Electrochemical Detection of Rapid Adenosine Changes in the Brain

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Abstract

Adenosine is an important molecule in the central nervous system that modulates neurotransmitter release and induces neuroprotective effects during physiological and pathological conditions. Rapid adenosine release has been discovered using fast-scan cyclic voltammetry, which is a paradigm shift for the understanding of the time course of adenosine. However, there are still questions associated with the mechanism of rapid adenosine formation and its functions on a rapid time scale. Here, pharmacological tests as well as transgenic animals were used to investigate the formation and functions of rapid adenosine release.

In **Chapter 2**, two modes of rapid adenosine release: (1) spontaneous adenosine release, with no stimulation and (2) mechanically-stimulated adenosine release in the prefrontal cortex, hippocampus, and striatum were characterized**.** The frequency of spontaneous adenosine release and the concentration of mechanically-stimulated adenosine release differ among regions. The hippocampus had higher frequency of spontaneous release events and higher concentration of stimulated release, whereas the prefrontal cortex had less frequent spontaneous release and lower concentrations of mechanically-stimulated release. Rapid adenosine release differs in brain regions and therefore its neuromodulator effects vary, which is important for the future development of novel therapeutic treatments for different diseases. In **Chapter** 3, the mechanism of adenosine formation for the two modes of adenosine was studied in hippocampus. To understand whether rapid adenosine release was from extracellular breakdown of ATP,

transgenic mice that lack of NTPDase1 (CD39), converting ATP or ADP to AMP or ecto-5′-nucleotidase (CD73), converting AMP to adenosine, were used. CD39 and CD73 knockout (KO) did not affect the concentration of spontaneous and mechanically-stimulated adenosine, indicating other pathways are involved in the rapid adenosine release. However, the frequency of spontaneous release events decreased in CD73KO and CD39KO mice, so these enzymes differentially regulate the frequency of spontaneous release. Thus, future pharmacological agents could target these enzymes and preferentially target just the spontaneous mechanism.

Chapters 4-5 described the function of spontaneous adenosine release. In **Chapter 4**, I developed a method to simultaneously detect rapid adenosine release and oxygen levels *in vivo* using a modified voltage waveform. Adenosine and oxygen events were correlated, as 34% of adenosine events were followed by an oxygen event. The frequency of rapid adenosine and oxygen release was regulated through A_{2A} receptors but not A_1 receptors. In **Chapter 5,** the correlated adenosine and oxygen events during ischemia and reperfusion (I/R) injury were further studied. Ischemia was induced with bilateral common carotid artery occlusion. The frequency of adenosine and oxygen events increased during I/R, indicating the local blood flow was transiently increased. However, blockade of A_{2A} receptors eliminated the increase of correlated adenosine and oxygen events. These studies provide an understanding that adenosine transiently changes local blood flow even during ischemia, thus, adenosine may exert local neuroprotective effects during I/R injury.

Overall, this thesis highlights the possibility of rapid adenosine release as a therapeutic candidate for treatment of various diseases, including stroke. By understanding how and where rapid adenosine releases, strategies to regulate the frequency of spontaneous release or increase local concentration of adenosine by stimulation would be practical for future treatment of adenosine-related diseases.

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Acknowledgments

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Chapter 1: Introduction

1.1 Overview of adenosine

1.1.1 Introduction of adenosine

Adenosine is an important neuromodulator and neuroprotector in the central nervous system (CNS). It is produced primarily from the metabolism of adenosine triphosphate (ATP) and distributed throughout the entire body. Adenosine was first identified by Drury and Szent-Gyorgyi in 1929 as a physiological regulator of coronary vascular tone.[1] In 1931, Bennet and Drury showed more actions of adenosine signaling, including a marked lowering of body temperature.[2] Since then, adenosine has been extensively studied. In the CNS, adenosine is considered as a neuromodulator affecting neurotransmitter release[3] and synaptic plasticity[4]. Adenosine plays a vital role in the regulation of many biological functions, including blood flow,[5] pain,[6] memory,[7] and sleep[8]. Adenosine signaling has also been implicated as a neuroprotector in multiple diseases such as stroke[9,10], ischemia[11], epileptic seizures[10], and oxidative stress events[12].

Traditional methods of adenosine measurement are on the slow time scale of minutes to hours. A recent analytical approach, fast-scan cyclic voltammetry (FSCV) using carbon fiber microelectrodes, provides sufficient selectivity and sensitivity to detect adenosine release with sub-second temporal resolution.[13,14] This method led to the discovery of a rapid mode of adenosine release; however, there are still many gaps in our understanding of the formation and function of rapid adenosine release.

1.1.2 Adenosine formation and metabolism

Adenosine is an endogenous purine nucleoside composed of adenine attached to a ribose sugar. Adenosine is formed at both intra- and extracellular sites by a variety of

mechanisms. The intracellular formation of adenosine relies on the catabolism of cytosolic ATP or ADP to adenosine monophosphate (AMP) during metabolic stress, which is linked to the maintenance of cellular energy balance.[15] The main source of intracellular adenosine formation is cytosolic 5′-nucleotidase, which dephosphorylates AMP to adenosine. Under normal oxygenated conditions, ATP catabolism and anabolism rates are equal, which maintains the cytosolic concentration of both ATP and ADP.[16] The intracellular concentration of ATP is about 50 times higher than AMP; therefore, a small variation of ATP concentration could lead to a significant change of AMP available for the subsequent formation of adenosine.[17] Under tissue stress or trauma, inadequate oxygen supply limits the oxidative phosphorylation of ADP to regenerate ATP, which leads to an increase in ADP concentration and acetylate kinase activity in the direction of AMP formation (Fig. 1.1). Subsequently, increased AMP is hydrolyzed to adenosine and adenosine is then released into the extracellular space by an equilibrative adenosine transporter (ENTs) due to the increased cytosolic concentration. After intracellular reuptake, adenosine can be phosphorylated directly to AMP by adenosine kinase or deaminated to inosine by adenosine deaminase as shown in Figure 1.1.[18]

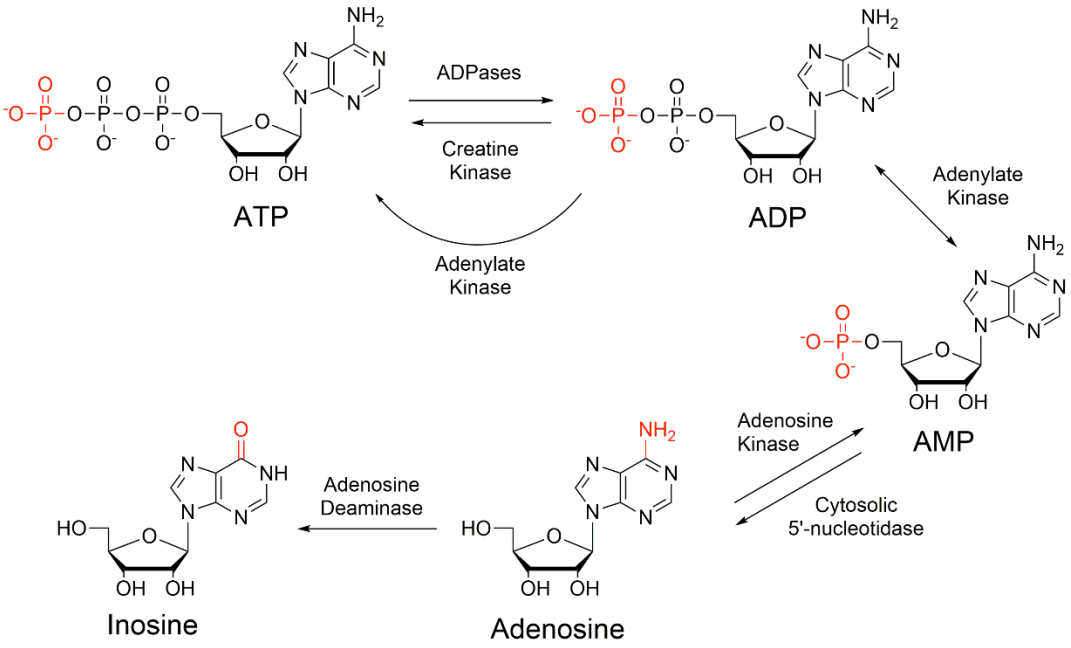

Figure 1.1 Metabolic pathways of ATP, ADP, AMP and adenosine. ATP can hydrolyze to ADP by ADPase and can be generated back from ADP by creatine kinase. Two ADP molecules can generate one ATP and one AMP molecule by adenylate kinase, and this process is reversible. Adenosine is produced by the hydrolysis of AMP by cytosolic 5'-nucleotidase. Adenosine can either generate AMP by adenosine kinase or produce inosine by adenosine deaminase.

Another pathway of intracellular adenosine formation is the hydrolysis of S-adenosylhomocysteine (SAH) via SAH hydrolase (SAHH), which is the transmethylation pathway and also produces L-homocysteine.[19] The reaction of hydrolysis of SAH is reversible, depending on the local concentration of adenosine and homocysteine. In the heart, the majority of adenosine is from hydrolysis of SAH under normoxic conditions. SAHH is also widely expressed in the brain and has high expression levels in cortex and cerebellum.[19] However, SAH synthesis has low impact on adenosine formation in brain.

In general, extracellular formation of adenosine is attributed to degradation of ATP, ADP and AMP.[18] ATP or ADP is first catalyzed to AMP by ecto-nucleoside triphosphate diphosphohydrolase (NTPDases; e.g., NTPDase 2, CD39) and then AMP is

dephosphorylated to adenosine by ecto-5′-nucleotidase (CD73).[20] Adenosine accumulation is also limited by its deamination to inosine by adenosine deaminase.[18] Another source of extracellular adenosine is through its release from synaptic vesicles by a Ca^{2+}-dependent excitation-secretion mechanism.[21]

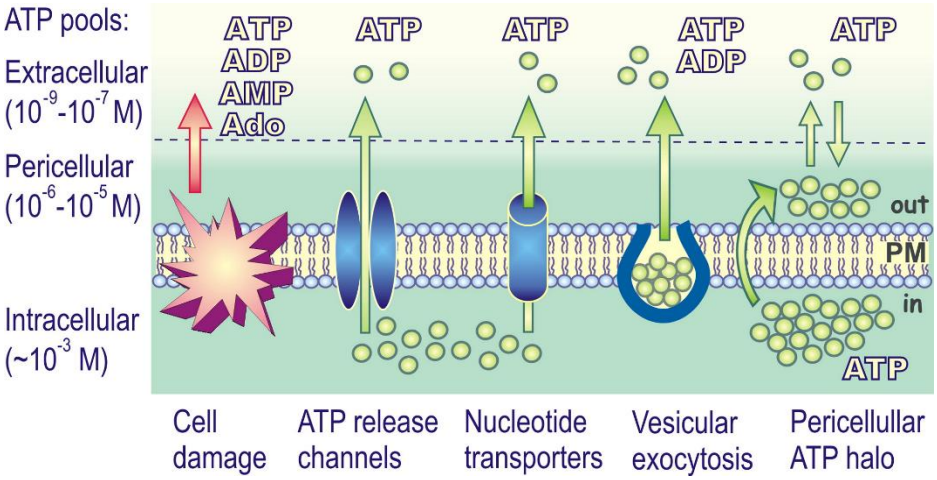

Figure 1.2 ATP release pathways. ATP can be released by cell damage such as mechanical perturbation and shear stress, ATP release channels, including pennexin 1, nucleotide transporters, or exocytosis. ATP in the pericellular space can also exchange with bulk extracellular ATP by certain dissociation-association mechanism. Figure reprinted with permission from Gennady G. Yegutkin. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Elsevier. 2008; 1783 (5): 673-694. Copyright 2008 Elsevier B.V.

Extracellular released ATP can rapidly degrade to adenosine within 200 milliseconds. Thus, knowing the pathways of ATP is important to reveal the mechanism of adenosine formation. Pathways for ATP release into extracellular spaces are diverse. Extracellular release of ATP could be mediated by (1) exocytosis,[22] (2) multiple types of channels,[23] including pannexin 1 and connexin hemichannels; or (3) nucleotide transporters (Fig. 1.2). Large amount of ATP release occurs in response to mechanical and other stimulations, such as mechanically pertubation,[24] swelling, [25] and shear stress[26]. In addition, micromolar concentration of “ATP halo” is found in the pericellular space,

which exchanges with bulk extracellular ATP by certain dissociation-association mechanism (Fig. 1.2).[27,28]

Adenosine metabolism is carried out by two enzymes: adenosine deaminase (ADA) and adenosine kinase (ADK), which reduce adenosine concentration by catalyzing the phosphorylation and deamination of adenosine to form AMP and inosine.[29] The estimated cytosolic concentration of adenosine is 0.01 – 0.1 μM at normoxic conditions.[19] The K_m of ADA for adenosine is 25-150 μM, however, the K_m of ADK for adenosine is much lower than that of ADA, about 1 μM. Therefore, ADK is considered to be the primary route of adenosine metabolism under physiological conditions. Recently, rapid adenosine release has been discovered in real-time, however, the mechanism of adenosine formation is not fully known. It is important to understand if rapidly formed adenosine is generated from metabolism of ATP or directly released as adenosine per se.

1.1.3 Adenosine receptors

Adenosine mediates its biological effects by interacting with four G protein-coupled receptors, namely the A_1, A_{2A}, A_{2B} and A_3.[30] These receptors are coupled to different G proteins: A_1 and A_3 (via Gi) inhibit the neurotransmission by blocking adenylyl cyclase to decrease cAMP level, whereas both A_{2A} and A_{2B} (via Gs) stimulate adenylyl cyclase and increase generation of cAMP.[31] The A_1 and A_{2A} are highly expressed in the brain and have high affinity for adenosine at approximately 70 nM and 150 nM, respectively.[32] However, the A_{2B} and A_3 are less abundant in the brain and have a relatively lower affinity for adenosine at 5100 nM and 6500 nM, respectively.[32] Therefore, the impact of adenosine on brain function may mainly depend on the signaling of A_1 and A_{2A} receptors.

