the right is from the large event around 65 seconds.

with FSCV to show multiple CVs in a single image. Three spontaneous adenosine release events are shown in Figure 4.1C. The green/purple circles in the center of the 3-D plot are the primary oxidation peaks, while the green/purple circles directly below at 1.0 V are the secondary oxidation peaks for adenosine. Using Principal Component Analysis (PCA), a concentration vs time plot is calculated and displayed directly above the color plots (Figure 4.1B) (Heien et al., 2004). The data show that large adenosine events are released and rapidly cleared from the extracellular space in less than five seconds.

4.3.2 Control Experiments

To examine any experiment effects from the procedure, DMSO, was injected in sham rats. The concentrations were placed into 30 minute bins in order to determine the effect of time (Figure 4.2A) with the pre-drug values in white bars and the post-DMSO injection concentration in gray bars. There was no significant difference between the concentration (one way ANOVA, n = 6 rats, p = 0.6955). Similarly, the inter-event time distribution did not change following DMSO injection (Figure 4.2B, KS test, p = 0679). Thus, frequency and concentration of adenosine transients do not change with time and any changes observed in drug experiments can be attributed to the drug.

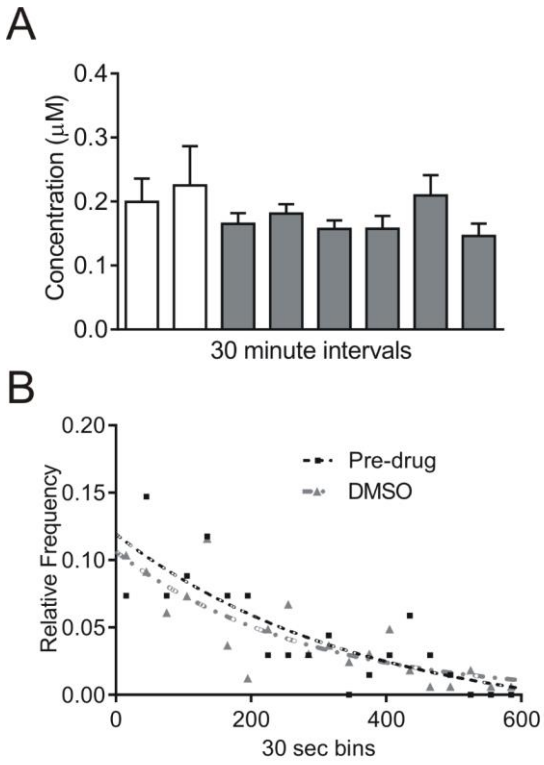

Figure 4.2 Control experiment with DMSO

A) Concentration of spontaneous adenosine release placed into 30 minute bins. Pre-drug values shown in white bars and post-DMSO injection is in gray bars. There was no significant change in concentration over time (n = 6 rats, p = 0.6955). B) Inter-event histogram with exponential decays fit for pre-drug (black) and post-DMSO (gray) show there is not significant change in underlying distribution (KS test, p = 0.679).

4.3.3 Glutamate NMDA receptors

CPP, a selective NMDA receptor antagonist, was injected at 6.25 mg/kg, i.p. a dose where it was previously established to be an effective antagonist in the brain (Lehmann et al., 1987;Starr and Starr, 1993). CPP did not significantly alter adenosine concentration (Figure 4.3A, unpaired *t*-test, $n = 6$ rats, $p = 0.1959$). Inter-event times, or the time between consecutive events, were examined to determine the effect of drugs on frequency. The inter-event times were placed into 30 second bins and the underlying distributions were compared before and after drug administration (Figure 4.3B&C). The NMDA receptor antagonist CPP significantly increased the frequency of transient adenosine release. CPP significantly changed the inter-event time distribution (KS test, $p = 0.0035$) and decreased the average time between consecutive events from 66 ± 5 seconds to 46 ± 5 seconds (unpaired t-test, $p = 0.0041$). Example data show the difference between pre-drug (Figure 4.3D) and post-CPP transients (Figure 4.3E). A corresponding color plot for pre-drug data shows five transients in an 80 second window, while nine transients are shown in a color plot following CPP administration. Concentration vs time traces are above the color plots.

Since the NMDA antagonist had an effect, we tested NMDA, a specific agonist of the NMDA receptor. The dose was 50 mg/kg i.p., a dosage low enough to not cause death or seizures (Leander et al., 1988;Irifune et al., 1995), but large enough to see effects in the brain (Guertin, 2004). NMDA did not significantly change the concentration from pre-drug values (Fig. 3A, unpaired *t*-test, $n = 6$ rats, $p = 0.2393$). NMDA did not significantly change the underlying distribution of inter-event times (Fig. 3C, KS test, $p = 0.5518$). Thus the antagonist increased the frequency of transient adenosine release, while the agonist did not have an effect on frequency.

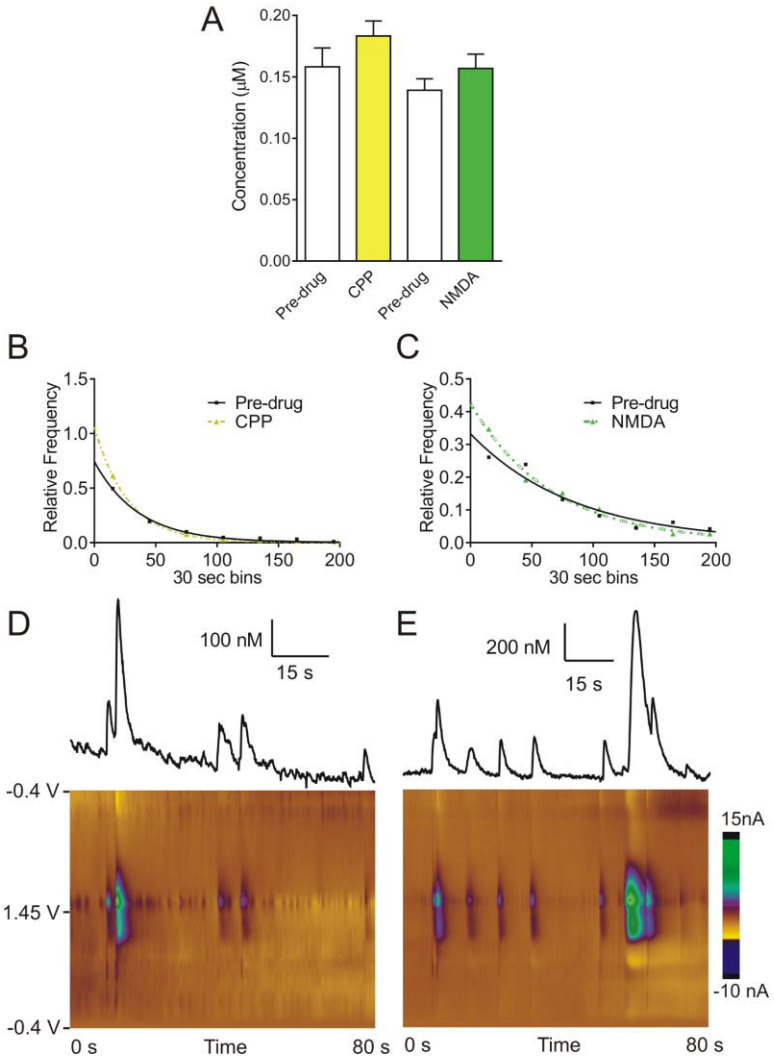

Figure 4.3 The effect of NMDA antagonist and agonist on transient adenosine release

A) Concentration before and after CPP (antagonist, 6.25 mg/kg, i.p.) and NMDA (agonist, 50 mg/kg, i.p.). Compared to the pre-drug value, CPP (yellow) and NMDA (green) do not significantly change the concentration (n = 6 rats, p = 0.1959; and n = 6 rats, p = 0.2393, respectively). B) Inter-event distribution for pre-drug (black squares) and CPP (yellow triangles). The x-axis is the time with the inter-event times placed into 30 second bins. The y-axis is the relative frequency of each time bin. The histogram is fit with an exponential decay for pre-drug (black line) and post-CPP (dashed yellow line). The underlying distribution between pre- and post-CPP is significantly different (KS test, p = 0.0035). C) Inter-event histogram for NMDA (green triangles and dashed line). The pre- and post-drug distributions are not significantly different (KS test, p = 0.5518). D) Example concentration vs time trace with corresponding 3-D color plot for pre-drug. Five transient appear in an 80 second window. E) Conc vs time with 3-D color plot after CPP administration. Nine transients are shown in an 80 second window.

4.3.4 Glutamate AMPA receptors

The AMPA receptor is another ionotropic, non-NMDA sensitive glutamate receptor that mediates synaptic signaling in the brain. NBQX is a selective antagonist of the AMPA receptor that provided potent anti-convulsant protection against seizures around 15 mg/kg i.p. (Chapman et al., 1991). Here we administered NBQX at 15 mg/kg i.p. and saw no change in the concentration of transient adenosine release (Figure 4.4A, unpaired *t*-test, n = 6 rats, p = 0.2568). The inter-event times remained unchanged following NBQX (Figure 4.4B, KS test, p = 0.3920). Since there was no change in frequency following AMPA antagonist administration, no AMPA agonist was examined.

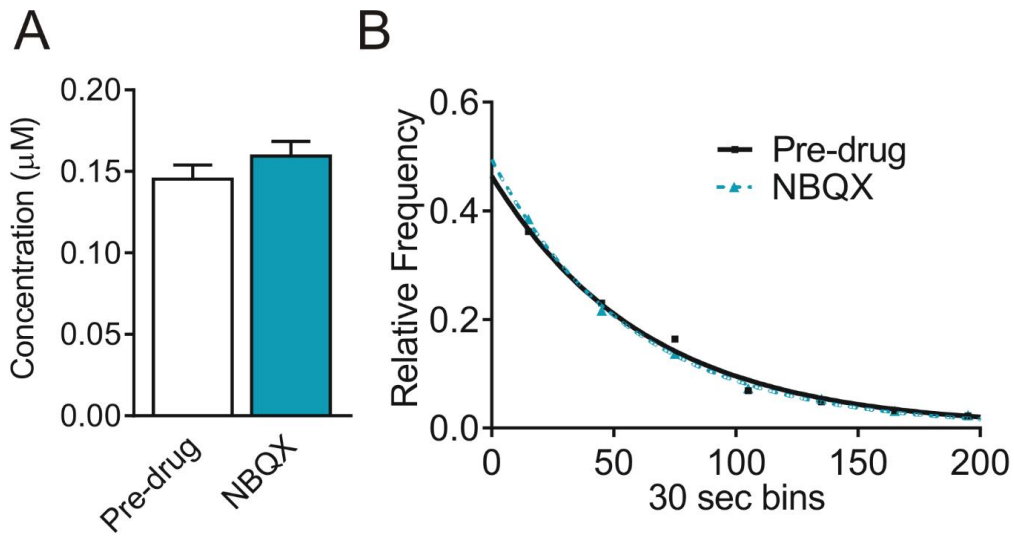

Figure 4.4 The effects of AMPA antagonist NBQX on transient adenosine

A) Concentration before (white) and after NBQX (blue, 15 mg/kg, i.p.). The concentration did not significantly change after AMPA antagonism (n = 6 rats, p = 0.2568). B) Inter-event time histogram. Pre-drug (black) and post-NBQX (blue) inter-event times placed into 30 second bins and there is no significant difference between underlying distributions (KS test, p = 0.3920).

4.3.5 Metabotropic glutamate receptors

There are multiple metabotropic glutamate receptors that are divided into different classes. However, here we investigated group 2 metabotropic glutamate receptors

(mGlu2/3) *in vivo* as both adenosine and mGlu2/3 have shown pre-synaptic inhibition in the CNS (Zhang and Schmidt, 1999). The specific mGlu2/3 antagonist, LY 341495 (LY), increased plasma corticosterone in mice at 1 mg/kg, i.p (Scaccianoce et al., 2003). LY also decreased the number of seizures in epileptic mice at 5 mg/kg i.p. (Ngomba et al., 2005) and thus we chose 5 mg/kg i.p. as our dose. The mGlu2/3 antagonist did not alter the concentration of spontaneous adenosine release (Figure 4.5A, unpaired *t*-test, $n = 6$ rats, $p = 0.8172$). The inter-event distribution following LY did not significantly change (Figure 4.5B, KS test, $p = 0.1411$). Thus, mGlu2/3 receptors do not regulate the frequency or concentration of spontaneous adenosine release.

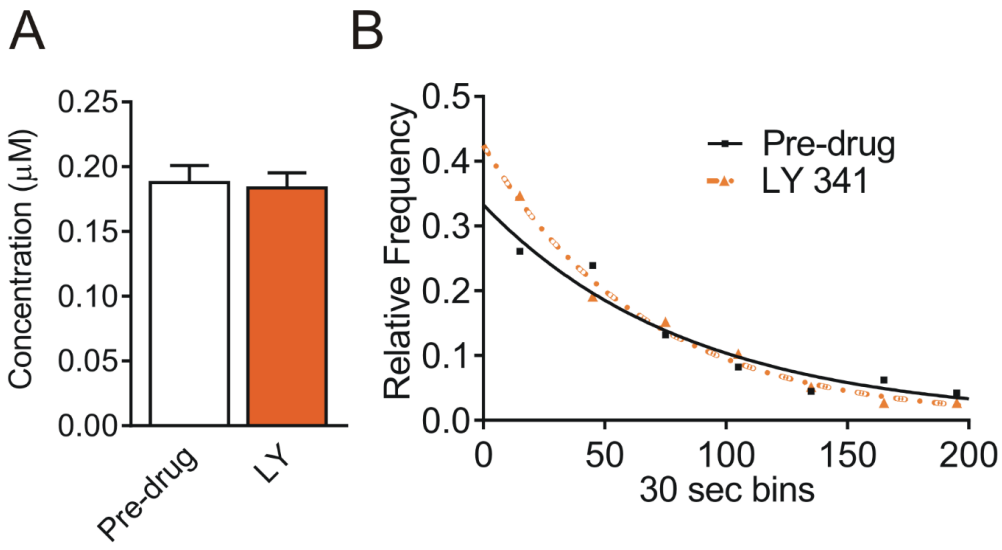

Figure 4.5 The effect of metabotropic glutamate mGlu2/3 receptor antagonist on transient adenosine release

A) Concentration of spontaneous, transient release following mGlu2/3 antagonism with LY 341495 (1 mg/kg, i.p.). The concentration did not significantly change following LY administration ($n = 6$ rats $p = 0.8172$). B) Inter-event histograms before (black) and after (orange) mGlu2/3 antagonism. The frequency or the underlying distribution between the two did not significantly change (KS test, $p = 0.1411$).

4.3.6 $GABA_A$ receptors

There are two main types of GABA receptors: $GABA_A$ receptors which are ionotropic, ligand gated ion channels and $GABA_B$ receptors which are metabotropic

receptors that indirectly open ion channels. The $GABA_A$ receptor antagonist bicuculline induces seizures at 7 mg/kg in adult rats (Zouhar et al., 1989) and so we chose a lower dose, 5 mg/kg i.p. to block the $GABA_A$ receptor without causing seizures. The $GABA_A$ antagonist bicuculline did not have an effect on the concentration of adenosine release (Figure 4.6A, unpaired *t*-test, n = 6 rats, p = 0.6266). The frequency of spontaneous adenosine release did not significantly change after $GABA_A$ receptor antagonism as the inter-event time distribution did not change after bicuculline injection (Figure 4.6B, KS test, n = 6 rats, p = 0.7537).

4.3.7 $GABA_B$ Receptors

The $GABA_B$ antagonist CGP 52432 (CGP) has been used previously in brain slices and it inhibited glutamate receptor-dependent adenosine release (Klyuch et al., 2012). Injected i.p. at 30 mg/kg, CGP showed anti-depressant properties in mice (Felice et al., 2012), thus we used CGP at 30 mg/kg i.p. The $GABA_B$ antagonist, CGP, did not significantly change the adenosine concentration (Figure 4.6C, unpaired *t*-test, n = 6 rats, p = 0.6836). Similarly, the inter-event distribution did not change after $GABA_B$ antagonism (Figure 4.6D, KS test, p = 0.4092), thus $GABA_B$ receptors do not regulate adenosine release.

4.3.8 A_{2a} receptors

We examined the effect of A_{2a} receptors on the concentration and frequency of release. SCH 442416 (SCH) is an A_{2a} antagonist that was administered at 3 mg/kg, a dose that previously increased locomotor activity (Lerner et al., 2010;Orru et al., 2011). The A_{2a} antagonist, SCH, had no effect on the concentration of spontaneous transient adenosine release. The concentration did not significantly change for SCH (Figure 4.7A,

unpaired *t*-test, *n* = 7 rats, *p* = 0.6171). SCH also decreased the underlying inter-event time distribution (Figure 4.7B, KS test, *p* = 0.0221).

At 3 mg/kg i.p., CGS 21680 (CGS) is an A_{2a} agonist that previously increased the affinity of D_2 receptors for dopamine in the rat brain (Hillefors-Berglund et al., 1995) and at lower doses decreased alcohol self-administration (Houchi et al., 2013). Thus, CGS

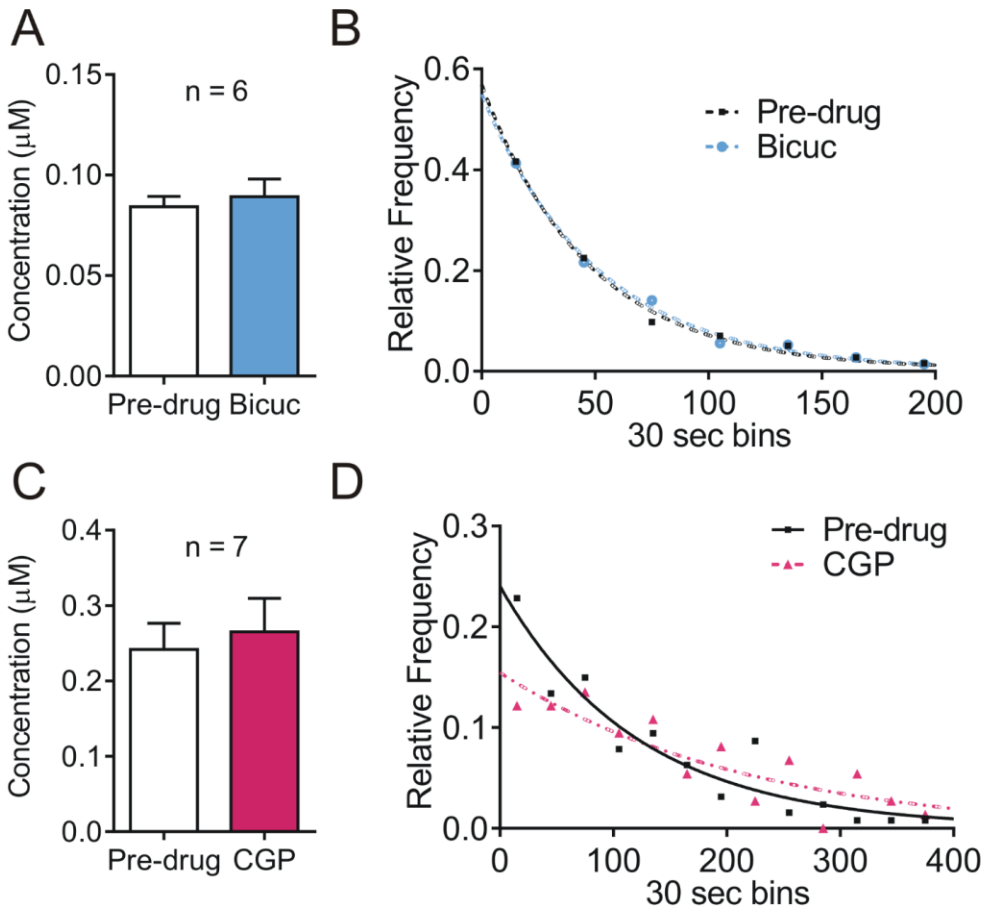

Figure 4.6 The effects of GABA receptor antagonists on spontaneous adenosine release

A) Before and after concentrations of $GABA_A$ antagonist, bicuculline (blue, 5 mg/kg, i.p.) and $GABA_B$ antagonist, CGP 52432 (pink, 30 mg/kg, i.p.). The pre-drug and post-bicuculline concentration values are not significantly different (*n* = 6 rats, *p* = 0.6266) nor are the values after CGP administration (*n* = 6 rats, *p* = 0.6836). B) Inter-event histograms for pre-drug (black) and post-bicuculline (blue) fit with exponential decays. The underlying distributions are not significantly different (KS test, p = 0.6711). C) Inter-event times for $GABA_B$ antagonism with CGP are not significantly different than pre-drug values (KS test, *p* = 0.4092).

was administered at 3 mg/kg, i.p. The transient adenosine concentration did not significantly change following CGS administration (Figure 4.7C, unpaired t-test, n = 6 rats, p = 0.1932). CGS had no effect on the frequency of transient adenosine release (Figure 4.7D, KS test, p = 0.7975). Thus, transients occur less frequently with blockade of A_{2a} receptors.

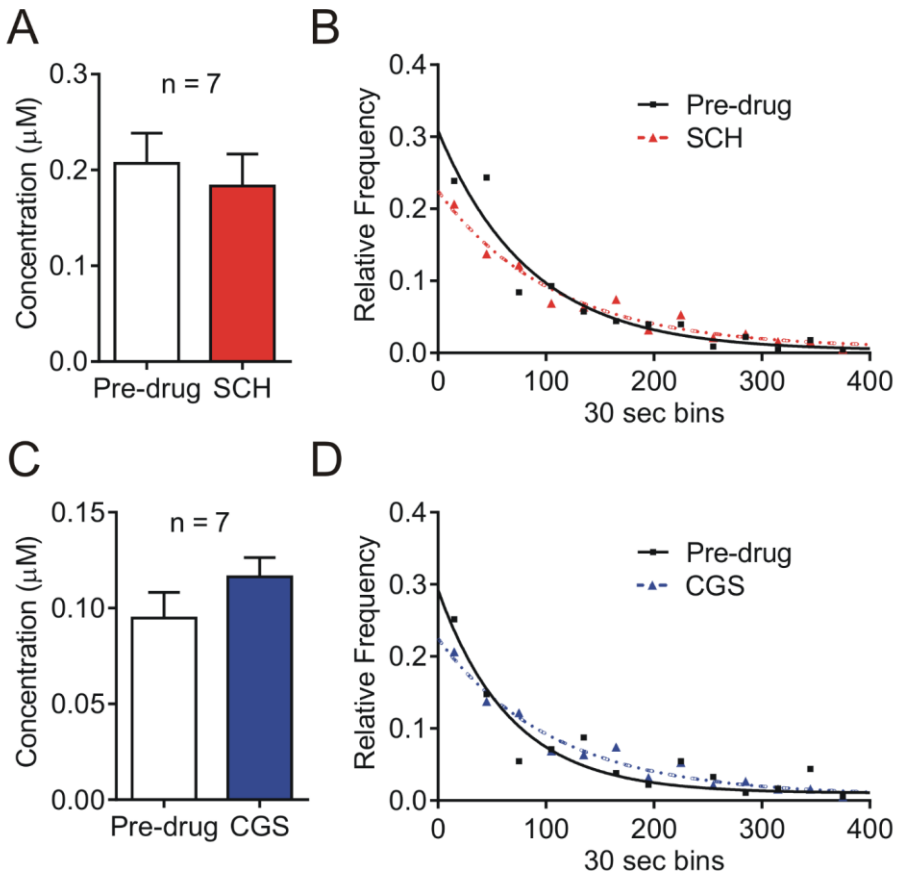

Figure 4.7 The effect of adenosine A2a receptor antagonist and agonist on transient adenosine

A) The concentration of spontaneous adenosine before and after A_{2a} antagonism (SCH 442416, 3 mg/kg, i.p., red) and agonism (CGS 21680, 3mg/kg, i.p., blue). There was no significant difference following SCH administration (n= 6 rats, p = 0.6171) or following CGS administration (n = 6 rats, p = 0.1932). B) Inter-event histogram with inter-event times placed into 30 second bins before (black) and after SCH (red). The underlying distribution was significantly different (KS test, p = 0.0221), with the frequency of spontaneous events increasing post-drug. C) Inter-event histograms with exponential decay for pre-drug (black) and post-drug (blue) were not significantly different following CGS administration (KS test, p = 0.7975).

4.4 Discussion

We investigated the role of ionotropic and metabotropic receptors in modulating spontaneous, transient adenosine release in the striatum. Spontaneous release of adenosine was identified in spinal cord slices of mice (Street et al., 2011) and the caudate-putamen and prefrontal cortex of anesthetized rats (Nguyen et al., 2014) suggesting an important signaling pathway conserved across species and locations. We studied ionotropic and metabotropic glutamate and GABA receptors as well as A_{2a} receptors. Spontaneous adenosine release is not ionotropic glutamate receptor dependent, as blocking NMDA or AMPA receptors did not decrease release. However, the NMDA antagonist, CPP, increased the frequency of release events demonstrating a feedback mechanism between adenosine and glutamate. The frequency of spontaneous adenosine release is not dependent on $GABA_A$ or $GABA_B$ receptors or metabotropic glutamate mGlu2/3 receptors. An A_{2a} receptor antagonist decreased the frequency of release demonstrating autoregulation of adenosine. Previous studies had also found that A_1 receptor antagonist increases release (Nguyen et al., 2014), so both A_1 and A_{2a} receptors regulate adenosine. Thus our work shows (1) that glutamate and adenosine are tightly regulated in a feedback loop involving NMDA receptors, and (2) that adenosine receptors are important for self-regulation. Transient adenosine release can quickly modulate neurotransmission (Ross and Venton, 2014), so understanding how transient adenosine signaling is regulated will facilitate future work in manipulating the neuromodulatory properties of adenosine.

4.4.1 Ionotropic receptors

Adenosine and glutamate have a concomitant relationship in the brain, where inhibitory effects of adenosine modulate the effects of glutamate and excitatory

glutamatergic receptors can cause release of adenosine. For example, adenosine inhibits excitatory glutamatergic transmission in the hippocampus (Masino et al., 2002) and in the cerebellar cortex (Kocsis et al., 1984). Similarly, glutamate administration also modulates adenosine release, as the application of NMDA to slices invokes a release of adenosine that acts at inhibitory A_1 receptors (Manzoni et al., 1994;Ferguson and Stone, 2010). However, not all studies have shown a correlation between adenosine and glutamate. Electrically-stimulated, transient adenosine release was partially dependent on ionotropic glutamate receptors in the striatum, but was independent of them in the hippocampus, nucleus accumbens, and cortex (Pajski and Venton, 2013). Similarly, train-evoked adenosine release was also independent of NMDA and AMPA receptors in cerebellar rat brain slices (Wall and Dale, 2007).