1.1.3.1 A_1 receptors

In the CNS, A_1 receptors are highly expressed in cerebral cortex, hippocampus and cerebellum.[30,33,34] These receptors are located presynaptically, postsynaptically and nonsynaptically[35] and modulate the release of neurotransmitters, such as glutamate, acetylcholine, serotonin and GABA[36]. Adenosine acting through A_1 receptor is known to exert an inhibitory effect on synaptic transmission.[37] A_1 receptors are mainly enriched in excitatory synapses.[38–40] The inhibition of synaptic transmission is largely due to presynaptic A_1 receptors[41–43] and is suggested to rely on the coupling of A_1 receptors to the inhibition of N-type calcium channels[44,45], which decrease stimulus-evoked release of glutamate in the central synapses.[46,47] Postsynaptic A_1 receptors can simultaneously control N-type calcium channels and NMDA receptors to influence the response of excitatory stimuli.[48,49] Nonsynaptic A_1 receptors can control potassium current leading to neuronal hyperpolarization.[50] A_1 receptors are also located on non-neuronal localizations and modulate the functions of astrocytes and glia in brain tissue.[51–53]

1.1.3.2 A_{2A} receptors

A_{2A} receptors are highly concentrated in olfactory bulb as well as in dorsal and ventral striatum.[54–59] A_{2A} receptors express at lower levels outside of striatum in hippocampus and cortex.[58,60] These receptors are mostly located in synapses.[61] Adenosine acting at A_{2A} receptors exert an excitatory effect on synaptic transmission.[62] Presynaptic A_{2A} receptors control the release of glutamate and also terminate the effect of A_1 receptor-mediated inhibition of synaptic transmission to increase neuronal activity.[63,64] A_{2A} receptors, which are located postsynaptically, can control NMDA receptors.[65–67] A_{2A}

receptors are also located in endothelial cell and vascular smooth muscle cells, where they play an important role in vasodilation.[68]

1.1.4 Relationship among adenosine, blood flow, and oxygen

Adenosine is a vasodilator, regulating blood flow to deliver oxygen and other metabolites to tissue. Adenosine-related vasodilation has been widely studied under a variety of conditions. Intravenous injection of adenosine increases regional cerebral blood flow,[69] reflected by increase in levels of oxygen. Adenosine-related vasodilation is attributed to the adenosine A_{2A} receptors, where activation of adenosine A_{2A} receptors on cerebral vascular smooth muscle and endothelial cells causes cerebral vasodilation and thus increases blood flow.[70,71] Caffeine, a nonselective adenosine antagonist, binds to adenosine receptors to result in decreased baseline of cerebral blood flow in humans as shown by various measurement techniques.[72–75] Adenosine A_{2A} receptor agonist, CGS 21680, enhanced cortical blood flow under hypoxic conditions, while A_{2A} receptor antagonist, ZM241385, reduced the increase in cortical blood flow induced by hypoxia.[76]

1.1.5 The role of adenosine in different brain diseases

Adenosine is an important neuromodulator and neuroprotector in the brain and influences many functions. The levels of adenosine change when there is unbalance between energy production and energy requirement. Adenosine levels were markedly increased with increased neuronal activity, particularly during hypoxia or ischemia.[77] The potential role of adenosine as an endogenous neuroprotective agent in different diseases, such as ischemia, epilepsy, Parkinson's disease, and Alzheimer's disease has been repeatedly emphasized, where its neuromodulatory role mainly relies on the A_1 and A_{2A}

receptors. Due to widespread involvement of adenosine in different brain diseases, it is important to understand its regulation in order to improve therapeutic strategies to treat diseases.

1.1.5.1 Epilepsy

A seizure is the clinical manifestation of epilepsy, which occurs due to an abnormal, excessive, hypersynchronous discharge of a population of cortical neurons.[78] Extracellular adenosine is a critical indicator to determine the susceptibility of the brain to seizure activity.[10] The concentration of extracellular adenosine increases upon seizure activity.[79] Overexpression of ADK in transgenic mice reduces extracellular adenosine level and therefore increases susceptibility to seizure.[80] However, transgenic mice with reduced expression of ADK were resistant to status epilepsy. Thus, adenosine may be considered as a potential anti-epileptic substance.

The effect of adenosine on epilepsy is mainly mediated by A_1 receptors as activation of A_1 receptors decreases the hyper-excitability associated epilepsy.[81] Several studies have confirmed the critical role of A_1 receptors in controlling epilepsy development. For example, activation of A_1 receptors by A_1 agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA), suppressed seizure activity.[82] Conversely, inhibition of A_1 receptors by A_1 antagonist, DPCPX, caused longer seizure durations.

1.1.5.2 Alzheimer's diseases

Alzheimer's disease is a common neurodegenerative disorder of the central nervous system that affects the elderly and is manifested by cognitive and memory deterioration. Diagnosis of Alzheimer's disease is based on clinical symptoms and is

confirmed post-mortem by the observation of the presence of neurofibrillary tangles and senile plaques, which are characterized by the extracellular accumulation of the amyloid-beta (Aβ) protein and the pathological accumulation of tau protein in neurons. Several studies investigated the role of A_1 and A_{2A} receptors in Alzheimer's disease. Pharmacological and genetic blockade of A_{2A} receptors were found to be protective by preventing Aβ-induced synaptotoxicity[83] and improving memory[84]. A_1 receptors are highly expressed in the CA1 region of hippocampus,[85] while a reduced level of A_1 receptors has been found in Alzheimer's disease.[86] Thus, manipulation of adenosine receptors may be beneficial for treatment of Alzheimer's disease.

1.1.5.3 Traumatic brain injury

Traumatic brain injury is immediate mechanical damage of brain tissue, which can cause lasting effect of chronic pain[87], anxiety[88], epilepsy[89], and other sleep disturbances[90]. Previous studies have shown that adenosine is markedly increased after experimental traumatic brain injury and the increase of adenosine concentration is correlated with injury serverity.[91] Modulation by adenosine receptors has shown beneficial effects on traumatic brain injury *in vivo*, where A_{2A} deficient mouse exhibited decreased cognitive impairment and neuropathological damage.[92] Chronic caffeine treatment as a non-specific adenosine receptor antagonist also attenuated brain injury by suppressing glutamate release and inhibiting inflammation.[93] However, blocking A_1 receptors with DPCPX after injury exacerbated neuronal death and attenuated motor function.[94] Similar to previous observations of a large increase in extracellular adenosine, lowering a carbon fiber microelectrode into brain tissue, which is used to study mechanosensitive adenosine release in real time, also evokes a rapid and large adenosine

release.[95] However, the source or formation of adenosine that is available for mechanically-stimulated release is unclear. More research is needed to determine the function and mechanism of formation of mechanically-evoked adenosine.

1.1.5.4 Ischemia

Adenosine plays an important role as an endogenous neuroprotector in brain ischemia. Adenosine concentration dramatically increases in the extracellular space after cerebral ischemia.[96,97] This increase of adenosine concentration can be explained by different mechanisms: (1) hydrolysis of extracellular ATP in the first 20 min after ischemia onset;[97] (2) adenosine outflow through equilibrative nucleoside transporter 2 (ENT2) following the concentration gradient as adenosine builds up due to hydrolysis of intracellular ATP;[97] or (3) inhibition of adenosine uptake by down-regulating concentrative nucleoside transporter 2 and 3 as well as ENT 1.[96] Adenosine acts at receptors, primarily A_1 and A_{2A}, located on cells and on blood vessels to exert its effects and these receptors are important targets in the treatment of ischemia.

Activation of A_1 receptors inhibits excitatory synaptic transmission during hypoxia/ischemia both *in vitro*[98] and *in vivo*[99], as activation of A_1 receptors can reduce Ca^{2+} influx and thus lower presynaptic neurotransmitter release.[100] In *in vitro* hypoxia/ischemia models, selective A_1 receptor agonists reduce cellular damage or death following hypoxia in cultured cerebellar neurons[101] and in brain slices[102]. In *in vivo* animal models, systemic administration of A_1 agonist, 2-chloro-N(6)-cyclopentyladenosine (CCPA), reduces oxidative stress and inflammation after a cerebral ischemic injury.[103] These findings support the neuroprotective role of the activation of A_1 receptor.

The activation of A_{2A} receptors shows a potential therapeutic role at different times in the treatment of stroke. Administering the selective A_{2A} receptor antagonist, ZM241385, prior to ischemia, was neuroprotective in hyperglycemic cerebral ischemia, as shown by reduced hippocampal injury and improved Morris water maze performance in rats.[104] Another study showed administration of A_{2A} receptor antagonist, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4,triazolo[1,5-c]pyrimidine (SCH58261), after hypoxia/ischemia reduced ischemic brain damage in rats.[105] Therefore, it is important to consider the time duration of drug exposure to activate adenosine A_{2A} receptors in the treatment of stroke. Spontaneous adenosine events increased in frequency by about 52% during ischemia-reperfusion injury and A_{2A} antagonist, SCH 442416 (3 mg/kg, *i.p.*) decreased the frequency of spontaneous events about 31%.[106] However, whether rapid adenosine release is able to cause blood flow increase during ischemia-reperfusion injury in unknown. More research is needed to determine how rapid adenosine release affects blood flow during stroke.

1.2 Analytical methods to detect adenosine

Electrochemical detection of neurotransmitters has become more popular as it enables researchers to monitor rapid neurotransmitters change, even within complex brain tissue. Fast-scan cyclic voltammetry (FSCV) at a carbon-fiber microelectrode has been successfully used to detect neurochemicals, such as adenosine,[14] dopamine,[107] hydrogen peroxide[108] and serotonin[109] *in vivo* as these neurotransmitters are electroactive and can be oxidized at specific voltages. Traditional methods of adenosine measurement, such as radiometric labeling with HPLC analysis and microdialysis, are on the minute time scale.[110,111] FSCV has been used to measure endogenous adenosine release in both

ex vivo and *in vivo* experiments on a sub-second time scale, indicating adenosine can be released and cleared within a few seconds.

1.2.1 Detection of adenosine using traditional methods

Several experimental tools have been developed to characterize brain function by measuring chemical and bioelectrical signals. The most widely used is microdialysis, which removes dialysate from the brain for subsequent analysis by HPLC.[112,113] However, its utility is limited by low temporal and spatial resolution. The probe size (200 – 500 μm) of microdialysis is larger than brain cell and blood vessels, which could cause cell damage.[114,115] Microdialysis requires longer sampling periods, ranging from minutes to an hour, and is not effective at measuring rapid changes in concentration of adenosine. Biological enzyme sensors with a diameter of 25 μm provide rapid measurements on the order of seconds and allow the measurement of rapid changes in adenosine concentration.[98] Using purine biosensors, adenosine changes have been observed by increasing extracellular glucose concentration, where adenosine levels increase 2-fold in response to glucose and 3-fold during the wakefulness period in mice.[116] Adenosine increases are also observed with acute hyperammonemia and systemic inflammation, but the time frame for this experiment is limited to 70-80 minutes due to loss of sensitivity of the biosensor *in vivo*.[117] The construction of enzyme biosensors requires three enzymes, adenosine deaminase, nucleoside phosphorylase, and xanthine oxidase to break down adenosine and produce hydrogen peroxide, which is detected by amperometry. A null sensor is also required to distinguish adenosine from inosine. Biosensors are useful when quantifying concentration of adenosine. However, their slower temporal response (2 s to

1 min), which is due to interferences of adenosine metabolites, cannot provide sufficient information to understand transient adenosine signals on a sub-second time scale.

1.2.2 Characterization of adenosine using fast-scan cyclic voltammetry (FSCV)

Fast-scan cyclic voltammetry (FSCV) with a carbon-fiber microelectrode is an electrochemical technique that measures changes in electroactive species *in vitro* and *in vivo*. With FSCV, the potential is ramped and the resultant current is measured. FSCV has many advantages, including excellent sensitivity, moderate selectivity, high spatial resolution, and sub-second time resolution.[118] To measure adenosine with FSCV, a triangle waveform scans from –0.4 V to 1.45 V *vs.* a Ag/AgCl reference electrode and back with a scan rate of 400 V/s at 10 Hz (Fig. 1.3A). The charging of the electrode surface results in a large, relatively stable background current, which is proportional to the capacitance of electrode and scan rate (Fig. 1.3B)[119]. The background current can be subtracted out to obtain a smaller faradic current change due to oxidation of adenosine. In the resulting cyclic voltammogram, the primary oxidation of adenosine occurs at 1.3 V on the cathodic scan and the secondary oxidation occurs around 1.2 V on the anodic scan, which is the characteristic electrochemical fingerprint to identify adenosine (Fig. 1.3C). Adenosine undergoes three oxidation steps (Fig. 1.4), but only the first and second 2-electron oxidations of adenosine are usually observed with carbon-fiber microelectrodes, and these two oxidation steps are irreversible.[14] The oxidations of adenosine can also be seen in the example 3D color plots of adenosine events in a 30 s window in each brain region (Fig. 1.3D). The green /purple circles in the center of the 3D color plot represent oxidative current of adenosine. The top circle is the primary oxidation of adenosine and the circle directly below is the secondary oxidation of adenosine, which is proportional to

concentration. Concentration vs. time traces show the primary oxidation of adenosine, which is derived from the color plots below (Fig. 1.3D).