In this study spontaneous, transient adenosine release is not ionotropic glutamate receptor dependent because blocking NMDA or AMPA receptors did not decrease the amount of transient adenosine released. The previous studies that found adenosine release was dependent on activation of NMDA or AMPA receptors, examined electrically-stimulated adenosine release. Spontaneous, transient adenosine release is independent of ionotropic glutamate receptor activation and thus it may be regulated differently than electrically-stimulated release.

Although the amount of adenosine release is independent of ionotropic glutamate receptors activation, the frequency of adenosine release was regulated by NMDA but not AMPA receptors. The NMDA antagonist, CPP, significantly increased the frequency of spontaneous adenosine release demonstrating a possible feedback mechanism between adenosine and glutamate. Inhibiting NMDA receptors causes a robust effect on the central nervous system decreasing the frequency of adenosine release, which is surprising since glutamate generally has an excitatory effect. The pre-synaptic location of NMDA receptors (Krebs et al., 1991;Wang, 1991;Petralia et al., 1994) further supports the hypothesis of a

feedback loop between adenosine and NMDA receptors. Glutamate and adenosine (or ATP, a precursor of adenosine) could be co-localized in vesicles and activation of pre-synaptic NMDA receptors by glutamate might signal to decrease the amount of release. In addition, A_1 and NMDA receptors are co-localized (Rebola et al., 2003;Ciruela et al., 2006) and antagonists of A_1 receptors also increase the frequency of adenosine release (Nguyen et al., 2014). Thus, the increase in frequency due to NMDA receptor antagonists may be due to NMDA receptors co-localized with A_1 receptors, which are inhibitory. Interestingly the agonist NMDA did not have an effect on the frequency of release. The agonist may not be effective because the tonic glutamate level is already sufficient to activate NMDA receptors and the receptors cannot be further activated. Alternatively, the dose of NMDA may not be high enough to see an effect; however higher doses increases the incident of stroke (Leander et al., 1988) and were avoided.

GABA is the primary inhibitory neurotransmitter in the brain and $GABA_A$ receptors are ionotropic receptors that gate chloride channels. Although there is no evidence of co-localization of GABA and adenosine receptors, cross-talk between $GABA_A$ and A_1 receptors has been demonstrated (Shrivastava et al., 2011). Adenosine decreased the hyperpolarization effects of $GABA_A$ through A_1 receptors (Li et al., 2004) and altered the response of $GABA_A$ receptors from inhibitory to excitatory after seizures (Ilie et al., 2012). Furthermore, A_1 receptors activation inhibited $GABA_A$ evoked currents in the hippocampus (Rombo et al., 2014). Those studies all focused on the effects of adenosine modulating GABA release. Here, we examined the $GABA_A$ antagonist bicuculline and found that it had no effects on transient adenosine release. Thus, spontaneous adenosine release is not regulated by ionotropic $GABA_A$ receptors.

4.4.2 Metabotropic receptors

Metabotropic receptors regulate signaling via G-protein coupled receptors. Here, we studied group II metabotropic receptors: mGlu2/3. These glutamate receptors are generally located pre-synaptically and inhibit neurotransmitter release (Niswender and Conn, 2010) by decreasing Ca^{2+} and cAMP concentrations (Coutinho and Knopfel, 2002). The mGlu2/3 receptor antagonist, LY 341495, had no effect on spontaneous adenosine release.

$GABA_B$ receptors are inhibitory metabotropic receptors and previous studies of single-spike evoked release found that $GABA_B$ antagonists slightly increased release (Klyuch et al., 2012). However, spontaneous, transient adenosine is not dependent on $GABA_B$ receptors as the antagonist, CGP, had no effect on the concentration or frequency of transients. Our studies were performed in the striatum, whereas the previous single-spike evoked release was performed in the cerebellum so there could be differences by brain region. Spontaneous, transient adenosine release was not affected by metabotropic receptors for glutamate (mGlu2/3) or GABA ($GABA_B$) demonstrating that adenosine is not dependent on metabotropic glutamate or $GABA_B$ heteroreceptors.

4.4.3 Adenosine receptors

A_{2a} receptors are excitatory G-protein coupled receptors that increase cAMP levels. A_{2a} antagonists are neuroprotective (Ongini et al., 1997) during ischemia (Bona et al., 1997) and reduce stroke injury (Gao and Phillis, 1994). Here, the A_{2a} antagonist, SCH 442416, significantly decreased the frequency of spontaneous adenosine release. The A_{2a} agonist, CGS 21680, had no effect on the frequency of spontaneous adenosine release, demonstrating that the A_{2a} receptor activation was saturated to a point of seeing

no additional response. Alternatively, the dose may not have been high enough to sufficiently activate A_{2a} receptors.

Previously, we found that A_1 receptor agonist decreased the frequency of release (Nguyen et al., 2014). A_1 and A_{2a} receptors have an opposite effect on each other, where A_{2a} activation can reduce the affinity of A_1 agonists for A_1 receptors (Ciruela et al., 2006). A_1 receptors have some auto-receptor characteristics (Cechova et al., 2010) and electrically-stimulated adenosine release causes auto-inhibition through the A_1 receptor (Wall and Dale, 2007). Thus, there is a feedback loop where increased adenosine release activates additional adenosine receptors, which then inhibits further release. Determining the extent that the adenosine A_1 or A_{2a} receptor is more important in self-regulating spontaneous adenosine release is difficult, especially since the overall expressions are unknown around the release sites. However, this study shows that both adenosine receptors modulate the frequency of release events through a complex mechanism.

4.5 Conclusion

The goal of this study was to determine the modulation of spontaneous, transient adenosine through ionotropic and metabotropic receptors. Spontaneous, transient adenosine frequency is dependent on adenosine receptors (A_1 and A_{2a}) and NMDA receptors; while metabotropic glutamate (mGlu2/3), $GABA_A$, $GABA_B$, and AMPA receptors do not modulate the frequency. None of the receptors affected the concentration of release events, demonstrating that the amount of release is fixed and not receptor dependent. The regulation of spontaneous, transient release is different than previous studies of electrically-stimulated adenosine release, which was both ionotropic and metabotropic glutamate, $GABA_B$, and adenosine receptor dependent. Thus different types of adenosine release have different modes of regulation. The modulation of adenosine is

complicated and spontaneous adenosine release should be examined in multiple brain regions to further understand adenosine signaling. By understanding which receptors regulate fast acting adenosine, we will be able to manipulate and control the rapid modulatory properties of adenosine.

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Chapter 5 Mechanical stimulation evokes rapid increases in adenosine concentration in the prefrontal cortex

“The only true wisdom is in knowing you know nothing”

Socrates

5 Abstract

Mechanical perturbations of live tissue can release ATP, which is broken down to adenosine. Here we used FSCV with carbon-fiber microelectrodes to measure mechanically evoked adenosine release in the rat brain by lowering the electrode 20-100 µM. Mechanically stimulated adenosine release was first found *in vivo*, however due to experimental limitations, further characterizations of mechanisms were completed in brain slices. Mechanical stimulation evoked adenosine *in vivo* (average: 3.3 ± 0.6 µM) and in brain slices (average: 0.8 ± 0.1 µM) in the prefrontal cortex. The release was transient, lasting 42 ± 5 s. Multiple mechanical stimulations were repeated and the concentration and duration did not change over time. Biosensors specific for adenosine and ATP were used to show that a portion of the release was from direct adenosine release. Lowering a 15 µm diameter glass pipette near the carbon-fiber microelectrode produced similar results as lowering the actual microelectrode. Multiple stimulations within a 50 µm region of a slice did not significantly change over time or damage cells. Thus, mechanical perturbations from inserting a probe in the brain causes rapid, transient adenosine signaling which might be neuroprotective. This chapter has been modified from a published version in the Journal of Neurochemistry (**Journal of Neurochemistry**. 2014, 130(1): 50-60).

5.1 Introduction

Mechanical damage can cause cell swelling, stretching, mechanical perturbation, shear stress and even cell death (Stalmans and Himpens 1997;Wan *et al.* 2008;Xia *et al.* 2012). Mechanical stimulation, or mechanosenstive release, is the release of neurotransmitters that has been observed in the eye (Stalmans and Himpens 1997), digestive system (Woo *et al.* 2008), lung (Ramsingh *et al.* 2011), bladder (Olsen *et al.* 2011), and the brain (Xia *et al.* 2012). The release of mechanosensitive neurotransmitters was found from astrocytes and was calcium dependent (Newman 2001). Mechanosensitive release of glutamate (Montana *et al.* 2004) and ATP (Xia *et al.* 2012) has been observed, however direct measurements of release on a rapid time scale has not been studied.

Adenosine is a neuroprotective agent that primarily works through the inhibitory A_1 receptor (Stone 2005). Adenosine is released during ischemia events, strokes, and traumatic brain injury (Rudolphi *et al.* 1992;Fredholm 1997). Direct rapid adenosine release has been discovered through electrical stimulation (Wall and Dale 2007;Cechova and Venton 2008) and spontaneous release on a rapid time scale (Street *et al.* 2011;Nguyen *et al.* 2014). Mechanically stimulated adenosine release was also observed in the brain. During deep brain stimulation, a microelectrode is implanted in the brain causing mechanical stimulation and an immediate reduction of tremors without electrical stimulation (Tasker 1998). In human clinical trials, the release of adenosine during electrode implantation was noticed within seconds (Chang *et al.* 2009;Tawfik *et al.* 2010) and concurrently prevented tremors (Chang *et al.* 2012). However, the majority of released adenosine is believed to come from ATP metabolism (Latini and Pedata 2001). The characterization of mechanosensitive ATP and adenosine on a second time scale has not been done for during probe implantation.

In this study, we characterized the rapid rise in adenosine concentration after mechanical stimulation in the prefrontal cortex *in vivo* and in brain slices. A carbon-fiber microelectrode was lowered to cause mechanical perturbation to the tissue and adenosine measured electrochemically using fast-scan cyclic voltammetry. Mechanical stimulation evoked an increase in adenosine concentration in anesthetized rats and in brain slices. Mechanically evoked adenosine did not change over time and multiple mechanical stimulations. Biosensors for adenosine and ATP were used to show that a portion of mechanically evoked release is directly from adenosine release. The pressure application of aSCF is not responsible for the release of adenosine. Similarly, cell death is not responsible for the release of mechanical ATP and adenosine. Thus, adenosine could provide transient neuromodulation during damage caused by electrode implantation.

5.2 Methods

5.2.1 Chemicals

All chemicals were from Fisher Scientific (Fair Lawn, NJ, USA) unless otherwise stated. Adenosine, ATP, and inosine were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.1 M $HClO_4$ for 10 mM stock solutions. Stock solutions were diluted daily in artificial cerebral spinal fluid (aCSF) to calibration concentrations for all brain slice experiments. The aCSF was 126 mM NaCl, 2.5 mMKCl, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$ dehydrate, 1.2 mM $MgCl_2$ hexahydrate, 25 mM $NaHCO_3$, 11 mM glucose, and 15 mM tris (hydroxymethyl) aminomethane, pH 7.4 was used in slices and for calibration of electrodes used in slices. Phosphate-buffered saline (PBS) containing 131.25 NaCl mM, 3.0 KCl mM, 10.0 NaH_2PO_4 mM, 1.2 $MgCl_2$ mM, 2.0 Na_2SO_4 mM, and 1.2 $CaCl_2$ mM with the pH adjusted to 7.4 was used to calibrate electrodes for *in vivo* experiments. Adenosine was tested at 1.0 µM for brain slice and *in vivo* experiments.

5.2.2 Electrochemistry

Carbon-fiber microelectrodes were fabricated from T-650 carbon-fibers as previously described (gift from Cytec Engineering Materials, West Patterson, NJ) (Swamy and Venton 2007). Cylinder electrodes, 50 µm long and 7 µm in diameter, were used with fast-scan cyclic voltammetry. Fast-scan cyclic voltammograms were collected using a ChemClamp (Dagan, Minneapolis, MN). Data was collected using Tarheel CV software (gift of Mark Wightman, UNC) using a homebuilt data analysis system and two computer interface boards (National Instruments PCI 6052 and PCI 6711, Austin TX). The electrode was scanned from -0.4 V to 1.45 V (vs Ag/AgCl) and back with a scan rate of 400 V/s and a repetition rate of 10 Hz. Electrodes were equilibrated for 30 min in tissue with the waveform being applied before measurements taken.

5.2.3 Brain slice preparation/experiments

All animal experiments were approved by the Animal Care and Use Committee of the University of Virginia. Male Sprague-Dawley rats (250-350 g, Charles River, Wilmington, MA) were housed in a vivarium and given food and water *ab libitum*. Rats were anesthetized with isoflurane (1 mL/100 g rat weight) in a desiccator and immediately beheaded. The brain was removed within 2 min and placed in 0-5°C aCSF for 2 min for recovery. Four hundred micrometer slices of the prefrontal cortex were prepared using a vibratome (LeicaVT1000S, Bannockburn, IL), transferred to oxygenated aCSF (95% oxygen, 5% CO_2), and recovered for an hour before the experiment. A pump (Watson-Marlo 205U, Wilmington, MA) was used to flow 35-37°C aCSF over the brain slice at 2 mL/min for all experiments. The electrodes were inserted 75 µm into the tissue. After equilibration, 60 sec of baseline data was collected and the brain slice was mechanically stimulated by lowering the electrode 50 µm with a micromanipulator.

5.2.4 Picospritzing method

Pressure ejection of aCSF onto brain slices was tested using a Parker Hannifin picospritzer (Picospritzer III, Cleveland, OH). A pulled glass pipette was filled with aCSF and placed 30 µm from the carbon-fiber microelectrode. The ejection parameters were 10 psi for 100 milliseconds and 100-200 nL of aCSF was delivered.