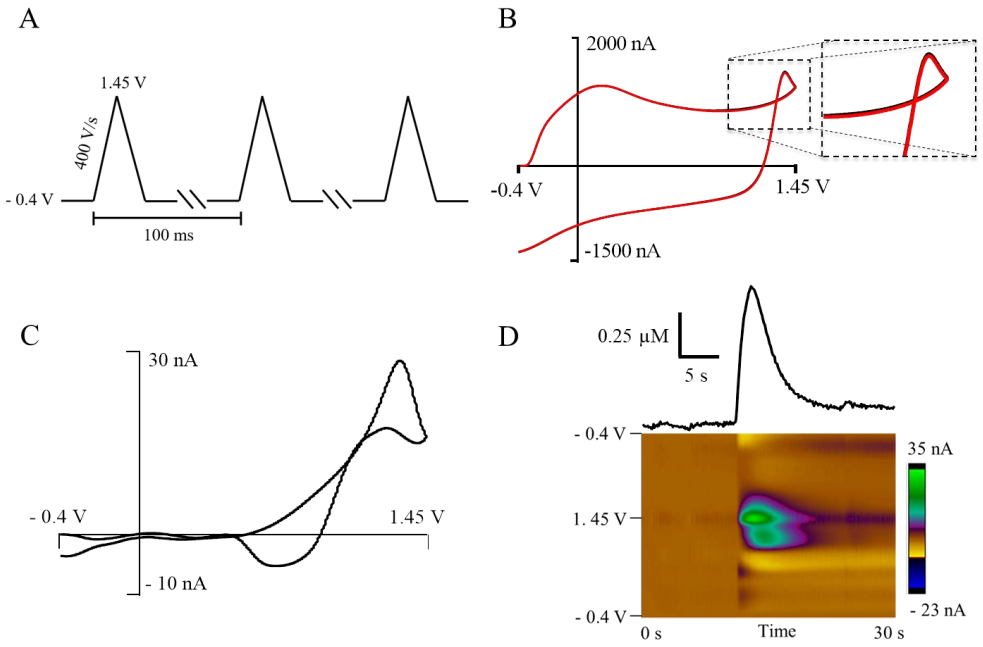

Figure 1.3 Detection of adenosine using FSCV. (A) Fast changes of adenosine were monitored using FSCV at a carbon fiber microelectrode by scanning from – 0.4 V to 1.45 V and back with a scan rate of 400 V/s at 10 Hz. (B) Fast scan rate produces a large and relatively stable background current (red line). The black line is the background current with oxidation current of adenosine. (C) A cyclic voltammogram for adenosine shows that the primary oxidation occurs at 1.3 V and the secondary oxidation occurs at around 1.2 V. (D) 3D color plot of adenosine in a 30 s window. Time is plotted on the abscissa, applied potential is plotted on the ordinate, and the current is displayed in false color. The green /purple circles in the center of the 3D color plot represent oxidative current of adenosine. The top circle is the primary oxidation. Concentration *vs.* time trace shows the primary oxidation of adenosine, which is derived from the color plots below.

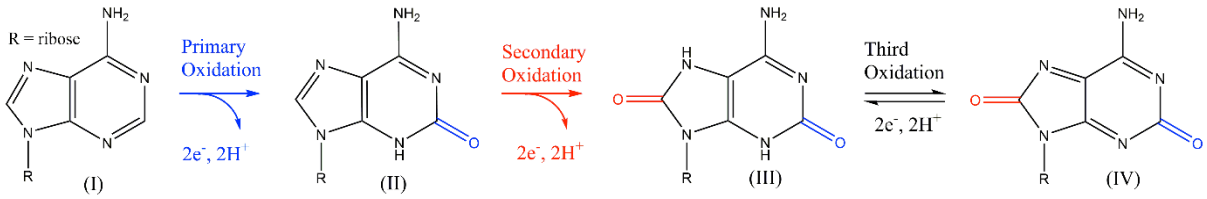

Figure 1.4 Electrochemical reaction of adenosine. Adenosine has three oxidation steps, where the first two steps are irreversible and are usually detectable using FSCV at a carbon fiber microelectrode. The third oxidation step is reversible and seldom observed with FSCV.

1.2.2.1 Electrically-stimulated adenosine release

Transient adenosine release was first characterized in caudate-putamen using FSCV after electrical stimulation of dopaminergic neurons in substantia nigra/ventral tegmental area of anesthetized rats.[120] The dopamine signal, which was also measured by FSCV, appeared immediately after electrical stimulation and was cleared from the extracellular space within 3-4 s, while adenosine signal was slightly delayed and cleared in about 15 s (Fig. 1.5). The average concentration of adenosine was 0.94 ± 0.09 μM. Pharmacological studies were also performed to confirm the identity of adenosine. Even though histamine has a similar cyclic voltammogram as adenosine, a histamine synthesis precursor, L-histidine, has no effect on adenosine signal. In addition, the concentration of adenosine was increased with a selective non-nucleoside adenosine kinase inhibitor, ABT-702, which inhibits the metabolism of adenosine. Administration of an adenosine receptor antagonist, theophylline, decreased the concentration of stimulated adenosine and oxygen, indicating stimulated adenosine release was correlated with an oxygen signal.

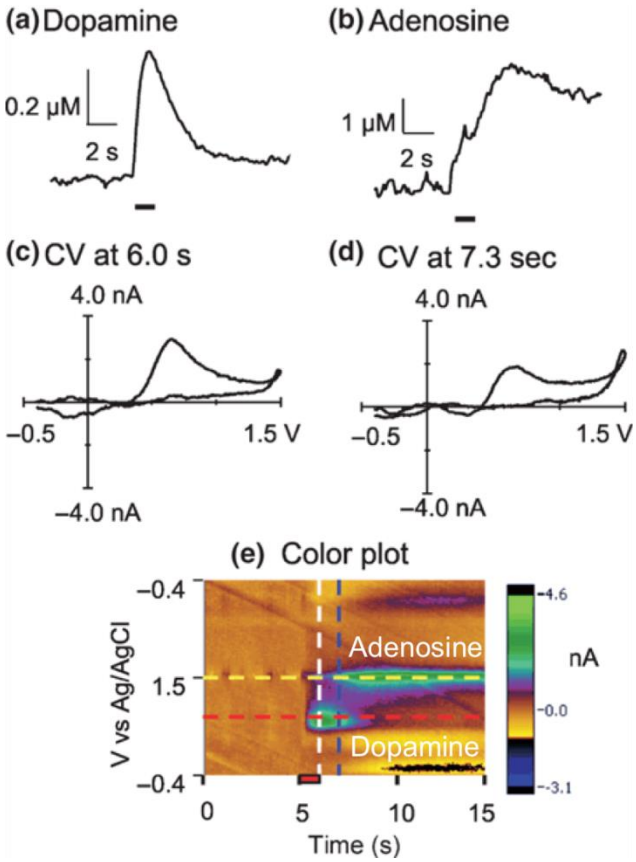

Figure 1.5 Electrically-stimulated adenosine and dopamine release in the caudate-putamen. Concentration *vs* time traces of (a) dopamine and (b) adenosine. The clearance of dopamine is faster than adenosine. Cyclic voltammograms (CV) of (c) dopamine and (d) adenosine were taken at 6.0 s and 7.3 s, receptively. The CV at 6 s shows clear oxygen oxidation peak at 0.6 V and small peak of adenosine at 1.5 V. However, the CV at 7.3 s shows a clear oxidation of adenosine peak. (e) The oxidations of dopamine and adenosine can also be seen in the example 3D color plot in a 15 s window. Dopamine was released immediately after stimulation but adenosine release appeared slightly delayed from the start of stimulation. Figure reprinted with permission from Cechova S, Venton BJ. Transient adenosine efflux in the rat caudate-putamen. J Neurochem. 2008; 105(4): 1253-1263.[120] Copyright 2008 John Wiley and Sons.

Adenosine A_1 receptors can self-regulate adenosine release.[121] An adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA), decreased both stimulated adenosine and dopamine release, while an A_1 antagonist, 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) increased stimulated adenosine release. These effects were partially mediated

by dopamine D_1 receptors but adenosine release was not a downstream action of dopamine release, indicating A_1 receptors can self-regulate adenosine release.

Electrically-stimulated adenosine release has been characterized in the caudate-putamen, nucleus accumbens, hippocampus and cortex with 5 pulses at 60 Hz.[122] The concentrations of stimulated adenosine vary between brain regions, where the stimulated adenosine is highest in the dorsal caudate putamen with average of 0.34 ± 0.08 μM and is lowest in the secondary motor cortex with concentration of 0.06 ± 0.02 μM. Pharmacological tests revealed that adenosine release was activity-dependent in all brain regions and was partially dependent on degradation of ATP in the nucleus accumbens, hippocampus and prefrontal cortex, while adenosine release was ionotropic glutamate receptor-dependent in the caudate-putamen. Thus, the electrically stimulated adenosine release varies in regions. It will be interesting to determine whether spontaneous release follows the similar pattern as stimulated release in each brain region.

The mechanism of electrically stimulated adenosine release was further investigated in striatal rat brain slices using low- and high-frequency stimulations.[123] Low-frequency stimulation with 5 pulses at 10 Hz evoked a lower degree of adenosine release with average measured concentration of 0.22 ± 0.02 μM, while high-frequency with 5 pulses at 60 Hz resulted in increased adenosine release with concentration of 0.36 ± 0.04 μM. Application of nucleoside transporter inhibitors, propentofylline (PPF, 50 μM) or *S*-(4-nitrobenzyl)-6-thioinosine (NBTI, 10 μM) did not decrease the concentration of adenosine, indicating other pathways, such as synaptic vesicle-mediated exocytotic release or extracellular breakdown of ATP may contribute to the electrically-stimulated release. Application of ARL-67156 (50 μM), which inhibits the degradation of ATP to

ADP and AMP, decreased low-frequency stimulated adenosine release. However, application of ARL-67156 (50 μM) alone or with AOPCP (100 μM), which inhibits the breakdown of AMP to adenosine, did not affect the concentration of adenosine resulting from high-frequency stimulation, indicating the high-frequency stimulated adenosine release does not depend on the metabolism of ATP. In addition, removing calcium ions or blocking ionotropic glutamate receptors attenuated both low- and high-frequency stimulated adenosine release, suggesting the stimulated adenosine release is activity-dependent. In summary, the mechanism of electrically-stimulated adenosine release is involved in multiple pathways.

1.2.2.2 Mechanically-stimulated adenosine release

Mechanically-stimulated adenosine release has been found by lowering a carbon fiber microelectrode or moving a small pipette near the recording electrode *in vivo* and in brain slices in the prefrontal cortex of rats.[95] The average concentration of mechanically stimulated adenosine release was 3.3 ± 0.6 μM *in vivo* by lowering the electrode 100 μm, and 0.8 ± 0.1 μM in brain slices by lowering the electrode 50 μm (Fig. 1.6). Multiple electrode stimulations did not change the concentration of adenosine resulting from mechanically perturbed adenosine release or significantly cause cell damage. However, application of tetrodotoxin (TTX, 0.5 μM) to block sodium channels or calcium chelation with EDTA decreased the concentration of adenosine, suggesting the release of adenosine is activity dependent. An AMPA receptor antagonist, CNQX (10 μM), did not significantly change the concentration of adenosine; therefore, mechanically-stimulated adenosine release was not dependent on AMPA receptors. A recombinant NTPDase 1, 2 and 3 inhibitor, POM-1 (100 μM), decreased the concentration of mechanical-evoked

adenosine, and thus, a portion of adenosine is from degradation of ATP. However, the mechanism of mechanically-evoked release is not fully understood. Whether mechanically-stimulated release varies between brain regions and if AMP is involved in the formation of release still needs to be investigated. In addition, it is important to determine whether spontaneous release occurs by the same or different mechanisms compared to stimulated release.

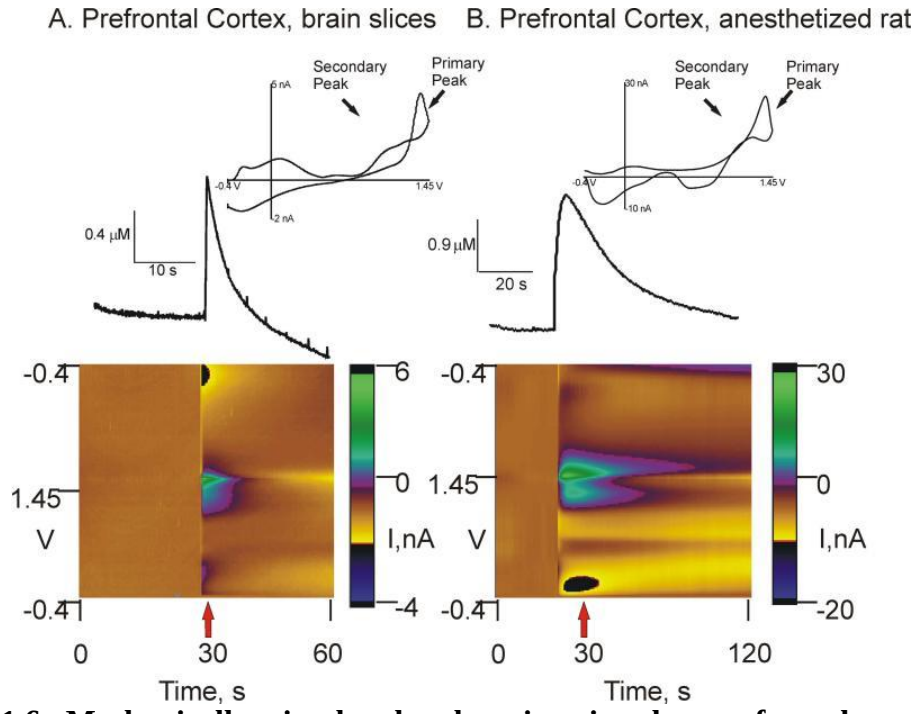

Figure 1.6 Mechanically-stimulated adenosine in the prefrontal cortex. (A) Mechanically-stimulated adenosine release by lowering a carbon fiber microelectrode 50 µm in brain slices. Cyclic voltammograms for adenosine show that the primary oxidation occurs at 1.3 V and the secondary oxidation occurs at around 1.2 V. Concentration vs time trace shows the primary oxidation of adenosine appears immediately after stimulation with a peak concentration of 2.4 µM. The green /purple circles in the center of the 3D color plot represent oxidative current of adenosine. (B) Mechanically-stimulated adenosine release by lowering a carbon fiber microelectrode 100 µm *in vivo* with a peak concentration of 0.77 µM. Figure reprint with permission from Ross AE, Nguyen MD, Privman E, Venton BJ. Mechanical stimulation evokes rapid increases in extracellular adenosine concentration in the prefrontal cortex. *J Neurochem.* 2014;130(1):50-60. Copyright 2014 John Wiley and Sons.