5.2.5 Staining Experiment

Slices were obtained and a CFME was inserted in the medial region of the prefrontal cortex (following coordinates from bregma: AP: 4.20 mm, ML: 0.4 to 0.8 mm, and DV: -3.4 to -4.0mm). After equilibration, the electrode was lowered 50 µm into the tissue, raised back up, and lowered again. Once all of the slices were treated in this manner, the slices were washed once with aCSF, then stained at 4 degrees Celsius with LIVE/DEAD fixable aqua dead cell stain kit (Life Technologies Invitrogen, Grand Island NY) (2uL/mL for 30 minutes) for cells with compromised cell membranes (necrosis) and DAPI (Sigma Aldrich, St. Louis MO) (0.5uL/mL for 15 minutes) for nuclear staining. The slices were washed twice with cold aCSF and then fixed with 4% paraformaldehyde (Sigma Aldrich, St. Louis MO) in PBS for 15 minutes. The slices were washed twice with aCSF, and then mounted into the imaging chamber of a Zeiss 780 NLO multiphoton imaging system (Thornwood, NY). Z stacks were acquired up to 100 µm into the tissue at the area of electrode insertion according to the coordinates. An additional Z stack was obtained at a control region in which no electrode had been inserted. Cell death was calculated by dividing the number of cells with compromised cell membranes (bright LIVE/DEAD staining) by the total number of cells (DAPI staining) using ImageJ64.

5.2.6 In vivo experiments

Male Sprague-Dawley rats (250-350 g, Charles River, Willmington, MA) were anesthetized with 50% wt urethane in saline solution (1.5 g/kg, i.p). Bupivicaine (0.25 mL,

s.c., APP Pharmaceuticals, LLC; Schaumburg, IL, USA) was administered for local analgesia. The rat's temperature was maintained at 37°C using a heating pad with a thermistor probe (FHC, Bowdoin, ME, USA). The working electrode was implanted in the prefrontal cortex: coordinates in mm from bregma: anterior-posterior (AP): +2.7, mediolateral (ML): +0.8, and dorsoventral (DV): -3.0 (Paxinos and Watson 2007). A Ag/AgCl reference electrode was inserted on the contralateral side of the brain. The electrode was lowered 100 µm to stimulate mechanosensitive release every 30 minutes for 2 hours.

5.2.7 Enzyme biosensors

Enzymatic biosensors for adenosine (SBS-ADO-20-50), ATP (SBS-ATP-20-50) and inosine (SBS-INO-20-50) were purchased from Sarissa Biomedical Ltd (Coventry, UK) and were prepared and conditioned as recommended by the manufacturer. These sensors have been previously characterized (Llaudet *et al.* 2003;Frenguelli *et al.* 2003). Inosine sensors lack adenosine deaminase which is crucial for adenosine detection and were therefore used as a null sensor in a separate set of experiments. Enzyme sensor data was collected using an Axon Axopatch 200B (Molecular Devices, Sunnyvale, CA) and data was collected and analyzed from a homebuilt data analysis system written for amperometry. The sensors were held at 500 mV and the collection frequency was 60 Hz for all measurements. Biosensors were calibrated based on guidelines by Sarissa prior to and after use in brain slices. Adenosine biosensors had a decrease in sensitivity of 57 ± 7 % (n=11) after implantation in brain slices, whereas carbon-fiber microelectrodes had a decrease of 40 ± 7 % (n=5) in sensitivity. On days where ATP sensors were used, 2 mM glycerol was added to the aCSF as recommended by the manufacturer. Biosensors were tested for cross-sensitivity with other analytes and the results were found to be negligible. Electrodes were lowered in buffer to verify that movement did not elicit a

significant current. Adenosine, ATP, and inosine enzyme sensors were calibrated at 1.0, 5.0, and 10 µM before and after slice experiments. For slice experiments, gravity flow was used due to noise from the pump and the experiments were performed in exactly the same manner for adenosine, ATP, and inosine biosensors as the carbon-fiber microelectrodes.

5.2.8 Statistics

All values are reported as the mean ± standard error of the mean (SEM). Paired t-test were performed to compare data before and after drugs in the same slices. All statistics were performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and considered significant at the 95% confidence level ($p < 0.05$). A one-way ANOVA with Bonferroni post-tests was performed to compare consecutive mechanical stimulations *in vivo and* across stimulations for both CFME and glass pipettes. A two-way ANOVA was used to compare the two stimulation techniques.

5.3 Results

5.3.1 Mechanically-stimulated adenosine in brain slices and in vivo

Fast-scan cyclic voltammetry was used to electrochemically detect adenosine in brain slices. A large background current is obtained because of the fast scan rate, but it is removed with background subtraction (Millar *et al.* 1985). However, basal levels cannot be measured and the technique instead measures fast changes in concentration. Initially, mechanically-stimulated adenosine was discovered by lowering the carbon-fiber microelectrode 100 µm in the prefrontal cortex of the anesthetized rat. However, in order to do pharmacological characterization, we used brain slices to further characterize mechanically stimulated adenosine release. The carbon fiber was lowered 50 µm in the prefrontal cortex of a brain slice. Electrically-stimulated adenosine release was previously elicited in the prefrontal cortex (Pajski and Venton 2013) and thus this region has the

capability for evoked adenosine release. Adenosine was observed immediately following carbon-fiber microelectrode movement 100% of the time (*n* = 5 rats *in vivo*, 20 mechanical stimulations; *n* = 25 slices, 39 mechanical stimulations).

Figure 5.1A shows a color plot of adenosine release after mechanical stimulation (red arrow) in an anesthetized rat. Applied voltage is on the *y*-axis, time is on the *x*-axis, and the green and purple colors represent oxidative current, which is proportional to concentration. Before the electrode was lowered, a background signal was obtained, denoted by the tan color representing no current change. The primary oxidation peak for adenosine occurs immediately after the mechanical stimulation at approximately 1.4 V and the secondary peak occurs at 1.1 V (denoted by the black arrows in Figure 5.1A and B). The peak positions and shape of the cyclic voltammogram (CV) are characteristic of two, two-electron oxidations for adenosine (Dryhurst 1977;Swamy and Venton 2007). The third peak observed at 0.1 V could be the third oxidation peak for adenosine, but that peak is not often observed at our carbon-fiber microelectrodes (Swamy and Venton 2007). That peak and the blue (negative) currents on the bottom of the color plot are likely due to other chemical and ionic changes that occur with mechanical stimulation. The current after adenosine is released often goes below baseline due to ionic changes (Pajski and Venton 2013). A current vs. time trace at the main oxidation potential for adenosine shows a rapid increase in current immediately following the mechanical stimulation of the electrode. In Figure 5.1A, the signal lasts about 80 seconds and the peak current corresponds to 2.4 µM adenosine.

Mechanically evoked adenosine was also observed in the prefrontal cortex of brain slices. Figure 5.1B shows an example of mechanically evoked adenosine in slices after the electrode is lowered 50 µm. The peak current corresponds to 0.77 µM adenosine and the duration is approximately 10 s. Average results for carbon-fiber microelectrodes *in*

vivo and in slices are summarized in Table 5.1. The concentration and duration of adenosine release is on average much larger *in vivo* compared to brain slices.

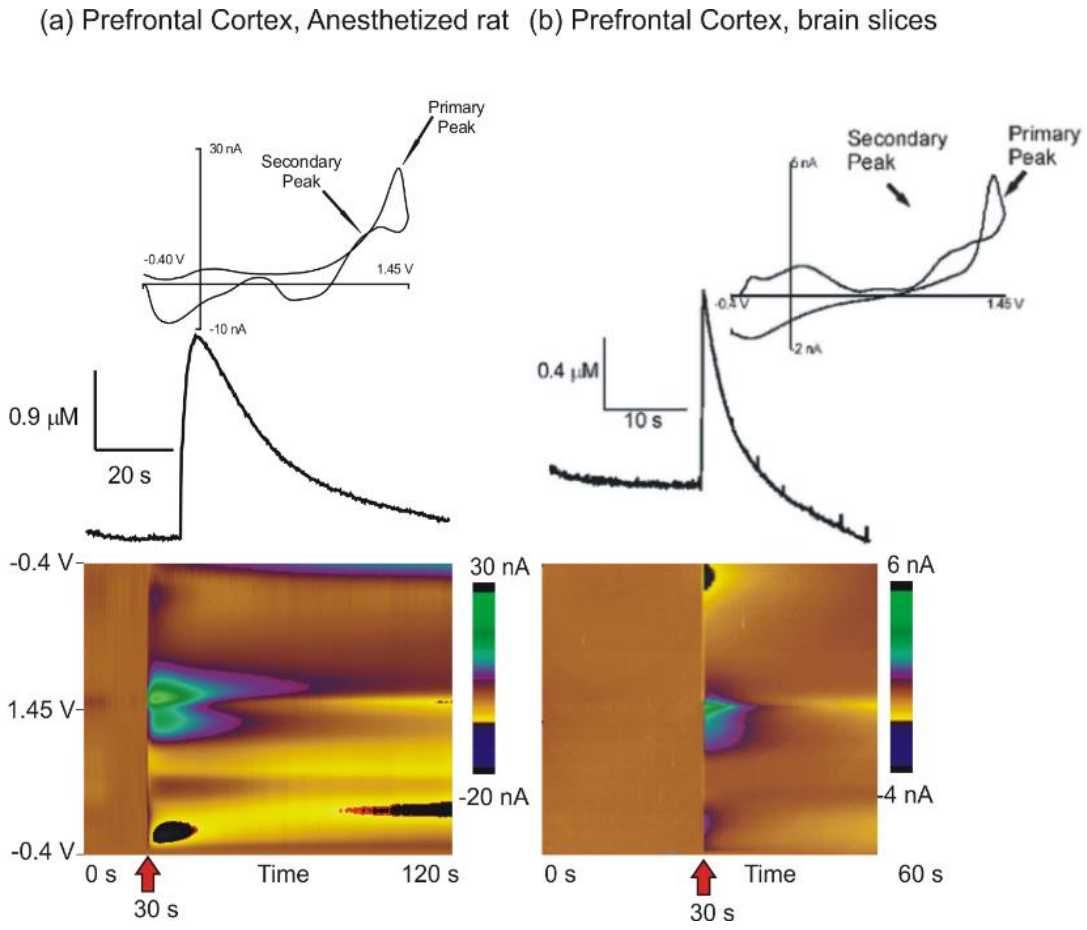

Figure 5.1 Adenosine after mechanical stimulation

The stimulation was quickly lowering a carbon-fiber microelectrode 100 µm in the prefrontal cortex of an anesthetized rat (Panel A) or 0.1 µm in a prefrontal cortex slice (Panel B) (red arrow denotes when electrode was lowered). A color plot with voltage on the y-axis, time on the x-axis and current in false color is shown. Adenosine occurs immediately after mechanical stimulation and is the green/purple region in the middle of the plot. Plots of the change in concentration for adenosine over time, located above the color plots, show that the adenosine signal lasted 80 s with a maximal concentration of 2.4 µM *in vivo* (A) and lasted approximately 10 seconds with a peak concentration of 0.77 µM for slices (B). The cyclic voltammograms have the typical primary and secondary adenosine oxidation peaks which confirm adenosine was detected.

	Conc. (µM)	Conc. Range (µM)	Duration (s)	Duration Range (s)	Percentage of slices detected
CFME *in vivo* n = 5	3.3 ± 0.6	0.3-10	42 ± 5	11-110	100
CFME in slices n = 25	0.8 ± 0.1	0.1-3.3	18 ± 2	2-46	100
Adenosine Sensor in slices n = 5	0.6 ± 0.03	0.4-0.8	10 ± 2	1.8-20	50

*All values are ± SEM

Table 5.1 Mechanically evoked adenosine

Figure 5.2A shows an example current versus time trace of consecutive mechanical stimulations every 30 minutes *in vivo.* The traces are similar, although there is variation that could be due to different areas of tissue being stimulated. The normalized current versus time trace in the inset shows that the temporal profile of adenosine is relatively stable. Figure 5.2B shows the average concentration for each successive mechanical stimulation as well as the average for all stimulations (blue bar). There was no significant effect of stimulation number on the concentration of adenosine evoked (n = 5 rats, p = 0.9716, one way ANOVA) or duration (Figure 5.2C, n = 5 rats, p = 0.9187, one way ANOVA). Thus, the concentration and duration of adenosine signaling is constant for multiple mechanical stimulations.

Mechanosensitive release has most often been attributed to ATP, which can then break down to form adenosine (Olsen *et al.* 2011;Ramsingh *et al.* 2011). ATP and adenosine have similar cyclic voltammograms because the adenine group is electroactive in both, although sensitivity for ATP at carbon-fiber microelectrodes is 3-6 fold less than for adenosine (Ross and Venton 2012). To confirm our sensors were measuring adenosine, mechanically evoked release was measured with selective biosensors for

adenosine, ATP, and inosine biosensors in brain slices (Llaudet *et al.* 2003). Inosine sensors lack adenosine deaminase, the first enzyme in the cascade that breaks adenosine down to inosine (Llaudet *et al.* 2003) and are used as a null sensor to ensure other compounds are not causing a false signal at the adenosine biosensor. While the sensitivity for the biosensors (adenosine sensitivity: 0.8 nA/µM, ATP: 0.25 nA/ µM, inosine: 0.7 nA/ µM) was not as good as with the carbon-fiber microelectrodes on our system,

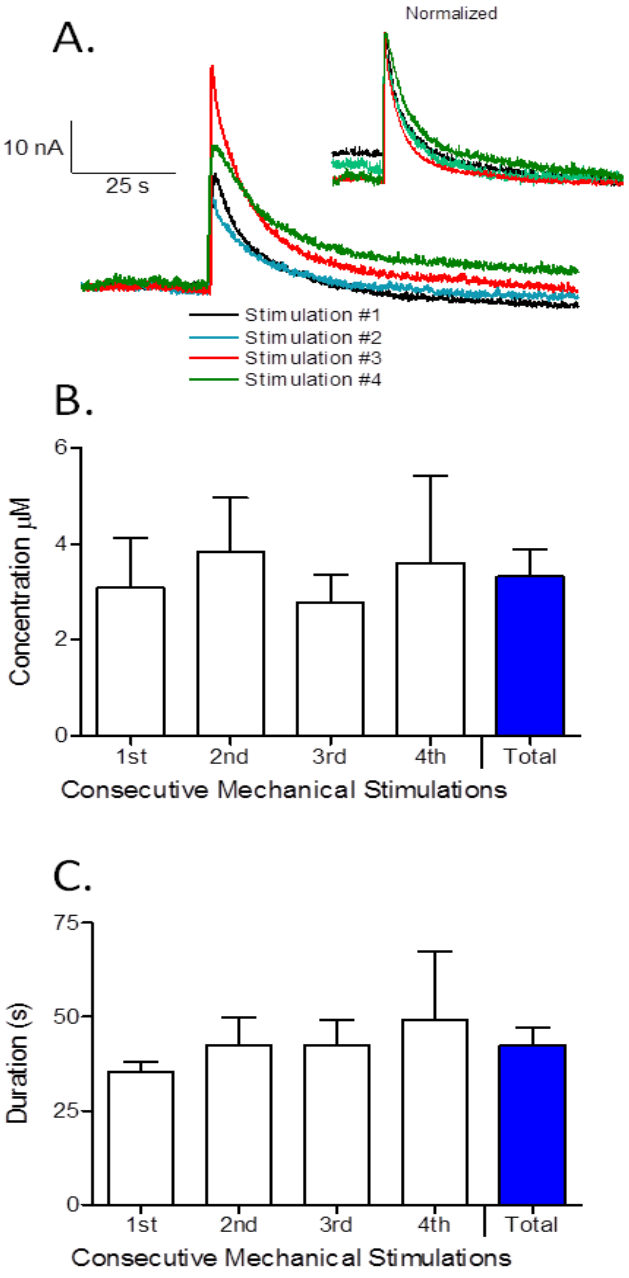

Figure 5.2 Four consecutive stimulations *in vivo*

In the prefrontal cortex of an anesthetized rat, 4 consecutive mechanical stimulations were performed every 30 minutes for 2 hours. A) Example current versus time traces are shown for four consecutive mechanical stimulations. There is no pattern of release decreasing over time. Normalized current versus time plot in the inset demonstrates that the temporal profile of the release is not changing. B) Average concentration of adenosine for each stimulation was not significantly different (n = 5 rats, p = 0.9716, one way ANOVA). The last (solid) bar shows the average for all 4 stimulations. C) The duration of adenosine signaling was also not significantly affected by the number of the stimulation (n = 5 rats, p = 0.9187, one way ANOVA). The last (solid) bar is the average for all 4 stimulations.

adenosine release was observed 50% of the time with mechanical stimulation (*n* = 5 slices, 10 mechanical stimulations, Figure 5.3 and Table 5.1. However, no changes were observed at ATP or inosine biosensors. Thus, the signal detected at carbon-fiber microelectrodes is likely to be adenosine. These data did not rule out ATP metabolism as a source of adenosine because ATP can breakdown to adenosine within 200 ms (Dunwiddie *et al.* 1997) and the response time of the ATP biosensors are on the magnitude of seconds (Llaudet *et al.* 2005). Thus, if ATP is rapidly metabolized to adenosine, then the biosensor is unlikely to detect much ATP.

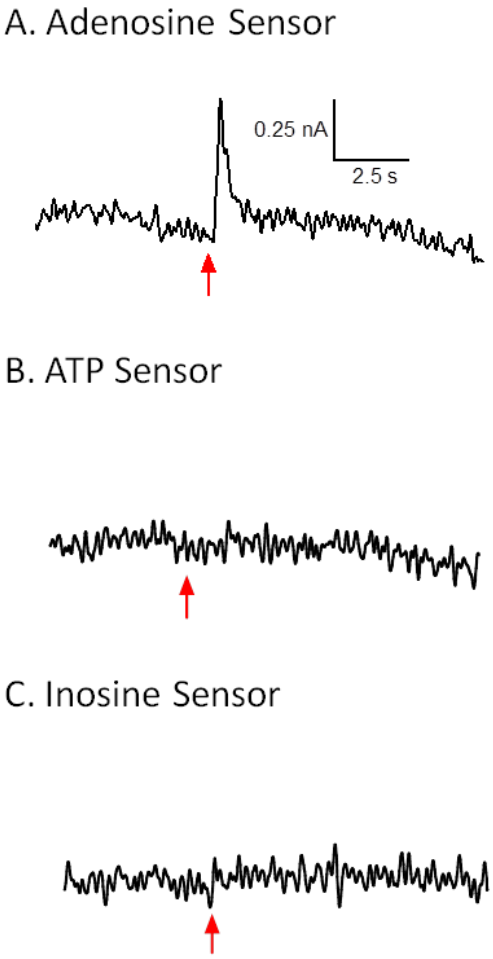

Figure 5.3 Enzyme biosensor measurements of mechanosenitive release

A.) Adenosine, B.) ATP, and C.) inosine enzyme biosensors were lowered 50 µm in prefrontal cortex slices. The arrow marks when the electrode was lowered. A. With the adenosine sensor, an increase in current was observed that lasts 6 seconds and corresponds to a peak concentration of 0.5 µM. B.) With the ATP sensor or C.) with the inosine sensor there was no increase in current for any slices tested (*n* = 5 slices, 10 total mechanical stimulations). The inosine sensor served as a null sensor to ensure other compounds were not causing a false signal at the biosensor. The scale bar is the same for all panels.

5.3.2 Evaluation of other methods for mechanical stimulation

Next, we examined the extent to which other mechanical stimulations evoked adenosine. An empty, pulled glass pipette approximately 15 µm in diameter was lowered 50 µm into the slice near the electrode. A similar increase in adenosine was detected at the carbon-fiber microelectrode when either the carbon-fiber microelectrode itself or a pipette near it was moved (Figure 5.4A and 4B). Repeated mechanical stimulations were also tested. The electrode or glass pipette was lowered 50 µm, moved back up 50 µm, and finally lowered again 50 µm, with 10 min intervals between stimulations for recovery. Figure 5.4A and 4B show CVs for both the carbon-fiber microelectrode and glass pipette stimulations, respectively. The CVs for adenosine were similar for all stimulations, which were performed in the same area of tissue. The magnitude of adenosine measured by repeated stimulations within each technique was not statistically different (repeated measures one-way ANOVA, $F = 0.3522$, $p = 0.7882$, $n = 5$). Carbon-fiber microelectrodes and glass pipettes were also compared to each other and there was no main effect of stimulation technique (repeated measures two-way ANOVA, $F(1,7) = 0.2229$, $p = 0.6512$) or stimulation number ($F(1,7) = 5.046$, $p = 0.0595$) and no interaction ($F(1,7) = 0.1402$, $p = 0.7192$). Thus, mechanically-evoked adenosine can occur by both physically lowering the working electrode or by moving something comparable in size nearby.

Local tissue damage associated with multiple electrode stimulations was characterized by staining for dead cells and was analyzed using multiphoton microscopy. A LIVE/DEAD stain was used to analyze cells with compromised cell membranes (necrosis) and a counter-stain, 4',6-diamidino-2-phenylindole (DAPI), stained for all nuclei. The first 50 µm of the slice tissue was found to be highly damaged, which is expected in slice experiments. A multiphoton Z-stack through the initial dead segment and into the underlying healthy tissue at the point of electrode insertion did not reveal a clear path for electrode insertion. There was no noticeable hole in the tissue and no track of cell death

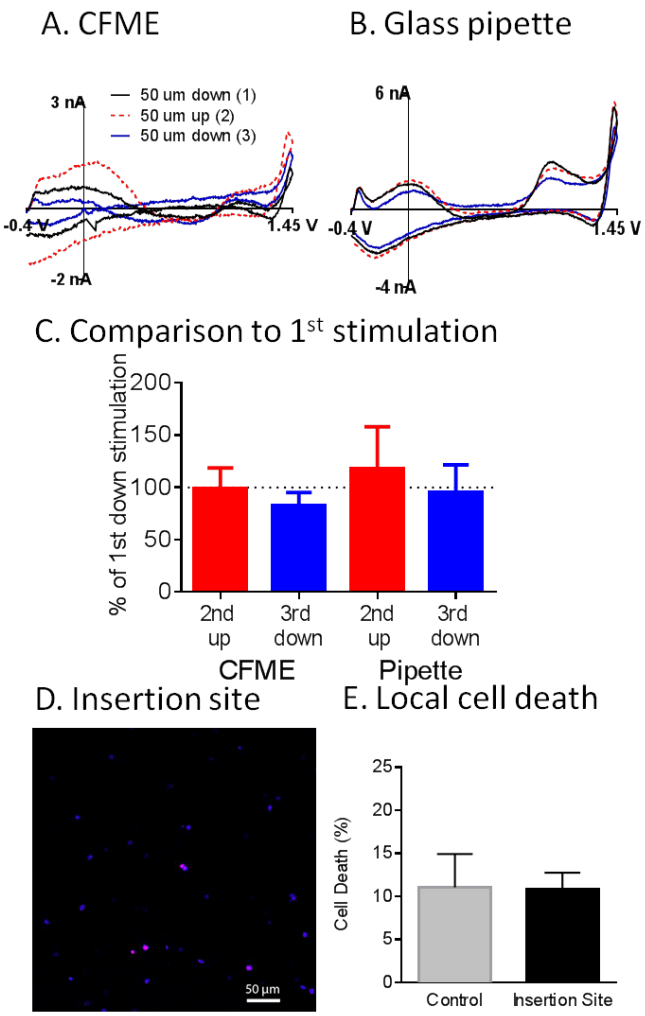

Figure 5.4 Mechanically stimulated adenosine does not cause cell death

Adenosine is mechanically evoked either by both lowering the working electrode or a glass pipette close to the working electrode. Multiple electrode stimulations did not cause significant cell death. Adenosine can be mechanically stimulated by A) moving the carbon-fiber microelectrode and B) moving a 15 µm-diameter glass pipette. Repeated stimulations were tested by moving the carbon-fiber microelectrode (A) or the glass pipette (B) 50 µm down into the tissue, 50 µm back up, and 50 µm back down. Ten minutes of recovery time was given between each move. C) The bar graph compares the second (up) and third (down) stimulation for both techniques. Data is graphed as a percentage of first stimulation and there are no significant differences between the two techniques (repeated measures two-way ANOVA, $F(1,7) = 0.2229$, $p = 0.6512$) or stimulation number ($F(1,7) = 5.046$, $p = 0.0595$) and no interaction ($F(1,7 = 0.1402$, $p = 0.7192$). Repeated adenosine measurements within each technique are not statistically different (repeated measures one-way ANOVA, $F = 0.3522$, $p = 0.7882$, $n = 5$). D) Cell imaging of the insertion site where there was multiple electrode stimulations reveals no obvious significant cell death. Blue represents DAPI staining, showing all nuclei. Red represents dead cells stained with a LIVE/DEAD cell assay and scale bar represents 50 µm. E) There was no significant difference in dead cell percentage between a control region (no electrode insertion) and the electrode insertion site (paired t-test, $p = 0.9061$, $n = 4$).

(Figure 5.4D and E), indicating that the electrode did not disrupt the cell membranes of the cells as it moved up and down through the tissue. Figure 5.4D shows all nuclei stained with DAPI and colored blue, and dead cells colored in red. The percentage of dead cells was counted and compared in the region of electrode implantation and a control region (Figure 5.4E) and there was no significant difference between these two regions (paired t-test, $p = 0.9096$, $n = 4$). This is consistent with previous work indicating that FSCV probe insertion does not significantly damage the tissue (Peters *et al.* 2004;Jaquins-Gerstl and Michael 2009).

5.4 Discussion

Mechanosensitive release of purines due to physical perturbation of cells has been mostly attributed to ATP release, which slowly builds up over time (Woo *et al.* 2008;Olsen *et al.* 2011;Ramsingh *et al.* 2011). In this study, we demonstrate that in the prefrontal cortex, mechanical stimulation elicits a rapid, transient release of adenosine. *In vivo*, peak adenosine release was on average 3.3 ± 0.6 µM and lasted 42 ± 5 s. These studies provide new insight that adenosine is rapidly released after electrode implantation and could act as a signaling molecule during tissue damage.

5.4.1 Mechanically evoked adenosine occurs via different methods

In this experiment, we mechanically stimulated brain tissue by lowering the working electrode 50-100 µm. This method of stimulation would be relevant to any type of probe implantation, for example electrochemical, electrophysiological, or deep brain stimulation (DBS) probes. Because lowering the carbon-fiber microelectrode could change the background current, the response was compared to lowering a glass pipette of similar size

near the stationary microelectrode. The evoked adenosine was similar for both techniques and thus the signal is not an artifact of moving the electrode.