1.2.2.3 Spontaneous, transient adenosine release

Spontaneous adenosine release was discovered in lamina II of sagittal spinal cord slices using FSCV by the Zylka group. These spontaneous transients lasted only 1-2 seconds in the extracellular space and resulated from the breakdown of AMP.[124] Spontaneous adenosine release events were characterized by the Venton group in the caudate-putamen (Fig. 1.7) and prefrontal cortex *in vivo*.[13] The average concentrations of adenosine in the caudate-putamen and prefrontal cortex were similar with 0.17 ± 0.01 μM and 0.19 ± 0.01 μM, respectively. However, the frequency of spontaneous adenosine release events in the prefrontal cortex was higher than in the caudate-putamen. The frequency of adenosine release events was modulated by adenosine A_1 receptors, where the A_1 antagonist, DPCPX (6 mg/kg *i.p.*) increased the frequency of adenosine events in both brain regions. However, activation of adenosine A_1 receptor with the A_1 agonist, CPA (1 mg/kg, i.p.), only decreased the release of adenosine events in the caudate putamen and had no effect in the prefrontal cortex. The average duration of spontaneously released adenosine is about 3 s in both brain regions and thus, this adenosine release can provide transient, local neuromodulation.

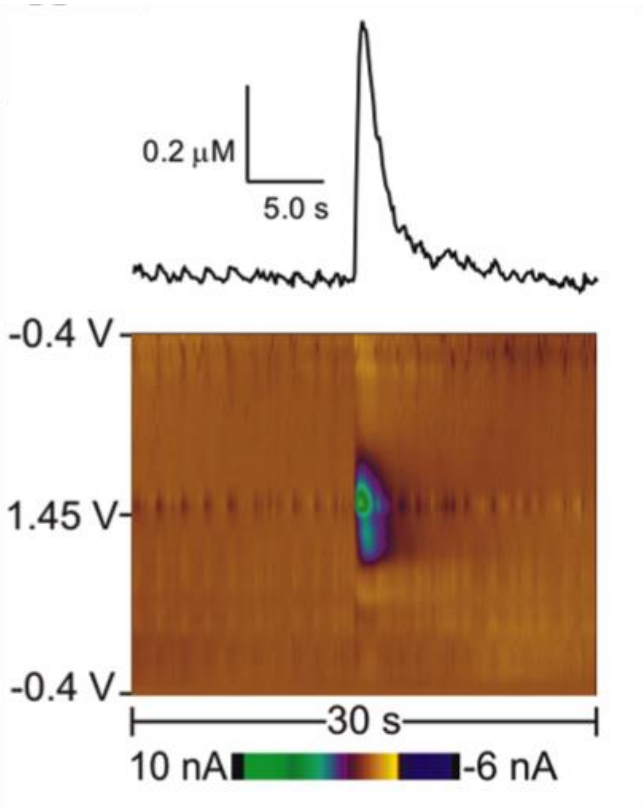

Figure 1.7 Spontaneous adenosine release in the caudate-putamen in vivo. 3-D color plot shows one spontaneous adenosine release event in a 30 second window. The concentration vs time trace shows the primary oxidation of adenosine with peak concentration about 0.6 μM, which was derived from the 3D color plot below. Figure reprint with permission from Nguyen MD, Lee ST, Ross AE, Ryals M, Choudhry VI, Venton BJ. Characterization of spontaneous, transient adenosine release in the caudate-putamen and prefrontal cortex. PLoS One. 2014;9(1):e87165.

The regulation of spontaneous adenosine release is also affected by glutamate and GABA receptors.[125] The glutamate NMDA antagonist, 3-(R-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, 6 mg/kg *i.p.*), increased the frequency of adenosine release events and resulting concentration of adenosine. However, the glutamate agonist, NMDA (50 mg/kg, *i.p.*), had no effect on frequency of adenosine release. The $GABA_B$ antagonist, CGP 52432 (30 mg/kg, *i.p.*), decreased the frequency of adenosine release events but the agonist, baclofen (5 mg/kg, *i.p.*), increased the frequency of release. Moreover, a selective antagonist of the AMPA receptor, NBQX (15 mg/kg, *i.p.*), a

specific metabotropic glutamate (mGlu2/3) antagonist, LY 341495 (5 mg/kg, *i.p.*), and a $GABA_A$ antagonist, bicuculline (5 mg/kg, *i.p.*), did not change the frequency of adenosine release events. Thus, spontaneous adenosine release events are modulated by NMDA and $GABA_B$ receptors.

A study of clearance of spontaneously released adenosine reveals an effect of both metabolism and uptake.[126] A specific ENTI inhibitor, NBTI (1 mg/kg or 40 mg/kg, *i.p.*), increased the duration of spontaneous adenosine release but had no effect on concentration and frequency. An adenosine kinase inhibitor, ABT-702 (5 mg/kg, *i.p*), or an adenosine deaminase inhibitor, EHNA (10 mg/kg, *i.p*), was used to inhibit metabolism of adenosine. The durations of spontaneous adenosine release were increased; therefore, the clearance rate of adenosine was decreased. Exogenously applied adenosine in brain slice, by pressure injecting adenosine before and after the brain slices were bathed in NBTI (10 μmol/L), ABT-702 (100 nmol/L), or EHNA (20 μmol/L), also decreased the duration of adenosine. Therefore, the clearance of extracellular spontaneously released adenosine is regulated by multiple mechanisms.

Spontaneous adenosine release events increase during cerebral ischemia and reperfusion injury in caudate-putamen of anesthetized rats.[106] During 30 minutes of ischemia, induced by bilateral common carotid artery occlusion (BCCAO), and 90 minutes of reperfusion, the frequency of spontaneous adenosine events increased. The number of adenosine events increased 52% during these periods compared to 2 hrs of normoxia. BCCAO does not cause cell death but cells exhibited shrinkage in the cell nucleus and swollen mitochondrial structure. Adenosine A_{2A} receptor antagonist, SCH 442416, decreased adenosine events by 27% during ischemia and reperfusion. Thus,

rapid adenosine release could provide local, rapid neuromodulation and neuroprotection during ischemia. Endogenous adenosine as a vasodilator increases cerebral blood flow during hypoxia.[127] A previous study shows a correlation of electrically stimulated adenosine and oxygen. Therefore, it is expected that spontaneous adenosine release may also affect oxygen changes in the brain. However, the extent to which spontaneously released adenosine has an effect on oxygen changes during ischemia reperfusion injury is unknown.

1.3 Concluding remarks

Previous studies on various diseases revealed long-term effects of adenosine, which could be mediated by targeting specific adenosine receptors. However, the time course of the real time signaling is difficult to obtain as measurement of adenosine previously occured on a slow time scale. Fast-scan cyclic voltammetry at a carbon fiber microelectrode has been successfully used to detect adenosine release *in vivo* and *ex vivo* in real time and provides a new understanding of the time course of extracellular adenosine signaling in the brain. However, more research is needed to understand the functions as well as the mechanisms of formation for adenosine release.

1.3.1 Mechanism of rapid adenosine formation

Previous studies have characterized spontaneous and mechanically-stimulated adenosine release *in vivo* and in brain slices in rats. Pharmacological studies also reveal how receptors regulate these events. However, the source and mechanism of formation of adenosine that is available for rapid release remains an open question. To better address the formation and mechanism of these modes of adenosine release, genetic mouse models

are needed. Therefore, I first characterized two modes of adenosine release in the mouse striatum, prefrontal cortex, and hippocampus in **Chapter 2**. This chapter provided fundamental information on the duration, frequency, and concentration of spontaneously released adenosine and the concentration of adenosine resulting from mechanically-stimulated release in various mouse brain regions. In **Chapter 3**, I examined whether these two modes of adenosine release depend on metabolism of extracellular ATP using CD39 knockout mice, which lacks nucleoside triphosphate diphospholydrolase 1 (NTDPase 1) to convert ATP or ADP to AMP, and CD73 knockout mice, which lacks ecto-5′-nucleotidase to convert AMP to adenosine. These two chapters provide information on regional variation and mechanism of rapid adenosine release and how adenosine could be modulated more effectively in the brain.

1.3.2 Function of rapid adenosine release

Adenosine regulates blood flow by activating A_{2A} receptors that are located on the blood vessels.[70] However, whether the rapid change of spontaneously released adenosine could regulate cerebral blood flow is unknown. Previous studies showed that oxygen could be reduced at a carbon fiber microelectrode around -1.2 V.[128] A modified FSCV waveform was used to detect pH and oxygen changes after stimulated dopamine release, where the electrode was scanned to -1.4 V to reduce oxygen.[21] I have further modified this waveform to be able to simultaneously detect both adenosine and oxygen release events in rat brain. In **Chapter 4**, I measured both spontaneous adenosine and oxygen release events using this modified voltage waveform in the caudate-putamen. The changes in cerebral blood flow are parallel with changes in oxygen. I found that spontaneous adenosine and oxygen release events are correlated. 34% of adenosine

events exhibited a correlated oxygen event. Higher concentrations of adenosine events were always correlated with oxygen events. Both adenosine and oxygen release events were modulated by adenosine A_{2A} receptors but not A_1 receptors. In **Chapter 5**, the relationship between adenosine and blood flow changes was further studied under ischemia/reperfusion (I/R) injury. Transient adenosine and oxygen events increased during ischemia induced by BCCAO and reperfusion periods. Blocking A_{2A} receptors with SCH 442416 (3 mg/kg, *i.p.*) eliminated the increase in adenosine and oxygen events that were caused by I/R injury, while blocking adenosine receptors with caffeine (100 mg/kg, *i.p.*) decreased adenosine and oxygen events. These two chapters characterized the relationship of spontaneous adenosine and oxygen changes. Spontaneously released adenosine could transiently increase local blood flow, even under ischemia and reperfusion injury. In **Chapter 6**, I briefly summarize my work and propose possible future research, including spontaneous adenosine release in freely moving animals, interaction between rapid adenosine and dopamine release, and combination of FSCV with optogenetics.

In conclusion, this thesis marks the continued steps of a journey studying rapid adenosine release in the brain. By understanding how and where rapid adenosine is released, new therapy on modulation of adenosine release will be promising for future treatment of various diseases, including stroke or traumatic brain injury.

1.4 References

1. Drury AN, Szent-Györgyi A. The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J Physiol*. 1929;68(3):213-237. doi:10.1113/jphysiol.1929.sp002608.

2. Bennet DW, Drury AN. Further observations relating to the physiological activity of adenine compounds. *J Physiol*. 1931;72(3):288-320. doi:10.1113/jphysiol.1931.sp002775.

3. Sebastião AM, Ribeiro JA. Fine-tuning neuromodulation by adenosine. *Trends Pharmacol Sci*. 2000;21(9):341-346. doi:10.1016/S0165-6147(00)01517-0.

4. De Mendonça A, Ribeiro JA. Adenosine and Synaptic Plasticity. *DRUG Dev Res*. 2001;52:283-290.

5. O'Regan M. Adenosine and the regulation of cerebral blood flow. *Neurol Res*. 2005;27(2):175-181. doi:10.1179/016164105X21931.

6. Sollevi A. Adenosine for pain control. *Acta Anaesthesiol Scand Suppl*. 1997;110:135-136. doi:10.1002/ddr.1125.

7. Zhou S, Zhu M, Shu D, et al. Preferential enhancement of working memory in mice lacking adenosine A2A receptors. *Brain Res*. 2009;1303:74-83. doi:10.1016/j.brainres.2009.09.082.

8. Bjorness TE, Greene RW. Adenosine and sleep. *Curr Neuropharmacol*. 2009;7(3):238-245. doi:10.2174/157015909789152182.

9. Gaudin A, Yemisci M, Eroglu H, et al. Squalenoyl adenosine nanoparticles provide neuroprotection after stroke and spinal cord injury. *Nat Nanotechnol*. 2014;9(12):1054-1062. doi:10.1038/nnano.2014.274.

10. Boison D. Adenosine kinase, epilepsy and stroke: mechanisms and therapies. *Trends Pharmacol Sci*. 2006;27(12):652-658. doi:10.1016/j.tips.2006.10.008.

11. Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB. Adenosine and brain ischemia. *Cerebrovasc Brain Metab Rev*. 1992;4:346-369.

12. Ferreira JM, Paes-De-Carvalhó R. Long-term activation of adenosine A receptors blocks glutamate 2a excitotoxicity in cultures of avian retinal neurons. *Brain Res*. 2001;900:169-176. doi:10.1016/S0006-8993(01)02279-X.

13. Nguyen MD, Lee ST, Ross AE, Ryals M, Choudhry VI, Venton BJ.

Characterization of spontaneous, transient adenosine release in the caudate-putamen and prefrontal cortex. *PLoS One*. 2014;9(1):e87165. doi:19326203.

14. Swamy BEK, Venton BJ. Subsecond detection of physiological adenosine concentrations using fast-scan cyclic voltammetry. *Anal Chem*. 2007;79:744-750. doi:10.1021/ac061820i.