Traditional mechanical perturbation techniques, including cell swelling (Xia *et al.* 2012), cell stretching (Ramsingh *et al.* 2011), and shear stress (Woo *et al.* 2008;Olsen *et al.* 2011), are modeled after normal biological functions. Our study does not directly mimic one of these processes, but an interesting phenomenon was observed in the experiments where the electrode or glass pipette was lowered and raised multiple times. The adenosine increase after pulling the pipette up was slightly larger than for lowering it down. While this phenomenon would need to be characterized much further, it suggests some cell stretching may be a cause of adenosine increase when raising the electrode. These repeated experiments also show that the tissue is not dying, as the same response can be measured repeatedly and staining reveals no significant increase in local cell death after multiple electrode stimulations. Thus, the electrode insertion is not causing a significant amount of permanent tissue damage, in line with previous reports of minimal tissue damage associated with carbon-fiber microelectrode insertion *in vivo* (Peters *et al.* 2004;Jaquins-Gerstl and Michael 2009).

An adenosine response to probe implantation has been suggested in the past but never fully characterized. A phenomenon known as the microthalamotomy effect occurs following the DBS electrode implantation in the thalamus of Parkinson's patients (Tasker 1998). The implantation causes an immediate relief from tremors before and deep brain stimulations are evoked (Chang *et al.* 2012). Adenosine was elevated immediately after electrode implantation, preceding the microthalamotomy effect in the ferret thalamus (Tawfik *et al.* 2010). Chang et al. implanted a DBS probe into seven human patients and also saw the microthalamotomy effect along with release of adenosine in all patients (Chang *et al.* 2012). Future studies could probe whether adenosine is a cause or effect of the microthalamotomy effect during implantation in DBS. Elevated levels of adenosine

were also discovered during and after acupuncture in the tibialis anterior muscle close to the knee (Goldman *et al.* 2010). However, the adenosine was measured using HPLC on a 10 minute time scale and would not be able to detect rapid adenosine release. Research has a hard time determining the reason for acupuncture treatment (Ernst and White 1997) as sham vs acupuncture needles provide inconclusive results (He *et al.* 2013). The indeterminate studies with control needles, could be that adenosine is mechanically stimulated through implantation, regardless of needle. Future studies with FSCV and acupuncture could determine the mechanism through which acupuncture treatment works. Thus, other forms of probe implantation for DBS and acupuncture cause increases in adenosine, which may be similar or complementary to the transient adenosine release observed in this study.

5.4.2 Mechanical stimulation evokes large, transient adenosine changes

Mechanically-stimulated adenosine varied widely *in vivo* and in both slices. The concentration of adenosine detected *in vivo* ranged from 0.3 to 10 µM and from 0.1 µM to 3.3 µM in slices. Larger amounts of adenosine were released *in vivo* but the stimulation was 100 µm compared to 50 µm in slices, which would result in more cells being stimulated. The large range is not surprising because the density of adenosine releasing cells around the electrode could vary based on electrode location. Also, speed of insertion was not tightly controlled which could cause varying tissue responses. The average amount of adenosine released by mechanical stimulation was higher than previously detected with electrical stimulation (220 nM) (Pajski and Venton 2010) and spontaneous release (170 nM) (Nguyen *et al.* 2014); thus, tissue perturbation causes higher amounts of release than electrical stimulation and spontaneous release.

The duration of adenosine release varied from 2 s to 110 s *in vivo* and in brain slices. The duration was correlated with the concentration, as larger amounts of adenosine lasted longer because more time was needed to clear the adenosine from the extracellular space. Mechanically evoked adenosine signaling is fast compared to other studies that measure mechanically evoked ATP release (Ramsingh *et al.* 2011). Mechanosensitive ATP release is usually detected using luciferin-luciferase assay techniques (Woo *et al.* 2008) which allow measurements to be taken only every 30 s. The duration of the adenosine response is similar to electrically evoked adenosine signaling that has been shown in multiple brain regions (Pajski and Venton 2010;Pajski and Venton 2013;Klyuch *et al.* 2011). Spontaneous adenosine is released and cleared within 3 seconds (Street *et al.* 2011;Nguyen *et al.* 2014), which is within the time scale of mechanically stimulated adenosine. Thus, there is evidence of multiple forms of rapid adenosine signaling in the brain. Mechanical evoked release experiments are simpler and faster than electrical stimulation and spontaneous adenosine release and could be used to study the function of transient adenosine signaling, including any neuroprotective effects.

5.4.3 Function of mechanically evoked adenosine release

This work demonstrates that a local mechanical stimulation causes an elevation of adenosine concentration for less than a minute; thus transiently activating adenosine receptors. The function of rapid adenosine signaling after probe implantation remains unknown; however, adenosine signaling resulting from various forms of damage has been well documented in the literature (Frenguelli *et al.* 2007;Shen *et al.* 2011;Haselkorn *et al.* 2008). In particular, activation of A_1 receptors is inhibitory, which could dampen neurotransmission that causes excitotoxicity in the brain (Sperlagh *et al.* 2007).

Adenosine could also stimulate blood flow after probe implantation, which would deliver nutrients for tissue repair. The immediate nature of the response allows for rapid activation of the pathway, but the fast duration would not allow long-term depression of neuronal activity. Further investigations of downstream effects of rapid adenosine signaling after mechanical stimulation as well as interactions of adenosine with other signaling molecules that are released would be interesting to better understand the function of adenosine as a rapid modulator in the brain.

5.5 Conclusions

Mechanically evoked adenosine was observed immediately following mechanical stimulations in the prefrontal cortex in both brain slices and *in vivo*. Adenosine could be evoked by moving the microelectrode or a glass pipette implanted near the microelectrode and stimulations could be repeated, indicating the tissue was not dying. The study shows that implantation of a probe into brain tissue causes a transient adenosine response which could be neuroprotective.

5.6 Reference List

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Chapter 6 Conclusions and Future Directions

“Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning”

Albert Einstein

6 Abstract

In this dissertation, I have discovered and characterized rapid adenosine signaling in the brain using fast-scan cyclic voltammetry with carbon-fiber microelectrodes. Further, I characterized the concentration, duration, and frequency as well as the clearance and receptor modulation of spontaneous, transient adenosine. I then briefly examined mechanically evoked adenosine release in the cortex. In this final chapter, I will summarize my work and then discuss its broad applicability in the field of rapid adenosine signaling.

6.1 Fast acting adenosine release

Chapters 2-4 characterizes the recently discovered spontaneous, transient adenosine release. The average concentration is sufficient to activate adenosine receptors and the short duration (less than three seconds) indicate the reason that this mode of adenosine was not discovered with traditional techniques of measuring adenosine. The clearance of adenosine is controlled by multiple processes, metabolism and re-uptake, showing that the modulation of transient adenosine release is tightly regulated. **Chapter 4** focuses on the receptors that are modulating spontaneous adenosine release. The frequency of release is regulated by adenosine receptors, A_1 and A_{2a}, as well as the ionotropic glutamate receptor NMDA. The concentration of transient adenosine release is independent of any enzymes or receptors studied, demonstrating that regulation of spontaneous adenosine is primarily through the frequency of events and not the amount released in a single event. Finally, in **Chapter 5**, I discovered and characterized another mode of rapid adenosine signaling, mechanically-stimulated adenosine release. Both new modes of rapid adenosine signaling are important for understanding the complex picture of how adenosine modulates neurotransmission in the

brain. Spontaneous and mechanically-stimulated adenosine demonstrate there is rapid control on neurotransmission and neuroprotection in the brain that can be further studied to understand the full potential of adenosine signaling.

6.2 Future Studies

Chapters 2 through 4 focus on spontaneous, transient adenosine release. Although this dissertation examines the underlying characteristics of naturally occurring adenosine release, two areas of research still need to be explored. The first issue is the cell type from which spontaneous adenosine is released from and the second issue is the function of transient adenosine.

The location of the release of spontaneous adenosine can be further broken down into two aspects: (1) the cells from which adenosine is originating, and (2) the extent to which the adenosine is coming from direct release or the release of ATP. To assess the physical location, we can use FSCV to directly measure adenosine release from specific cells and locations. Using Ca^{2+} imaging techniques, adenosine release has been identified from astrocytes (Martin *et al.* 2007;Schmitt *et al.* 2012;Wall and Dale 2013). Transgenic mice with green fluorescent protein expressed in astrocytes have been developed (Zhuo *et al.* 1997) and spontaneous adenosine release has been found in mouse spinal cord slices (Street *et al.* 2011). Thus, future studies could be devoted to placing FSCV electrodes next to fluorescently labeled astrocytes in brain slices to determine the location of rapid adenosine release. Additionally, FSCV can be used to measure exocytosis directly from single cells, so future studies could examine adenosine release from neurons and astrocytes to better understand the mechanism and location of adenosine release.

A more complicated question is the extent to which spontaneous adenosine is actually a breakdown product of ATP or if adenosine is directly released as a neurotransmitter. ATP is released as a neuronal co-transmitter with dopamine, serotonin, GABA, and glutamate (Burnstock 2004), so adenosine release could be a result of ATP metabolism. Extensive research from multiple groups has concluded that rapid adenosine release is activity dependent and not released through nucleoside transporters (Wall and Dale 2007;Wall and Dale 2008;Pajski and Venton 2010;Street *et al.* 2011;Pajski and Venton 2013;Ross *et al.* 2014). In some studies, a portion of the rapid adenosine released is due to ATP metabolism, but in most instances, some of the adenosine does not originate from ATP. Because there are multiple enzymes that break down ATP (Barsotti and Ipata 2004;Street *et al.* 2013) and some of the inhibitors of these enzymes are inefficient (Wall *et al.* 2008), it is difficult to rule out ATP as the sole source of adenosine. However, adenosine was recently discovered in vesicles (Corti *et al.* 2013), so future work should examine the possibility of direct release of adenosine from exocytosis versus breakdown of exocytotically released ATP. These studies on spontaneous, transient adenosine release would be performed in brain slices, as activity dependent tools are more readily available such as Ca^{2+} removal and tetrodotoxin to inhibit action potentials. ATP is also released into the extracellular space through pannexin channels (Baranova *et al.* 2004;Chekeni *et al.* 2010;MacVicar and Thompson 2010;Billaud *et al.* 2015) and thus should be considered as a possible source of rapidly released adenosine. Carbenoxolone is an inhibitor of pannexin channels (Bruzzone *et al.* 2005) and is an ideal candidate for future studies determining if rapid adenosine release is from ATP breakdown and release through pannexin channels.

The second major issue to address is the biological function of spontaneous, transient adenosine release, as this has yet to be completely understood. Although my research found a frequency dependency on NMDA and adenosine receptors, this only

answers how release is regulated. Most likely, rapid adenosine signaling functions in a similar manner to the slow buildup of adenosine in the extracellular space. In the brain, adenosine regulates blood flow, neurotransmission, and neuroprotection over extended periods of time. Thus, future studies should examine these functions in the arena of short time scale release. As transiently applied adenosine release has already been shown to regulate evoked dopamine release (Ross and Venton 2014), future studies could be devoted to examining how adenosine regulates this process. Glutamate application on brain slices has increased evoked adenosine release (Hoehn and White 1990;Manzoni *et al.* 1994;Klyuch *et al.* 2012), thus future studies can examine spontaneous adenosine release in the presence of glutamate in brain slices.

Alternatively, we can utilize glutamate biosensors to directly measure glutamate neurotransmission and how it is affected by spontaneous adenosine release. Similar to adenosine and ATP biosensors (Dale 1998;Llaudet *et al.* 2005), glutamate biosensors use specific enzymes to metabolize glutamate to an electrochemically active compound that can be quantitated with amperometry on a rapid time scale (Pomerleau *et al.* 2003;Day *et al.* 2006;McLamore *et al.* 2010;Ozel *et al.* 2014). Carbon-fiber microelectrodes with FSCV detection of adenosine combined with glutamate biosensors with amperometry would be able to directly measure adenosine and glutamate release simultaneously. Similarly, electrophysiology studies that measure synaptic transmission could be combined with FSCV to measure the effect of spontaneous adenosine on neurotransmission. Electrophysiology with FSCV has been used to measure dopamine simultaneously with neural activity (Belle *et al.* 2013), thus the technique could be applied to measure rapid adenosine signaling. In the future, the effect that rapid adenosine signaling has on glutamate release and synaptic transmission could be examined by combining multiple techniques.

The second probable function of fast acting adenosine in the brain is regulation of blood flow. Previously, our laboratory studied the effect of evoked adenosine on oxygen levels in the brain using FSCV (Cechova and Venton 2008). Using a modified waveform that detects oxygen reduction, both adenosine and oxygen can be co-detected. Similarly, there are numerous techniques available to monitor heart rate and breathing in rodents (Anderson *et al.* 1999;Radespiel-Troger *et al.* 2003;Zehendner *et al.* 2013) that could be used to examine any relationship between spontaneous adenosine and blood flow. Future studies could examine transient adenosine release and determine whether there is a correlation between rapid adenosine and oxygen levels.

FSCV will prove useful in the understanding of rapid adenosine release as it relates to disease, especially in terms of epilepsy. For example, during epileptic events, adenosine decreased excitatory glutamate release and hyperpolarized neurons, which is desirable during hyper-excitatory seizures (During and Spencer 1992). FSCV was used to detect adenosine release during epilepsy and seizures in pigs and was safe in human experiments (Van Gompel *et al.* 2014). Adenosine was released prior to seizure termination and remained elevated following the epileptic event, which confirms that adenosine is involved in seizure expiration (Boison 2008). FSCV has rapid time resolution so it could be used to monitor adenosine during the rapid onset of seizures. FSCV could help distinguish if adenosine release is constant during all seizures and determine if adenosine is released before or after the onset of seizures.