15. Latini S, Pedata F. Adenosine in the central nervous system: Release mechanisms and extracellular concentrations. *J Neurochem*. 2001;79:463-484. doi:10.1046/j.1471-4159.2001.00607.x.

16. Park J, Gupta RS. Adenosine Metabolism, Adenosine Kinase, and Evolution. In: *Adenosine*. New York, NY: Springer New York; 2013:23-54. doi:10.1007/978-1-4614-3903-5_2.

17. Pedata F, Corsi C, Melani A, Bordoni F, Latini S. Adenosine extracellular brain concentrations and role of A2A receptors in ischemia. *Ann N Y Acad Sci*. 2001;939:74-84. doi:10.1111/j.1749-6632.2001.tb03614.x.

18. Sachdeva S, Gupta M. Adenosine and its receptors as therapeutic targets: An overview. *Saudi Pharm J SPJ Off Publ Saudi Pharm Soc*. 2013;21(3):245-253. doi:10.1016/j.jsps.2012.05.011.

19. Masino S, Boison D. *Adenosine : A Key Link between Metabolism and Brain Activity*. Springer; 2013.

20. Gödecke A. cAMP: fuel for extracellular adenosine formation? *Br J Pharmacol*. 2008;153(6):1087-1089. doi:10.1038/bjp.2008.7.

21. Corti F, Cellai L, Melani A, Donati C, Bruni P, Pedata F. Adenosine is present in rat brain synaptic vesicles. *Neuroreport*. 2013;24(17):982-987. doi:10.1097/WNR.0000000000000033.

22. Imura Y, Morizawa Y, Komatsu R, et al. Microglia release ATP by exocytosis. *Glia*. 2013;61(8):1320-1330. doi:10.1002/glia.22517.

23. Taruno A. ATP Release Channels. *Int J Mol Sci*. 2018;19(3). doi:10.3390/ijms19030808.

24. Newman EA. Propagation of intercellular calcium waves in retinal astrocytes and Müller cells. *J Neurosci*. 2001;21(7):2215-2223. doi:10.1523/JNEUROSCI.21-07-02215.2001.

25. Darby M, Kuzmiski JB, Panenka W, Feighan D, MacVicar BA. ATP Released From Astrocytes During Swelling Activates Chloride Channels. *J Neurophysiol*. 2003;89(4):1870-1877. doi:10.1152/jn.00510.2002.

26. Yamamoto K, Furuya K, Nakamura M, Kobatake E, Sokabe M, Ando J. Visualization of flow-induced ATP release and triggering of Ca2+ waves at caveolae in vascular endothelial cells. *J Cell Sci*. 2011;124(Pt 20):3477-3483. doi:10.1242/jcs.087221.

27. Yegutkin GG. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta - Mol Cell Res*. 2008;1783(5):673-694. doi:10.1016/J.BBAMCR.2008.01.024.

28. Falzoni S, Donvito G, Di Virgilio F. Detecting adenosine triphosphate in the pericellular space. *Interface Focus*. 2013;3(3):20120101. doi:10.1098/rsfs.2012.0101.

29. Herrling P. *Neural Plasticity and Repair : Special Issue: Neurodegenerative Diseases 2007*. S Karger Pub; 2007. doi:10.1159/isbn.978-3-318-01473-0.

30. Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W. Structure and function of adenosine receptors and their genes. *Naunyn Schmiedebergs Arch Pharmacol*. 2000;362(4-5):364-374. doi:10.1007/s002100000313.

31. Bono MR, Fernández D, Flores-Santibáñez F, Rosemblatt M, Sauma D. CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression. *FEBS Lett*. 2015;589(22):3454-3460. doi:10.1016/J.FEBSLET.2015.07.027.

32. Dunwiddie T V., Masino SA. The Role and Regulation of Adenosine in the Central Nervous System. *Annu Rev Neurosci*. 2001;24(1):31-55. doi:10.1146/annurev.neuro.24.1.31.

33. Mahan LC, McVittie LD, Smyk-Randall EM, et al. Cloning and expression of an A1 adenosine receptor from rat brain. *Mol Pharmacol*. 1991;40(1):1-7. doi:10.1016/0169-328X(92)90248-A.

34. Reppert SM, Weaver DR, Stehle JH, Rivkees SA. Molecular Cloning and Characterization of a Rat A_1 -Adenosine Receptor that is Widely Expressed in Brain and Spinal Cord. *Mol Endocrinol*. 1991;5(8):1037-1048. doi:10.1210/mend-5-8-1037.

35. Fredholm BB, Chen J-F, Cunha RA, Svenningsson P, Vaugeois J-M. Adenosine and Brain Function. In: *International Review of Neurobiology*. Vol 63. ; 2005:191-270. doi:10.1016/S0074-7742(05)63007-3.

36. Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem Int*. 2001;38(2):107-125. doi:10.1016/S0197-0186(00)00034-6.

37. Wei CJ, Li W, Chen J-F. Normal and abnormal functions of adenosine receptors in the central nervous system revealed by genetic knockout studies. *Biochim Biophys Acta - Biomembr*. 2011;1808(5):1358-1379. doi:10.1016/j.bbamem.2010.12.018.

38. Rebola N, Pinheiro PC, Oliveira CR, Malva JO, Cunha RA. Subcellular localization of adenosine A(1) receptors in nerve terminals and synapses of the rat hippocampus. *Brain Res*. 2003;987(1):49-58. doi:10.1016/S0006-8993(03)03247-5.

39. Rebola N, Rodrigues RJ, Lopes LV, Richardson PJ, Oliveira CR, Cunha RA. Adenosine A1 and A2A receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. *Neuroscience*. 2005;133(1):79-83. doi:10.1016/j.neuroscience.2005.01.054.

40. Tetzlaff W, Schubert P, Kreutzberg GW. Synaptic and extrasynaptic localization of adenosine binding sites in the rat hippocampus. *Neuroscience*. 1987;21(3):869-875. doi: 10.1016/0306-4522(87)90043-1.

41. Thompson SM, Haas HL, Gähwiler BH. Comparison of the actions of adenosine at pre- and postsynaptic receptors in the rat hippocampus in vitro. *J Physiol*. 1992;451:347-363. doi:10.1113/jphysiol.1992.sp019167.

42. Lupica CR, Proctor WR, Dunwiddie T V. Presynaptic inhibition of excitatory synaptic transmission by adenosine in rat hippocampus: analysis of unitary EPSP variance measured by whole-cell recording. *J Neurosci*. 1992;12(10):3753-3764. doi:10.1523/JNEUROSCI.12-10-03753.1992.

43. Scholz KP, Miller RJ. Inhibition of quantal transmitter release in the absence of calcium influx by a G protein-linked adenosine receptor at hippocampal synapses. *Neuron*. 1992;8(6):1139-1150. doi:10.1016/0896-6273(92)90134-Y.

44. Wu LG, Saggau P. Adenosine inhibits evoked synaptic transmission primarily by reducing presynaptic calcium influx in area CA1 of hippocampus. *Neuron*. 1994;12(5):1139-1148. doi:10.1016/0896-6273(94)90321-2.

45. Yawo H, Chuhma N. Preferential inhibition of oω-conotoxin-sensitive presynaptic Ca2+ channels by adenosine autoreceptors. *Nature*. 1993;365(6443):256-258. doi:10.1038/365256a0.

46. Dunwiddie T V, Haas HL. Adenosine increases synaptic facilitation in the in vitro rat hippocampus: evidence for a presynaptic site of action. *J Physiol*. 1985;369:365-377. doi:10.1113/jphysiol.1985.sp015907.

47. Ambrósio AF, Malva JO, Carvalho AP, Carvalho CM. Inhibition of N-,P/Q- and other types of Ca2+ channels in rat hippocampal nerve terminals by the adenosine A1 receptor. *Eur J Pharmacol*. 1997;340(2-3):301-310. doi:10.1016/S0014-

2999(97)01451-9.

48. de Mendonça A, Sebastião AM, Ribeiro JA. Inhibition of NMDA receptor-mediated currents in isolated rat hippocampal neurones by adenosine A1 receptor activation. *Neuroreport*. 1995;6(8):1097-1100.

49. Klishin A, Lozovaya N, Krishtal O. A1 adenosine receptors differentially regulate the N-methyl-D-aspartate and non-N-methyl-D-aspartate receptor-mediated components of hippocampal excitatory postsynaptic current in a Ca2+/Mg(2+)-dependent manner. *Neuroscience*. 1995;65(4):947-953. doi:10.1016/0306-4522(94)00518-A.

50. Greene RW, Haas HL. The electrophysiology of adenosine in the mammalian central nervous system. *Prog Neurobiol*. 1991;36(4):329-341. doi:10.1016/0301-0082(91)90005-L.

51. van Calker D, Biber K. The Role of Glial Adenosine Receptors in Neural Resilience and the Neurobiology of Mood Disorders. *Neurochem Res*. 2005;30(10):1205-1217. doi:10.1007/s11064-005-8792-1.

52. Tsutsui S, Schnermann J, Noorbakhsh F, et al. A1 Adenosine Receptor Upregulation and Activation Attenuates Neuroinflammation and Demyelination in a Model of Multiple Sclerosis. *J Neurosci*. 2004;24(6):1521-1529. doi:10.1523/JNEUROSCI.4271-03.2004.

53. Haselkorn ML, Shellington DK, Jackson EK, et al. Adenosine A $_1$ Receptor Activation as a Brake on the Microglial Response after Experimental Traumatic Brain Injury in Mice. *J Neurotrauma*. 2010;27(5):901-910. doi:10.1089/neu.2009.1075.

54. Fink JS, Weaver DR, Rivkees SA, et al. Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Brain Res Mol Brain Res*. 1992;14(3):186-195. doi:10.1016/0169-328X(92)90173-9.

55. Rosin DL, Robeva A, Woodard RL, Guyenet PG, Linden J. Immunohistochemical localization of adenosine A2A receptors in the rat central nervous system. *J Comp Neurol*. 1998;401(2):163-186. doi:10.1002/(SICI)1096-9861(19981116)401:2<163::AID-CNE2>3.0.CO;2-D.

56. Schiffmann SN, Jacobs O, Vanderhaeghen JJ. Striatal restricted adenosine A2 receptor (RDC8) is expressed by enkephalin but not by substance P neurons: an in situ hybridization histochemistry study. *J Neurochem*. 1991;57(3):1062-1067. doi:10.1111/j.1471-4159.1991.tb08257.x.

57. Schiffmann SN, Libert F, Vassart G, Vanderhaeghen JJ. Distribution of adenosine

A2 receptor mRNA in the human brain. *Neurosci Lett*. 1991;130(2):177-181. doi: 10.1016/0304-3940(91)90391-6.

58. Svenningsson P, Hall H, Sedvall G, Fredholm BB. Distribution of adenosine receptors in the postmortem human brain: An extended autoradiographic study. *Synapse*. 1997;27(4):322-335. doi:10.1002/(SICI)1098-2396(199712)27:4<322::AID-SYN6>3.0.CO;2-E.

59. Svenningsson P, Le Moine C, Kull B, Sunahara R, Bloch B, Fredholm BB. Cellular expression of adenosine A2A receptor messenger RNA in the rat central nervous system with special reference to dopamine innervated areas. *Neuroscience*. 1997;80(4):1171-1185. doi:10.1016/S0306-4522(97)00180-2.

60. Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ, Freeman TC. Tissue distribution of adenosine receptor mRNAs in the rat. *Br J Pharmacol*. 1996;118(6):1461-1468. doi:10.1111/j.1476-5381.1996.tb15561.x.

61. Rebola N, Canas PM, Oliveira CR, Cunha RA. Different synaptic and subsynaptic localization of adenosine A2A receptors in the hippocampus and striatum of the rat. *Neuroscience*. 2005;132(4):893-903. doi:10.1016/j.neuroscience.2005.01.014.

62. Marchi M, Raiteri L, Risso F, et al. Effects of adenosine A1 and A2A receptor activation on the evoked release of glutamate from rat cerebrocortical synaptosomes. *Br J Pharmacol*. 2002;136(3):434-440. doi:10.1038/sj.bjp.0704712.

63. Lopes L V., Cunha RA, Ribeiro JA. Cross Talk Between A_1 and A_{2A} Adenosine Receptors in the Hippocampus and Cortex of Young Adult and Old Rats. *J Neurophysiol*. 1999;82(6):3196-3203. doi:10.1152/jn.1999.82.6.3196.

64. Ciruela F, Casadó V, Rodrigues RJ, et al. Presynaptic Control of Striatal Glutamatergic Neurotransmission by Adenosine A1-A2A Receptor Heteromers. *J Neurosci*. 2006;26(7):2080-2087. doi:10.1523/JNEUROSCI.3574-05.2006.

65. Wirkner K, Gerevich Z, Krause T, et al. Adenosine A2A receptor-induced inhibition of NMDA and GABAA receptor-mediated synaptic currents in a subpopulation of rat striatal neurons. *Neuropharmacology*. 2004;46(7):994-1007. doi:10.1016/j.neuropharm.2004.01.008.

66. Rebola N, Lujan R, Cunha RA, Mulle C. Adenosine A2A Receptors Are Essential for Long-Term Potentiation of NMDA-EPSCs at Hippocampal Mossy Fiber Synapses. *Neuron*. 2008;57(1):121-134. doi:10.1016/j.neuron.2007.11.023.

67. Higley MJ, Sabatini BL. Competitive regulation of synaptic Ca2+ influx by D2 dopamine and A2A adenosine receptors. *Nat Neurosci*. 2010;13(8):958-966. doi:10.1038/nn.2592.

68. Ibarra-Alvarado C, Galle J, Melichar VO, et al. Phosphorylation of Blood Vessel Vasodilator-Stimulated Phosphoprotein at Serine 239 as a Functional Biochemical Marker of Endothelial Nitric Oxide/Cyclic GMP Signaling. *Mol Pharmacol*. 2002;61(2):312-319. doi:10.1124/mol.61.2.312.

69. Højer-Pedersen E, Postiglione A, Cuocolo A, et al. Effect of acetazolamide on cerebral blood flow in subacute and chronic cerebrovascular disease. *Stroke*. 1995;18(5):887-891. doi:10.1161/01.str.26.9.1572.

70. Phillis JW. Adenosine in the control of the cerebral circulation. *Cerebrovasc Brain Metab Rev*. 1989;1(1):26-54.

71. Ibayashi S, Ngai AC, Meno JR, Winn HR. Effects of Topical Adenosine Analogs and Forskolin on Rat Pial Arterioles in vivo. *J Cereb Blood Flow Metab*. 1991;11(1):72-76. doi:10.1038/jcbfm.1991.8.

72. Mathew RJ, Wilson WH. Caffeine induced changes in cerebral circulation. *Stroke*. 16(5):814-817. doi:10.1161/01.STR.16.5.814.

73. Cameron OG, Modell JG, Hariharan M. Caffeine and human cerebral blood flow: A positron emission tomography study. *Life Sci*. 1990;47(13):1141-1146. doi:10.1016/0024-3205(90)90174-P.

74. Liu TT, Behzadi Y, Restom K, et al. Caffeine alters the temporal dynamics of the visual BOLD response. *Neuroimage*. 2004;23(4):1402-1413. doi:10.1016/j.neuroimage.2004.07.061.

75. Bendlin BB, Trouard TP, Ryan L. Caffeine attenuates practice effects in word stem completion as measured by fMRI BOLD signal. *Hum Brain Mapp*. 2007;28(7):654-662. doi:10.1002/hbm.20295.

76. Coney AM, Marshall JM. Role of adenosine and its receptors in the vasodilatation induced in the cerebral cortex of the rat by systemic hypoxia. *J Physiol*. 1998;509 (Pt 2(2):507-518. doi:10.1111/J.1469-7793.1998.507BN.X.

77. Newby AC. Adenosine: origin and clinical roles. *Adv Exp Med Biol*. 1991;309A:265-270. doi:10.1007/978-1-4899-2638-8_60.

78. Bromfield EB, Cavazos JE, Sirven JI. Basic Mechanisms Underlying Seizures and Epilepsy. *American Epilepsy Society*. 2006.

79. During MJ, Spencer DD. Adenosine: A potential mediator of seizure arrest and postictal refractoriness. *Ann Neurol*. 1992;32(5):618-624. doi:10.1002/ana.410320504.

80. Li T, Ren G, Lusardi T, et al. Adenosine kinase is a target for the prediction and

prevention of epileptogenesis in mice. *J Clin Invest*. 2008;118(2):571-582. doi:10.1172/JCI33737.

81. de Mendonça A, Sebastião AM, Ribeiro JA. Adenosine: does it have a neuroprotective role after all? *Brain Res Brain Res Rev*. 2000;33(2-3):258-274. doi:10.1016/S0165-0173(00)00033-3.

82. Gouder N, Fritschy J-M, Boison D. Seizure Suppression by Adenosine A1 Receptor Activation in a Mouse Model of Pharmacoresistant Epilepsy. *Epilepsia*. 2003;44(7):877-885. doi:10.1046/j.1528-1157.2003.03603.x.

83. Canas PM, Porciúncula LO, Cunha GMA, et al. Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by beta-amyloid peptides via p38 mitogen-activated protein kinase pathway. *J Neurosci*. 2009;29(47):14741-14751. doi:10.1523/JNEUROSCI.3728-09.2009.

84. Laurent C, Burnouf S, Ferry B, et al. A2A adenosine receptor deletion is protective in a mouse model of Tauopathy. *Mol Psychiatry*. 2016;21(1):97-107. doi:10.1038/mp.2014.151.

85. Jaarsma D, Sebens JB, Korf J. Reduction of adenosine A1-receptors in the perforant pathway terminal zone in Alzheimer hippocampus. *Neurosci Lett*. 1991;121(1-2):111-114. doi:10.1016/0304-3940(91)90661-C.

86. Kalaria RN, Sromek S, Wilcox BJ, Unnerstall JR. Hippocampal adenosine A1 receptors are decreased in Alzheimer's disease. *Neurosci Lett*. 1990;118(2):257-260. doi:10.1016/0304-3940(90)90641-L.

87. Nampiaparampil DE. Prevalence of Chronic Pain After Traumatic Brain Injury. *JAMA*. 2008;300(6):711. doi:10.1001/jama.300.6.711.

88. Moore EL, Terryberry-Spohr L, Hope DA. Mild traumatic brain injury and anxiety sequelae: A review of the literature. *Brain Inj*. 2006;20(2):117-132. doi:10.1080/02699050500443558.

89. Bruns J, Hauser WA. The epidemiology of traumatic brain injury: a review. *Epilepsia*. 2003;44 Suppl 10:2-10. doi:10.1046/j.1528-1157.44.s10.3.x.

90. Verma A, Anand V, Verma NP. Sleep disorders in chronic traumatic brain injury. *J Clin Sleep Med*. 2007;3(4):357-362.

91. Kochanek PM, Verrier JD, Wagner AK, Jackson EK. The Many Roles of Adenosine in Traumatic Brain Injury. In: *Adenosine*. New York, NY: Springer New York; 2013:307-322. doi:10.1007/978-1-4614-3903-5_15.

92. Ning Y-L, Yang N, Chen X, et al. Adenosine A2A receptor deficiency alleviates

blast-induced cognitive dysfunction. *J Cereb Blood Flow Metab*. 2013;33(11):1789-1798. doi:10.1038/jcbfm.2013.127.

93. Li W, Dai S, An J, et al. Chronic but not acute treatment with caffeine attenuates traumatic brain injury in the mouse cortical impact model. *Neuroscience*. 2008;151(4):1198-1207. doi:10.1016/j.neuroscience.2007.11.020.

94. Varma MR, Dixon CE, Jackson EK, et al. Administration of adenosine receptor agonists or antagonists after controlled cortical impact in mice: effects on function and histopathology. *Brain Res*. 2002;951(2):191-201. doi:10.1016/S0006-8993(02)03161-X.

95. Ross AE, Nguyen MD, Privman E, Venton BJ. Mechanical stimulation evokes rapid increases in extracellular adenosine concentration in the prefrontal cortex. *J Neurochem*. 2014;130(1):50-60. doi:10.1111/jnc.12711.

96. Melani A, Pugliese AM, Pedata F. Adenosine Receptors in Cerebral Ischemia. *Int Rev Neurobiol*. 2014;119:309-348. doi:10.1016/B978-0-12-801022-8.00013-1.

97. Melani A, Corti F, Stephan H, et al. Ecto-ATPase inhibition: ATP and adenosine release under physiological and ischemic in vivo conditions in the rat striatum. *Exp Neurol*. 2012;233(1):193-204. doi:10.1016/J.EXPNEUROL.2011.09.036.

98. Dale N, Pearson T, Frenguelli BG. Direct measurement of adenosine release during hypoxia in the CA1 region of the rat hippocampal slice. *J Physiol*. 2000;526 Pt 1:143-155. doi:10.1111/J.1469-7793.2000.00143.X.

99. Fowler JC, Gervitz LM, Hamilton ME, Walker JA. Systemic Hypoxia and the Depression of Synaptic Transmission in Rat Hippocampus after Carotid Artery Occlusion. *J Physiol*. 2003;550(3):961-972. doi:10.1113/jphysiol.2003.039594.

100. Andiné P. Involvement of adenosine in ischemic and postischemic calcium regulation. *Mol Chem Neuropathol*. 18(1-2):35-49. doi:10.1007/BF03160021.

101. Logan M, Sweeney MI. Adenosine A1 receptor activation preferentially protects cultured cerebellar neurons versus astrocytes against hypoxia-induced death. *Mol Chem Neuropathol*. 1997;31(2):119-133. doi:10.1007/BF02815237.

102. Mori M, Nishizaki T, Okada Y. Protective effect of adenosine on the anoxic damage of hippocampal slice. *Neuroscience*. 1992;46(2):301-307. doi:10.1016/0306-4522(92)90052-4.

103. Hu S, Dong H, Zhang H, et al. Noninvasive limb remote ischemic preconditioning contributes neuroprotective effects via activation of adenosine A1 receptor and redox status after transient focal cerebral ischemia in rats. *Brain Res*. 2012;1459:81-90. doi:10.1016/j.brainres.2012.04.017.

104. Higashi H, Meno JR, Marwaha AS, Winn HR. Hippocampal injury and neurobehavioral deficits following hyperglycemic cerebral ischemia: effect of theophylline and ZM 241385. *J Neurosurg*. 2002;96(1):117-126. doi:10.3171/jns.2002.96.1.0117.

105. H BEAUGEFBH. Neonatal cerebral hypoxia-ischemia: the effect of adenosine receptor antagonists. 1997;36(9):1327-1338. doi:10.1016/S0028-3908(97)00139-1.

106. Ganesana M, Venton BJ. Early changes in transient adenosine during cerebral ischemia and reperfusion injury. *PLoS One*. 2018;13(5):e0196932. doi:10.1371/journal.pone.0196932.

107. Yorgason JT, Zeppenfeld DM, Williams JT. Cholinergic Interneurons Underlie Spontaneous Dopamine Release in Nucleus Accumbens. *J Neurosci*. 2017;37(8):2086-2096. doi:10.1523/JNEUROSCI.3064-16.2017.

108. Spanos M, Gras-Najjar J, Letchworth JM, Sanford AL, Toups JV, Sombers LA. Quantitation of Hydrogen Peroxide Fluctuations and Their Modulation of Dopamine Dynamics in the Rat Dorsal Striatum Using Fast-Scan Cyclic Voltammetry. *ACS Chem Neurosci*. 2013;4(5):782-789. doi:10.1021/cn4000499.

109. Wood KM, Hashemi P. Fast-scan cyclic voltammetry analysis of dynamic serotonin reponses to acute escitalopram. *ACS Chem Neurosci*. 2013;4(5):715-720. doi:10.1021/cn4000378.

110. Sims RE, Wu HHT, Dale N. Sleep-Wake Sensitive Mechanisms of Adenosine Release in the Basal Forebrain of Rodents: An In Vitro Study. Mongrain V, ed. *PLoS One*. 2013;8(1):e53814. doi:10.1371/journal.pone.0053814.

111. Wang Y, Guo Y, Zhang L, Wu J. Characterizations of a new Cordyceps cicadae isolate and production of adenosine and cordycepin. *Braz J Microbiol*. 2012;43(2):449-455. doi:10.1590/S1517-83822012000200004.

112. Melani A, De Micheli E, Pinna G, Alfieri A, Corte L Della, Pedata F. Adenosine extracellular levels in human brain gliomas: an intraoperative microdialysis study. *Neurosci Lett*. 2003;346(1-2):93-96. doi:10.1016/S0304-3940(03)00596-2.

113. Sharma R, Engemann SC, Sahota P, Thakkar MM. Effects of ethanol on extracellular levels of adenosine in the basal forebrain: an in vivo microdialysis study in freely behaving rats. *Alcohol Clin Exp Res*. 2010;34(5):813-818. doi:10.1111/j.1530-0277.2010.01153.x.

114. Nesbitt KM, Jaquins-Gerstl A, Skoda EM, Wipf P, Michael AC. Pharmacological mitigation of tissue damage during brain microdialysis. *Anal Chem*. 2013;85:8173-8179. doi:10.1021/ac401201x.

115. Kennedy RT. Emerging trends in in vivo neurochemical monitoring by microdialysis. *Curr Opin Chem Biol*. 2013;17(5):860-867. doi:10.1016/j.cbpa.2013.06.012.

116. Scharbarg E, Daenens M, Lemaître F, et al. Astrocyte-derived adenosine is central to the hypnogenic effect of glucose. *Sci Rep*. 2016;6(1):19107. doi:10.1038/srep19107.

117. Bjerring PN, Dale N, Larsen FS. Acute Hyperammonemia and Systemic Inflammation is Associated with Increased Extracellular Brain Adenosine in Rats: A Biosensor Study. doi:10.1007/s11064-014-1357-4.

118. Keithley RB, Takmakov P, Bucher ES, et al. Higher sensitivity dopamine measurements with faster-scan cyclic voltammetry. *Anal Chem*. 2011;83(9):3563-3571. doi:10.1021/ac200143v.

119. Robinson DL, Venton BJ, Heien MLA V, Wightman RM. Detecting subsecond dopamine release with fast-scan cyclic voltammetry in vivo. *Clin Chem*. 2003;49(10):1763-1773. doi:10.1373/49.10.1763.

120. Cechova S, Venton BJ. Transient adenosine efflux in the rat caudate-putamen. *J Neurochem*. 2008;105(4):1253-1263. doi:10.1111/j.1471-4159.2008.05223.x.

121. Cechova S, Elsobky AM, Venton BJ. A1 receptors self-regulate adenosine release in the striatum: evidence of autoreceptor characteristics. *Neuroscience*. 2010;171(4):1006-1015. doi:http://dx.doi.org/10.1016/j.neuroscience.2010.09.063.

122. Pajski ML, Venton BJ. The mechanism of electrically stimulated adenosine release varies by brain region. *Purinergic Signal*. 2013;9(2):167-174. doi:10.1007/s11302-012-9343-2.

123. Pajski ML, Venton BJ. Adenosine Release Evoked by Short Electrical Stimulations in Striatal Brain Slices is Primarily Activity Dependent. *ACS Chem Neurosci*. 2010;1(12):775-787. doi:10.1021/cn100037d.