Furthermore, all of the studies in this dissertation were done in anesthetized rats or brain slices of rats. While the anesthetized rat is a good place to begin studies on fast acting adenosine release, eventually transient adenosine should be monitored in awake animals. Studies on spontaneous adenosine release in rats in a natural environment, socially interacting with each other would provide further insight into the modulation of fast acting adenosine signaling.

6.3 Final conclusions

FSCV allows adenosine detection on a sub-second time scale and has been used to uncover a rapid mode of adenosine signaling that lasts only a few seconds. Spontaneous and mechanically-stimulated, rapid adenosine release were measured in anesthetized rats. In the future, these two modes of transient adenosine release will be further characterized to determine the intricate role that adenosine plays in the central nervous system. Future studies could use FSCV in conjunction with pharmacology, electrophysiology, and biosensors to fully understand the mechanism and function underlying this mode of rapid adenosine signaling.

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Appendix: Identifying and quantitating random adenosine transients

“Be on your guard; stand firm in the faith; be men of courage; be strong”

1 Corinthians 16:13

7 Introduction

Spontaneous, transient adenosine release was discovered in the caudate-putamen and prefrontal cortex of anesthetized rats using Fast-scan Cyclic Voltammetry (FSCV). The release events follow a random distribution (Nguyen *et al.* 2014). The concentration of spontaneous adenosine release is around 170 nM, which is large enough to activate adenosine receptors (Latini and Pedata 2001). The range of adenosine concentrations is large, with transients as large as 4 µM seen. The transient adenosine release and clearance is short, lasting only a couple of seconds. The goal of this project is to develop a program that can find, identify, and quantitate the peak height and clearance of random adenosine release.

When using FSCV a unique background subtracted cyclic voltammogram (CV) is generated for a molecule that is oxidized. The characteristic CV for adenosine has a primary and secondary oxidation peak. Large transients were easy to identify. However when quantitating close to the limit of detection (20-40 nM), the task was more difficult. The random nature of spontaneous release adds to the difficulty in identifying adenosine release. Additionally, as with any method of human analysis there is a problem of subjectivity. There is a possibility that a previously formed hypothesis could motivate how the data is analyzed. Currently we use principal component analysis (PCA) to objectively identify and characterized spontaneous, transient adenosine release, however we are working towards an automated system to detect and quantitate random release.

7.1 Principal Component Analysis

Principal Component Analysis, described in **Chapter 2**, was used to find adenosine transients. A brief overview of PCA is that the unique CV of adenosine was input into a training set and every CV in the data (10 per second of data collection) was compared to the trained CV to see if the principal components were matched. Any CV

that is significantly different than the principal components of the trained CV, will be above the Q-score generated from the training set and is significantly different than adenosine. The method was first developed for dopamine and is excellent at parsing out differences between dopamine and pH shifts (Heien *et al.* 2005). However, there are a few disadvantages for using PCA with adenosine analysis. The first is that unlike dopamine or pH shifts, the CV of adenosine changes over time (Nguyen *et al.* 2014). Adenosine undergoes multiple oxidations (Dryhurst 1977), none of which are reversible at carbon-fiber microelectrodes at low concentrations (Swamy and Venton 2007). Instead of a molecule of adenosine being oxidized and reduced multiple times at the electrode, adenosine is oxidized once or twice, desorbs, and diffuses away. This makes for a CV that changes for each subsequent scan because the concentration of adenosine, oxidation product I, and oxidation product II at the electrode are constantly in flux. The trained CV input into PCA does not account for this and can fail larger transients.

The second disadvantage of PCA is the amount of time required to perform data analysis. Each rat was its own control group and the pre-drug data was compared to the post-drug data for each rat. Data was collected for four total hours and the drug was injected at the halfway point. Such large amounts of data were beneficial to tease out significant differences pre- and post-drug. For each animal five of the largest pre-drug transients were first identified for input into the PCA training set. The next step was to go through each file, identify a potential transient by doing a manual check on the CV to ensure a similar shape to adenosine. Then the data was input through the PCA script which generated a residual color plot and concentration vs time. From the conc vs time plot, the duration and peak height were manually extracted. This procedure was repeated for each individual transient. In some cases, an individual animal had a couple hundred transients each taking a significant amount of time to analyze. Thus we developed a computer program written in MatLAB programming language to objectively identify

adenosine transients using FSCV, that will significantly reduce the time needed to analyze data.

7.2 Automated peak detector for Fast-scan Cyclic Voltammetry

The goal of this project is to develop a working program that can identify and quantitate transient peaks from FSCV data. To accomplish our goal we recruited the help of computer scientists from the University of Virginia. The development of the MatLAB code for detecting adenosine transients was performed as a “Tiger Team” project through the UVASCE (University of Virginia Alliance of Computation Science and Engineering). The code has four separate functions corresponding to separate steps in the data analysis workflow. Briefly, the four functions are: (1) read the FSCV files from FSCV into MatLAB, (2) remove background drift and filter the data to remove noise, (3) identify the beginning, climax, and end of the peak, and (4) generate data for each peak (such as duration, peak height, rise time, etc.). Thus with a working computer program, we are able to identify and quantitate peaks from FSCV data with an automated, unbiased script that will save countless hours of data mining.

The initial step is to read 3-D text files from a three minute FSCV data collection into MatLab. The first function displays the data files available to be imported and imports the user-selected data set into Matlab. The second function corrects for any background drift that is occasionally observed in carbon electrodes with FSCV analysis *in vivo*. The program then filters the data with a weighted average moving filter to remove high frequency noise and displays the smoothed data as a color-mapped intensity image. Currently, background subtracted text files are input into MatLAB. However, the program is anticipated to background subtract the files in future versions. A 3D color plot collected directly from LabView HDCV (UNC Department of Chemistry, NC) is shown in Figure 7.1

right above the intensity image generated by MatLAB. MatLAB uses a thermal heat map color scheme.

Next, the program allows the user to select a region of interest (ROI) for the code to analyze and displays just the ROI as a second intensity image. This has a couple of advantages, with the first being that the program is not analyzing the full data, thus reducing any outside noise or background drift that may interfere with peak analysis. Second, the user-defined data window, allows the program to analyze peaks that are in the potential range of adenosine. The two oxidations of adenosine occur at 1.0 and 1.4 V. However other molecules have different potential windows. Dopamine is oxidized at 0.6 V and reduced at -0.2 V using FSCV (Wightman *et al.* 1988), while serotonin is oxidized and reduced at 0.8 and -0.1V respectively (Xiao *et al.* 2014). Thus the program has the ability to identify and quantify other molecules with FSCV. Figure 7.2 shows the intensity image, along with the selected window and the subsequently magnified ROI below.

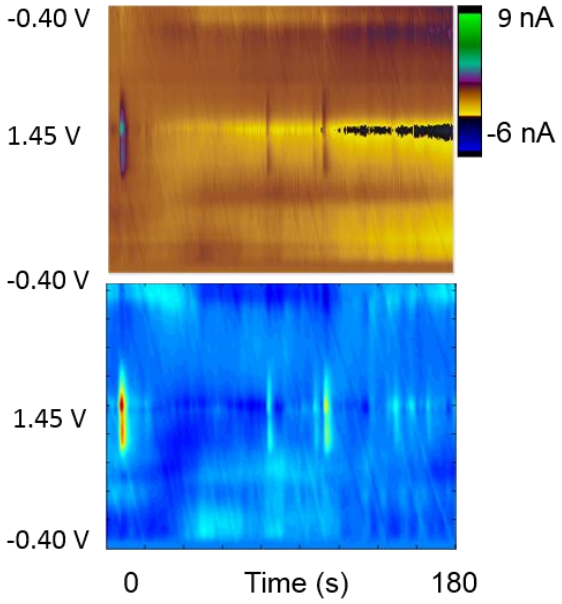

Figure 7.1 Three dimensional color plots from two different programs

(Top) three dimensional color plot from generated from HDCV Analysis from LabView. (Below) Heat map generated from MatLab from the exact same data.

Once the ROI is selected, the user has the option of selecting a specific row or voltage in order to get a current vs time trace. Multiple rows may be selected, allowing for flexibility in the molecule being detected. Figure 7.3 shows the selected rows as white lines, one through the primary peak at 1.4 V and one through the secondary peak at 1.0 V. After the rows are selected, the program gives a current vs time trace along with the output of a differencing filter convolved with the smoothed data. The second plot provides an approximation to the derivative of the time trace, which allows the user to determine a threshold for detecting peak transition events (Figure 7.4). If the output of the differencing filter exceeds the threshold, it is due to the signal trace rising steeply toward a peak. Detection of this event is designated as the transition to a peak and is used as a starting point in subsequent data analysis.

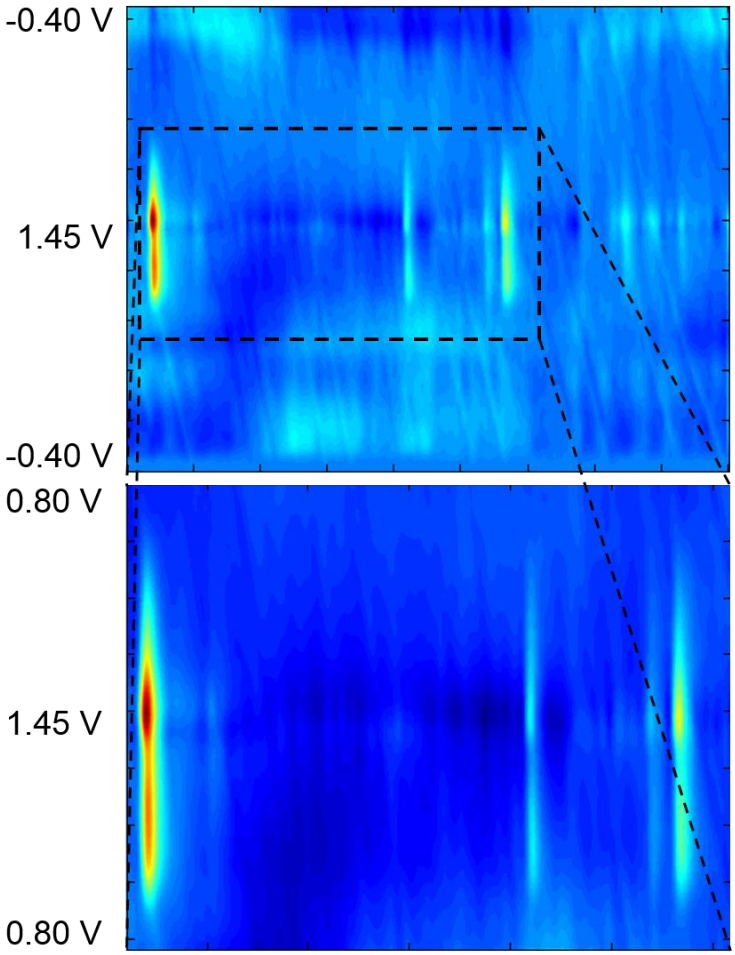

Figure 7.2 User defined window for peak analysis

3-D heat map of in vivo data. The top window shows the unaltered file, while the bottom window shows the user designated area for analysis.

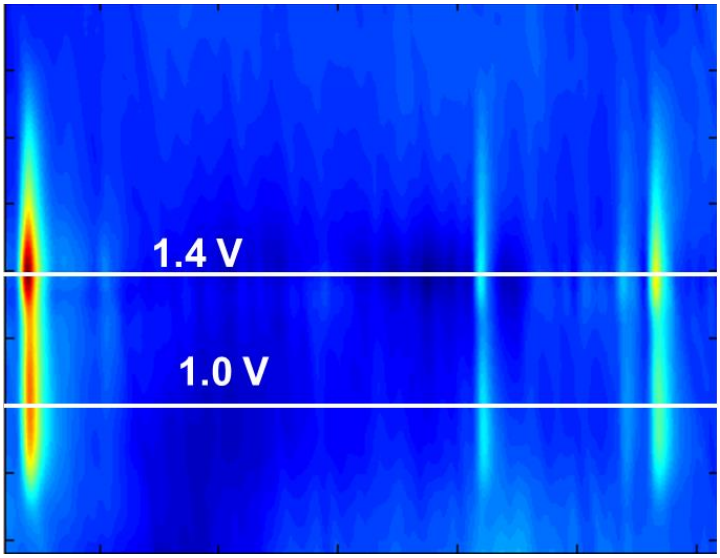

Figure 7.3 Selection of row for peak analysis

User defined window of 3-D heat map with selected rows at the primary oxidation peak voltage (1.4 V) and the secondary oxidation peak voltage (1.0 V)

The filter widths used in the data smoothing and differencing operations can be adjusted as inputs to the function to adapt the filtering operations to specific features in a data set. The default filter widths for the moving average and differencing filters are 13 and 7 respectively. The plot of the differencing filter output is normalized to 1.0 for the highest value. The default value for detecting a peak transition is 0.2, but again the default can be changed as an input to the function so that only peaks of interest are detected.

Once a threshold is chosen (or the default value is used), the third function processes each row of the smoothed data to first detect any peak transitions and then determines the start of the peak, the peak apex, and the end of the peak for each detection event. The column locations of the start, apex, and end of each detected peak are determined with reference to the peak transition event using the differencing filter output and simple heuristic algorithm described below.

The column (time) location of the peak transition is used as a starting point to look backward in time, through the differenced data, to determine when the differences approach zero within a preset threshold from a positive direction. Once the scan has crossed the threshold, the minimum of the smoothed data over the span of the differencing

filter is designated the peak starting point corresponding to the start of the adenosine transient. The span of the differencing filter is 2*k + 1 columns, where typically k = 7.