124. Street SE, Walsh PL, Sowa NA, et al. PAP and NT5E inhibit nociceptive neurotransmission by rapidly hydrolyzing nucleotides to adenosine. *Mol Pain*. 2011;7:80. doi:10.1186/1744-8069-7-80.

125. Nguyen MD, Wang Y, Ganesana M, Venton BJ. Transient Adenosine Release Is Modulated by NMDA and GABAB Receptors. *ACS Chem Neurosci*. 2017;8(2):376-385. doi:10.1021/acschemneuro.6b00318.

126. Nguyen MD, Ross AE, Ryals M, Lee ST, Venton BJ. Clearance of rapid adenosine release is regulated by nucleoside transporters and metabolism. *Pharmacol Res*

Perspect. 2015;3(6). doi:10.1002/prp2.189.

127. Winn HR, Rubio R, Berne RM. Brain adenosine concentration during hypoxia in rats. *Am J Physiol*. 1981;241:H235-H242. doi:10.1152/ajpheart.1981.241.2.H235.

128. Zimmerman JB, Wightman RM. Simultaneous electrochemical measurements of oxygen and dopamine in vivo. *Anal Chem*. 1991;63:24-28. doi:10.1021/ac00001a005.

Chapter 2: Regional variation of spontaneous and mechanically-stimulated adenosine release in mice

Abstract

Rapid adenosine release has been discovered in the brain that can modulate neurotransmission or blood flow. Adenosine is released spontaneously or after a mechanical stimulation, but these modes of adenosine release have not been compared. Here, we measured spontaneous and mechanically-stimulated adenosine release in anesthetized mouse prefrontal cortex, striatum, and hippocampus. For spontaneous adenosine release, the number of adenosine events in the prefrontal cortex (40 ± 4 per hour) was significantly lower than in the striatum (54 ± 3) or hippocampus (56 ± 3). However, the concentration (about 0.3 μM) and $t_{1/2}$ (about 2 s) of each event was similar between regions. Thus, spontaneous adenosine release differs regionally in frequency but not the resulting concentration. For mechanically-stimulated adenosine, the peak concentrations in the prefrontal cortex (8 ± 2 μM) and striatum (8 ± 1 μM) were significantly lower than in the hippocampus (16 ± 3 μM) but there was no difference in $t_{1/2}$ (8 s). Comparing the two modes of release, the hippocampus had high mechanically-stimulated concentrations and high spontaneous frequency, while the prefrontal cortex had lower spontaneously released adenosine frequency and mechanically-stimulated release. Because concentration differs for mechanically-stimulated release but not for spontaneous adenosine release, there may also be different pools or mechanisms of formation of adenosine for these different modes. Adenosine signaling, and thus the neuromodulatory effects, varies by region, which may be important for designing adenosine-based treatments for neurological disorders.

2.1 Introduction

Adenosine is an endogenous nucleoside that functions as a neuromodulator and neuroprotector in the central nervous system, particularly during stroke[1] and traumatic brain injury.[2] While adenosine may build up slowly during pathological events, our lab has discovered rapid adenosine release, lasting only a few seconds[3], that modulates phasic dopamine release and causes transient oxygen increases[4,5]. One mode of rapid adenosine release is spontaneous release, with no stimulation applied, and the frequency of this mode increases during ischemia-reperfusion injury[6], where spontaneously released adenosine acts as a local neuromodulator. Another mode of rapid adenosine release is mechanical stimulation, where small trauma, such as moving an electrode or probe near the electrode, causes transient adenosine release.[7] The hypothesis is that these modes of release may vary by region,[7,8] and that would cause regional variation in neuromodulation. Spontaneous and mechanically-stimulated adenosine release have been separately studied in the rat, but they have not been compared. Here, we use mice, because of the future possibilities of studying transgenics, to characterize spontaneous adenosine release events in the prefrontal cortex, striatum, and hippocampus using fast-scan cyclic voltammetry. Spontaneous adenosine release varied regionally in frequency, but not concentration, while mechanically-stimulated adenosine varied regionally in resulting concentration of adenosine. Thus, the two modes of adenosine release may produce different neuromodulatory effects in different regions.

2.2 Materials and methods

2.2.1 Animals and surgery

Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 6-8 weeks old and housed on a 12:12-h light/dark cycle with food and water provided *ad libitum*. Mice were anesthetized with 4% isoflurane in 100% oxygen for induction and then transferred to a stereotaxic frame (Stoelting, Wood Dale, IL, USA). Anesthesia was maintained with 1.5-3% isoflurane in 100% oxygen delivered via a facemask (Stoelting, Wood Dale, IL, USA), tail pinch to ensure that anesthesia is complete and substained. Isoflurane levels were adjusted to until loss of righting reflex was observed. A heating pad was used to maintain mouse body temperature around 37 °C. The surgical site was shaved and bupivacaine (0.10 mL) (Sensorcaine® MPF; APP Pharmaceuticals, LLC, Schaumburg, IL, USA) was administered under the skin for local anesthesia. The skull was exposed and holes were drilled to allow the placement of the electrode in the prefrontal cortex (AP +1.3 mm, ML + 0.2 mm, and DV -1.5 mm), striatum (AP +1.1 mm, ML + 1.5 mm, and DV -3.0 mm), and hippocampus (AP -2.5 mm, ML + 2.4 mm, and DV -1.8 mm) based on the altas of Paxinos and Franklin.[9] All experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia.

2.2.2 Chemicals

Phosphate-buffered saline (PBS) solution (all components purchased from Fisher Scientific (Fair Lawn, NJ, USA)) was used for electrode calibration and consisted of 3.0 mM KCl, 10.0 mM NaH_2PO_4, 2.0 mM $MgCl_2$, 131.25 mM NaCl and 1.2 $CaCl_2$, with pH adjusted to 7.4. Adenosine was purchased from Sigma Aldrich (Milwaukee, WI, USA).

A 10.0 mM stock solution of adenosine was prepared in 0.1 mM $HClO_4$ and this was diluted daily in PBS solution to 1μM for calibration of the electrodes. All aqueous solutions were prepared using deionized water (Milli-Q Biocel; Millipore, Billerica, MA, USA).

2.2.3 Electrochemistry

Fabrication of carbon fiber microelectrode with T-650 carbon fiber was previously described.[5] Cylinder electrodes, which were 150-200 μm long and 7 μm in diameter were used. Fast-scan cyclic voltammetry was used to detect adenosine as previously described.[5] For detection of adenosine, the electrode was scanned from -0.4 V to 1. 45 V and back to – 0.4 V with a frequency of 10 Hz at 400 V/s.

2.2.4 Data Analysis and Statistics

Transient adenosine events were firstly identified and analyzed using a new automated algorithm,[10] and adenosine events were then confirmed by an analyst to exclude any signals that were not adenosine. The primary oxidation peaks of adenosine in Figure 1a-1c were filtered using a Fourier transform 1 Hz filter to reduce noise. All statistics were performed in GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). All data are shown as mean ± SEM. A one-way ANOVA with post hoc Tukey's test was performed on the number of events, concentration and $t_{1/2}$ of transient adenosine to assess differences among brain regions. The distribution of inter-event time of adenosine events was analyzed using Kruskal-Wallis test with post-hoc Dunn's test. Some data points for adenosine inter-event time are not shown in order to better show the

lower inter-event time where changes are more obvious. However, all data for inter-event time were used for statistical analyses. Statistical significance was designated at $p < 0.05$.

2.3 Results and Discussion

The goal of this study was to characterize regional differences in spontaneous and mechanically-stimulated adenosine release *in vivo* in anesthetized mice. The brain regions chosen were the Cg1 of prefrontal cortex (PFC), striatum (STR), and CA1 of hippocampus (HPC) because these are common brain regions for both traumatic brain injury and memory studies. The prefrontal cortex participates in working memory,[11] the striatum is associated with procedural memory,[12] and the hippocampus plays an important role in memory formation.[13] Rapid changes of adenosine were monitored in anesthetized mice using fast-scan cyclic voltammetry at an implanted carbon-fiber microelectrode. Adenosine is identified by its two characteristic oxidation peaks in the cyclic voltammogram (top, Fig. 2.1a-c) and false color plots (bottom, Fig. 2.1a-c).[3]

Spontaneous neurotransmitter release is a common mode of synaptic release [14] and spontaneous adenosine release events have been reported *in vivo* and in brain slices in rat[3,5,15]. For spontaneous adenosine release (Fig. 2.1), adenosine transients were measured continuously in anesthetized mice for 4 hours. Multiple, short adenosine release events are depicted in the color plot, and marked on the concentration *vs.* time traces, which show how adenosine varies over time. In the prefrontal cortex, there are only 2 adenosine transient events in this example 80 s window (Fig. 2.1a) while in the striatum and hippocampus, there are 3 events in an 80 s window (Fig.2.1b and 2.1c). The number of adenosine events varied by region; on average, there were 40 ± 4 adenosine release events per hour in the prefrontal cortex, 54 ± 3 events in the striatum and 56 ± 3

events in the hippocampus, an overall significant effect of brain region (Fig. 2.1d, one-way ANOVA, n = 8 animals/region, $p = 0.0033$). The number of adenosine events in the prefrontal cortex was significantly lower than in the striatum or hippocampus (Tukey's post-test, $p = 0.011$ and $p = 0.0056$, respectively). To examine the frequency, the time was calculated between two consecutive adenosine events, called the inter-event time. The mean inter-event time was 87 ± 3 s in the prefrontal cortex, 66 ± 2 s in the striatum and 64 ± 2 s in the hippocampus. There was a significant effect of brain region on the underlying distributions of inter-event times (Fig. 2.1e, Kruskal-Wallis test, $p < 0.0001$) and the prefrontal cortex frequency was significantly lower than the striatum or hippocampus (post-hoc Dunn's test, $p < 0.0001$ and $p < 0.0001$, respectively). The frequency of adenosine transients observed in these mouse experiments is higher than the corresponding rat brain regions.[3,15]

The mean concentration of spontaneously released adenosine per event was 0.24 ± 0.04 μM in the prefrontal cortex, 0.31 ± 0.03 μM in the striatum, and 0.27 ± 0.03 μM in the hippocampus. Thus, there was no significant effect of brain region on concentration (Fig. 2.1f, one-way ANOVA, n=8 animals/group, $p = 0.35$). The concentration of spontaneously released adenosine ranged from 0.023 to 2.80 μM, sufficient to activate inhibitory A_1 receptors and excitatory A_{2A} receptors, which have high affinities in the low nanomolar range.[16] These are the first *in vivo* measurements of adenosine in mice using FSCV and concentrations were larger than similar regions in rats,[3,15] even though the basal concentrations of adenosine in mice are reportedly lower. [8,17]

To measure duration, $t_{1/2}$, the peak width at half peak height was used. The mean $t_{1/2}$ was 1.8 ± 0.1 s in the prefrontal cortex, 1.9 ± 0.1 s in the striatum, 1.7 ± 0.1 s in the

hippocampus, and these values were not significantly different from each other (Fig. 2.1g one-way ANOVA, $p = 0.14$). The short $t_{1/2}$ indicates spontaneously released adenosine is only available in the extracellular space for a few seconds and thus functions locally. Overall, the concentration and $t_{1/2}$ of spontaneous adenosine release do not vary by region but the frequency does, demonstrating that the concentration of spontaneously released adenosine is regulated in different regions through the number of release events and not the size or duration of each event.

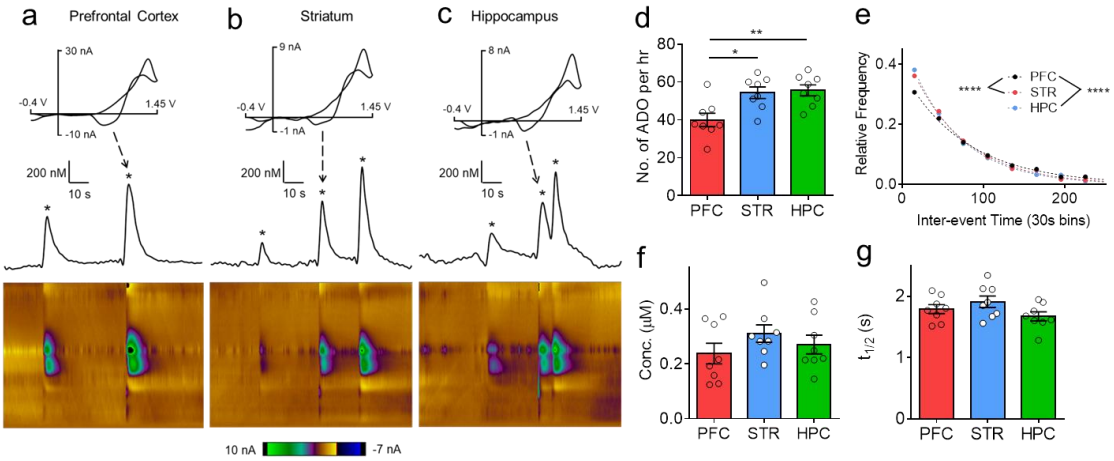

Figure 2.1 Spontaneous, transient adenosine release in various brain regions. Example of adenosine release in the (a) prefrontal cortex (PFC), (b) striatum (STR), and (c) hippocampus (HPC). Cyclic voltammograms of adenosine have the two characteristic oxidation peaks at 1.3 V on the cathodic scan and 1.2 V on the anodic scan. Concentration *vs.* time traces derived from primary oxidation of adenosine; adenosine transients marked with stars. Example 3-D color plot shows release events in an 80 s time window. Adenosine oxidation is the green/purple area in the middle of the plot. (d) Number of adenosine events per hour (main effect, one-way ANOVA, n = 8 animals, $p = 0.0033$). The number of adenosine events was significantly lower in the prefrontal cortex than the striatum and hippocampus (Tukey's test, $p = 0.011$ and $p = 0.0056$, respectively). (e) Inter-event time histogram. The underlying distributions of adenosine inter-event times were significantly different (Kruskal-Wallis test, $p < 0.0001$). (F) Mean event concentrations were not significantly different (one-way ANOVA, n = 8 animals/group, $p = 0.35$). (g) Mean $t_{1/2}$ was not different among the brain regions (one-way ANOVA, n = 8 animals/group, $p = 0.14$).