Next, starting again from the time location of the peak transition, the code looks forward in time through the differenced data to threshold when the differences approach zero. At that point, the differencing filter support will be spanning the peak of the signal and the maximum within that span is designated as the peak apex.

Finally, the code uses the location of the differencing filter support farthest after the peak location as the starting point. From this point, the code scans forward in time through the differenced data to threshold when the differences approach zero from the negative direction. This point is used to bracket the position of the smoothed data to designate the peak end. If the amplitude of the signal drops more than 90% from its peak value, then it is designated the peak endpoint.

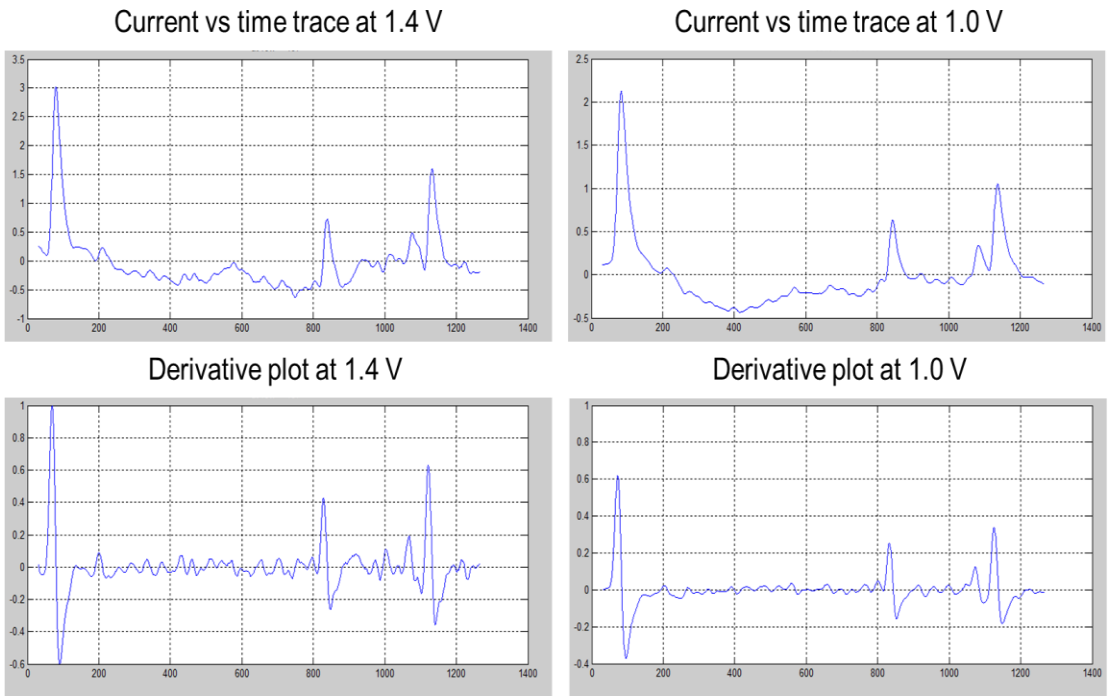

Figure 7.4 Current vs time traces and derivative plots

The user designated rows at 1.4 V on the left side and 1.0 V on the right side. The top two charts are the current vs time traces. The plots on the bottom are the derivative of the current vs time plots.

The function then displays the column (time) locations of the three peak parameters across all rows of the data as a color coded image. This shows the contours of the detected peak parameters, forming what could be described as a ridge in the intensity data, possibly with multiple peaks in said ridge (Figure 7.5). The line of starting points for the detected peaks are denoted in red, the line of peaks in the ridge are in white, and the line of end points of the peaks are in yellow. From this image, the user then selects which rows of the smoothed data and the associated peak start, apex, and stop events that are to be displayed. In this case 1.4 V and 1.0 V marked in blue dashed lines.

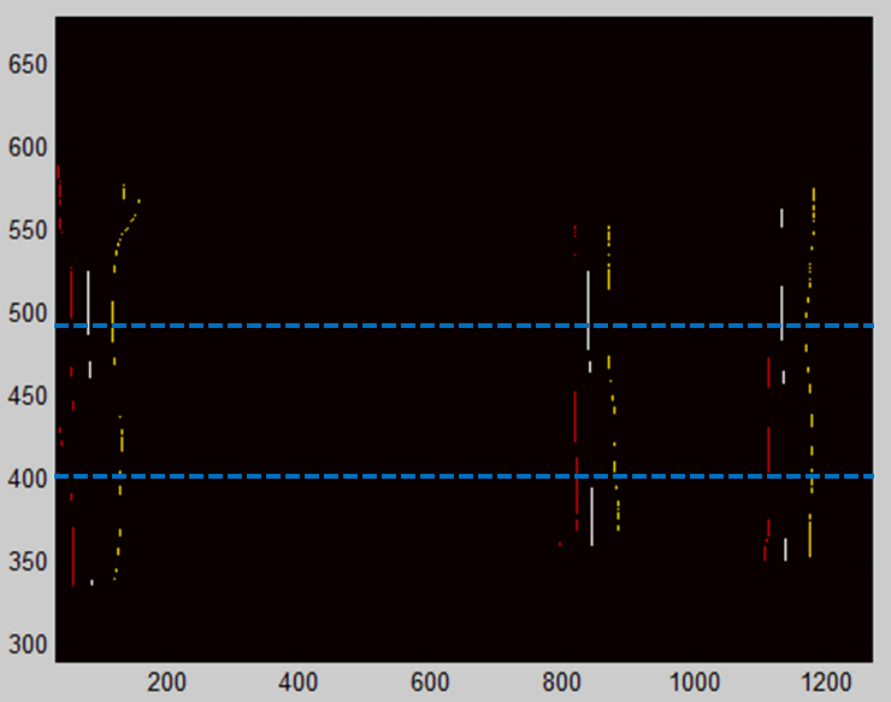

Figure 7.5 Contour map of peak ridges

A black plot of the different ridges detected in the user defined window. The red lines mark the beginning of the, the white lines the apex, and the yellow the end of the peak. Blue dashed lines are at 1.4 V and 1.0 V.

The code then plots a current vs time trace for each selected row, marking the start, apex, and end of each peak. The beginning and end of each peak are marked with red crosses and the summit is marked with a red circle (Figure 7.6).

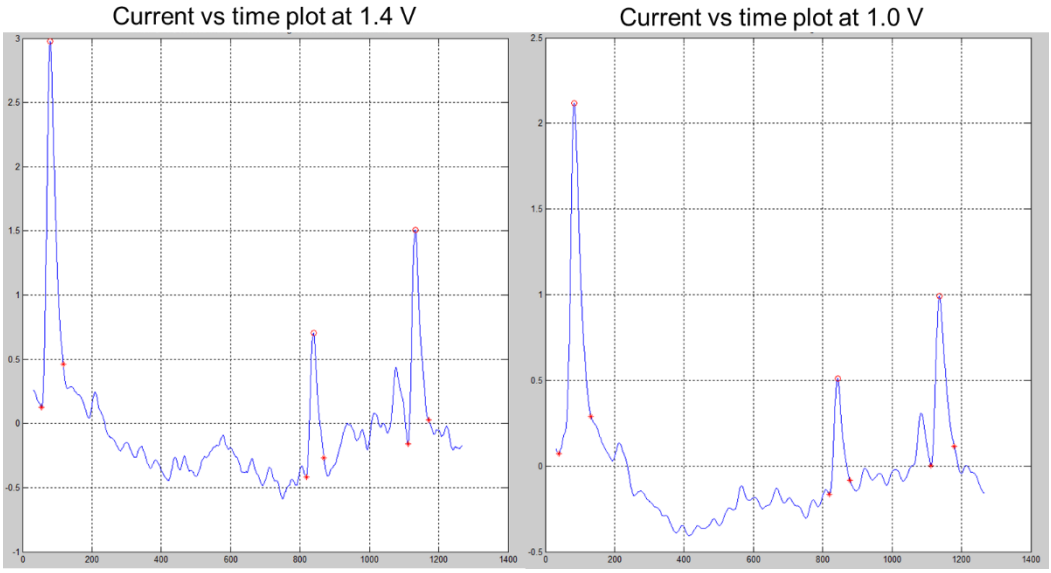

Figure 7.6 Current vs time plot

Current vs time plots with the beginning, apex, and end of each identified peak.

The fourth function uses the contours generated by the peak apex locations (i.e. the ridgeline) to determine the number and locations of multiple peaks in the ridge, along with the associated start and end transitions. To accomplish this, the code applies a differencing filter to the peak value data (indexed by rows) and uses the same techniques as applied to the row (time) data to determine the row locations of multiple peaks in that ridgeline. The code then outputs this data in a table format. The characteristics are peak height (in current), the potential of the maximal point (in voltage), the start, stop, and maximum of the peak, as well as the duration (Table 7.1).

The fourth function displays each detected ridge as a 3-D surface plot that can be rotated. There are three initial ridges in the example data, thus three 3D color plots were generated, however only the first is shown (Figure 7.7). The color plot shows the unique

CVs for adenosine transients in three dimensions, thus allowing identification of adenosine events.

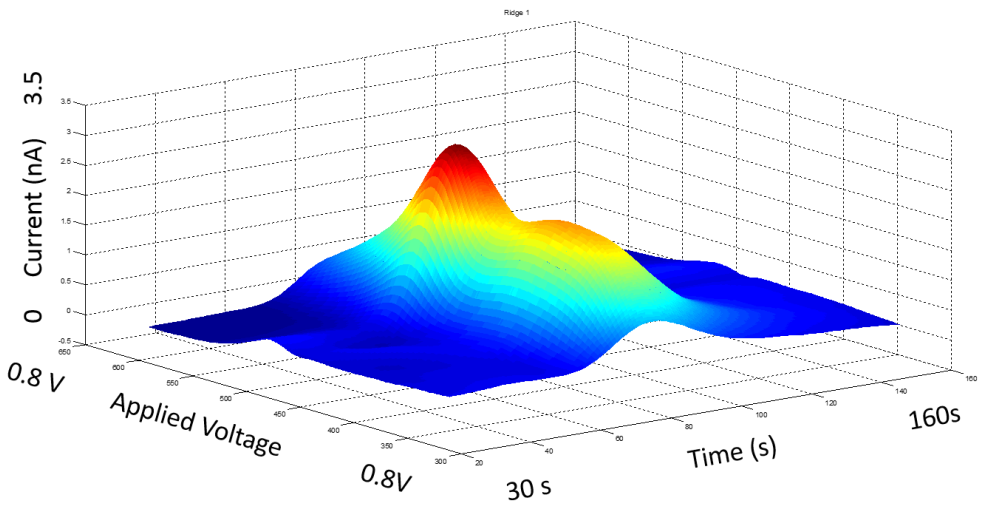

Figure 7.7 3-D heat map of an identified transient event

Heat map of the first identified transient. The program generates a plot of every detected event.

	Peak #	Height (nA)	Row (V)	Time (s)	Start (s)	End (s)	Duration
Ridge 1	1	2.1	1.3*	8.5	4.2	13.2	9.0
	2	3.0	1.3	8.1	5.6	11.8	6.2
Ridge 2	1	0.6	1.1*	84.5	82.3	88.5	6.2
	2	0.7	1.3	84.0	81.9	88.7	5.1
Ridge 3	1	1.1	1.2*	113.8	111.4	117.8	6.4
	2	1.6	1.3	113.3	111.2	116.9	5.7

* Denotes cathodic scan

Table 7.1 Data generated from MatLAB

For a comparison, the values collected manually using PCA are shown in Table 2

	Peak #	Height (nA)	Row (V)	Time (s)	Start (s)	End (s)	Duration
Ridge 1	1	3.3	1.3	7.8	x	x	4.2
Ridge 2	1	1.5	1.4	83.5	x	x	3.0
Ridge 3	1	2.2	1.3	113.2	x	x	4.4

x - not all values were collected manually, such as secondary peak information

Table 7.2 Data generated from manual analysis with HDCV

7.3 Comparison between human and computer analysis

The differences between the program (Table 7.1) and human analysis (Table 7.2) are noticeable, specifically in the peak heights and the durations. However, this is the earliest rendition of the code that has been compared to manually calculated values and the script is within range of the expected values. The peak times are slightly off, however the computer program is averaging every 13th data point accounting for some of the differences. The peak potentials are not exact matches; however they are sufficient for identification of adenosine, especially since the program generates 3-D plot of the event. Finally, the durations are longer from the MatLab analysis. This can be accounted for by multiple reasons. The first is that all durations are higher in the automated program which demonstrates the computer is choosing a beginning point and end point different from manual analysis. The start of the peak is more easily detected as the shape of most peaks have a sharp increase at the beginning, followed by a gradual decay. Thus the most probable discrepancy in the peak duration from automated and manual is in the analysis of the end of the peak. The detection of the end point of the peak can be modified in the program to match the manual analysis. The second reason comes from the filtering of seven data points, which could result in a longer peak duration. Regardless of the reason

for the increase in duration, the program will quantify peak duration more uniformly than any manual analysis could.

7.4 Future directions and conclusions

The computer program is capable of aiding in the detection of spontaneous, transient adenosine events, albeit some modifications need to be made going forward. The modifications include, but are not limited to, automatically background subtracting files, correcting electrode drift, a more streamlined approach, and a feature to examine reduction peaks as well as oxidation peaks. The program has the ability to pick out transient events that are not specific to adenosine, such as dopamine. Additionally, the automatic detection more uniform in the analysis of peaks than any manual detection. Thus, we have a new automatic analysis program that can detect spontaneous, transient adenosine events, saving countless hours of manual data analysis.

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