The second mode of adenosine release studied was mechanically-stimulated adenosine release. Other studies have demonstrated ATP release, which could be a precursor to adenosine release, due to mechanical perturbation,[18] swelling,[18] shear stress[19] or cell stretching[20] in both neurons[18] and astrocytes[21]. Traumatic brain injury, where there is mechanical damage of brain tissue, triggers an acute surge in adenosine to induce neuroprotective activity.[22,23] Similarly, lowering a carbon-fiber microelectrode into brain tissue also evokes rapid adenosine release, and this method of release was used here to study regional variation in mechanosensitive adenosine release.[7] A rapid rise in adenosine concentration after mechanical stimulation was observed in the prefrontal cortex, striatum, and hippocampus of mice (Fig. 2.2). A carbon-fiber microelectrode was lowered 0.1 mm to mechanically perturb the tissue, and mechanical stimulations were repeated every 15 min for 1 hour, which means new tissue was stimulated each time (although we have shown previously the same tissue can be mechanically-stimulated multiple times).[7] Fig. 2a shows an example of mechanically-stimulated adenosine release in the hippocampus, where adenosine increases immediately after lowering the electrode (at 30 s) with a peak concentration of 21 μM, and a $t_{1/2}$ of 4.8 s. The extra peaks in the color plot might be background subtraction errors due to ionic changes on the double layer charging current.

Fig. 2.2b-i compare the average concentration and $t_{1/2}$ of mechanically-evoked adenosine for each successive stimulation and for all stimulations. Overall, there was no significant effect of stimulation number on the concentration (Fig. 2.2b-d, one-way ANOVA, PFC: n = 8 animals, p = 0.20; STR: n = 9 animals, p = 0.61; HPC: n = 7 animals, p = 0.68) or $t_{1/2}$ (Fig. 2.2f-h, one-way ANOVA, PFC: p = 0.65; STR: p = 0.87;

HPC: p = 0.39). The concentration of mechanically-evoked adenosine varied from 0.65 to 46 µM but was on average 8 ± 2 µM in the prefrontal cortex, 8 ± 1 µM in the striatum and 16 ± 3 µM in the hippocampus, thus showing a significant effect of region (Fig. 2.2e, one-way ANOVA, p = 0.0005). The concentration in the hippocampus was significantly higher than in the striatum and prefrontal cortex (post-hoc Tukey's test, p = 0.012 and p = 0.0004, respectively). Mechanically-stimulated adenosine release was previously compared in the rat PFC *in vivo* (3.3 ± 0.6 µM) and *ex vivo* (0.8 ± 0.1 µM brain slices)[7] and the concentrations were smaller than those observed here in the mouse PFC, 8 ± 2 µM. However, the electrode was also slightly larger (150 -200 µm in mice compared to 50 -100 µm in rats), so the amount of perturbation and adenosine detected may be larger.

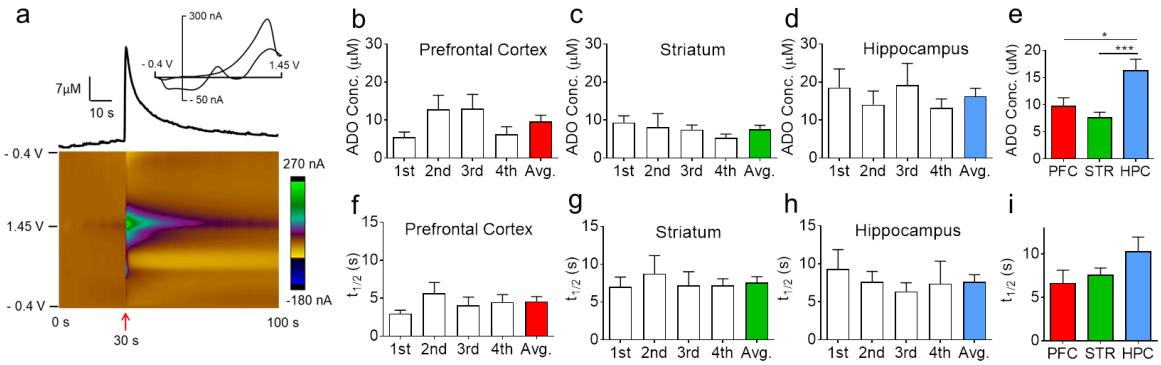

Figure 2.2 Mechanically-stimulated adenosine. (a) Example of mechanically-evoked adenosine in the hippocampus. Electrode was lowered 0.1 mm at 30 s (arrow). Concentration *vs.* time trace shows adenosine peak concentration was 21 µM and $t_{1/2}$ was 4.8 s. Four consecutive stimulations were performed every 15 min for a total of 4 stimulation in the (b) prefrontal cortex (PFC), (c) striatum (STR) and (d) hippocampus (HPC) *in vivo*. There was no effect of stimulation number in any region (one-way ANOVA, PFC: n = 8 animals, p = 0.20; STR: n = 9 animals, p = 0.61; HPC: n = 7 animals, p = 0.68). (e) Average concentrations significantly vary by region (one-way ANOVA, p = 0.0005) and concentration in the hippocampus was significantly higher than in the prefrontal cortex and striatum (post hoc Tukey's test, p = 0.012 and p = 0.0004, respectively). Average $t_{1/2}$ of adenosine did not differ by stimulation number in the (f) prefrontal cortex (p = 0.65), (g) striatum (p = 0.87) and (h) hippocampus (p = 0.39). (i) Average $t_{1/2}$ did not vary among brain regions (one-way ANOVA, p = 0.14).

The average $t_{1/2}$ of mechanically-evoked adenosine for all stimulations was 7 ± 1 s in the prefrontal cortex, 8 ± 1 s in the striatum and 10 ± 2 s in the hippocampus, but there was no significant effect of brain region on $t_{1/2}$ (Fig. 2.2i, one-way ANOVA, $p = 0.14$).

Mechanically-stimulated adenosine release varied by brain region in resulting concentration, but not duration. In the prefrontal cortex, A_1 and A_{2A} receptors modulate cortical acetylcholine,[24] but in the striatum and hippocampus, they also regulate other neurotransmitters, including glutamate and GABA.[25] The higher adenosine release in the hippocampus indicates larger pools of adenosine for mechanosensitive release and greater ability to provide rapid neuromodulation. The hippocampus is a critical area for memory formation and retrieval, so it may be necessary for it to have high neuromodulation.

These data allow a direct comparison of spontaneous and mechanically-stimulated adenosine release. The concentration of mechanically-stimulated adenosine is on average 10-fold higher than that resulting from spontaneous adenosine release events, likely because many cells are activated and release adenosine all at once. Mechanically-stimulated adenosine is sufficient to activate adenosine A_{2B} and A_3 receptors,[26] which have neuroprotective effects. Thus, mechanically-stimulated release may be able to provide more rapid neuroprotection by activating additional adenosine receptors. In addition, the clearance of mechanically-stimulated adenosine takes longer, so the neuroprotective adenosine is available longer for signaling in the extracellular space.[7] Spontaneously released adenosine on the other hand is regulated by frequency and not by concentration. It would provide lower levels of neuroprotection and neuromodulation, but on a more frequent basis.

The hippocampus had both the highest amount of mechanically-stimulated release and the greatest frequency of spontaneous adenosine release. Thus, it has the greatest releasable pool of adenosine, but we do not know if the mechanism of release is the same for both modes of adenosine release. Mechanism of release varies by type of stimulation and brain region. For example, electrically-stimulated adenosine release is partially activity-dependent[27] while mechanically-stimulated adenosine release is calcium dependent, activity dependent, and formed from rapid degradation of extracellular ATP[7]. Spontaneous adenosine release may be due to spontaneous exocytosis, independent of action potentials,[28] or pannexin or connexin channels. Thus, future research is needed to determine if these two modes are released by similar or different mechanisms and to examine how this rapid release contributes to neuroprotection and neuromodulation.

2.4 References

1. Sweeney MI. Neuroprotective effects of adenosine in cerebral ischemia: window of opportunity. *Neurosci Biobehav Rev*. 1997;21(2):207-217. doi:10.1016/S0149-7634(96)00011-5.

2. Lusardi TA. Adenosine neuromodulation and traumatic brain injury. *Curr Neuropharmacol*. 2009;7(3):228-237. doi:10.2174/157015909789152137.

3. Nguyen MD, Lee ST, Ross AE, Ryals M, Choudhry VI, Venton BJ. Characterization of spontaneous, transient adenosine release in the caudate-putamen and prefrontal cortex. *PLoS One*. 2014;9(1):e87165. doi:19326203.

4. Ross AE, Venton BJ. Adenosine transiently modulates stimulated dopamine release in the caudate-putamen via A1 receptors. *J Neurochem*. 2015;132(1):51-60. doi:10.1111/jnc.12946.

5. Wang Y, Venton BJ. Correlation of transient adenosine release and oxygen changes in the caudate-putamen. *J Neurochem*. 2017;140(1):13-23. doi:10.1111/jnc.13705.

6. Ganesana M, Venton BJ. Early changes in transient adenosine during cerebral ischemia and reperfusion injury. *PLoS One*. 2018;13(5):e0196932. doi:10.1371/journal.pone.0196932.

7. Ross AE, Nguyen MD, Privman E, Venton BJ. Mechanical stimulation evokes rapid increases in extracellular adenosine concentration in the prefrontal cortex. *J Neurochem*. 2014;130(1):50-60. doi:10.1111/jnc.12711.

8. Porkka-Heiskanen T, Strecker R., McCarley R. Brain site-specificity of extracellular adenosine concentration changes during sleep deprivation and spontaneous sleep: an in vivo microdialysis study. *Neuroscience*. 2000;99(3):507-517. doi:10.1016/S0306-4522(00)00220-7.

9. Watson C, Paxinos G. *Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, Fourth Edition*.; 2012.

10. Borman RP, Wang Y, Nguyen MD, Ganesana M, Lee ST, Venton BJ. Automated Algorithm for Detection of Transient Adenosine Release. *ACS Chem Neurosci*. 2017;8(2):386-393. doi:10.1021/acschemneuro.6b00262.

11. Lara AH, Wallis JD. The Role of Prefrontal Cortex in Working Memory: A Mini Review. *Front Syst Neurosci*. 2015;9:173. doi:10.3389/fnsys.2015.00173.

12. Packard MG, Knowlton BJ. Learning and Memory Functions of the Basal Ganglia. *Annu Rev Neurosci*. 2002;25(1):563-593. doi:10.1146/annurev.neuro.25.112701.142937.

13. Kogan JH, Franklandand PW, Silva AJ. Long-term memory underlying hippocampus-dependent social recognition in mice. *Hippocampus*. 2000;10(1):47-56. doi:10.1002/(SICI)1098-1063(2000)10:1<47::AID -HIPO5>3.0.CO;2-6.

14. Ramirez DMO, Kavalali ET. Differential regulation of spontaneous and evoked neurotransmitter release at central synapses. *Curr Opin Neurobiol*. 2011;21(2):275-282. doi:10.1016/j.conb.2011.01.007.

15. Lee ST, Venton BJ. Regional Variations of Spontaneous, Transient Adenosine Release in Brain Slices. *ACS Chem Neurosci*. 2018;9(3):505-513. doi:10.1021/acschemneuro.7b00280.

16. Sachdeva S, Gupta M. Adenosine and its receptors as therapeutic targets: An overview. *Saudi Pharm J SPJ Off Publ Saudi Pharm Soc*. 2013;21(3):245-253. doi:10.1016/j.jsps.2012.05.011.

17. Delaney SM, Geiger JD. Brain regional levels of adenosine and adenosine nucleotides in rats killed by high-energy focused microwave irradiation. *J Neurosci Methods*. 1996;64(2):151-156. doi:10.1016/0165-0270(95)00119-0.

18. Xia J, Lim JC, Lu W, et al. Neurons respond directly to mechanical deformation with pannexin-mediated ATP release and autostimulation of P2X $_7$ receptors. *J Physiol*. 2012;590(10):2285-2304. doi:10.1113/jphysiol.2012.227983.

19. Wan J, Ristenpart WD, Stone HA. Dynamics of shear-induced ATP release from red blood cells. *Proc Natl Acad Sci*. 2008;105(43):16432-16437. doi:10.1073/pnas.0805779105.

20. Stalmans P, Himpens B. Confocal imaging of Ca2+ signaling in cultured rat retinal pigment epithelial cells during mechanical and pharmacologic stimulation. *Invest Ophthalmol Vis Sci*. 1997;38(1):176-187.

21. Newman EA. Propagation of intercellular calcium waves in retinal astrocytes and Müller cells. *J Neurosci*. 2001;21(7):2215-2223. doi:10.1523/JNEUROSCI.21-07-02215.2001.

22. Clark RS, Carcillo JA, Kochanek PM, et al. Cerebrospinal fluid adenosine concentration and uncoupling of cerebral blood flow and oxidative metabolism after severe head injury in humans. *Neurosurgery*. 1997;41(6):1284-92; discussion 1292-3. doi:10.1097/00006123-199712000-00010.

23. Pignataro G, Maysami S, Studer FE, Wilz A, Simon RP, Boison D. Downregulation of Hippocampal Adenosine Kinase after Focal Ischemia as Potential Endogenous Neuroprotective Mechanism. *J Cereb Blood Flow Metab*. 2008;28(1):17-23. doi:10.1038/sj.jcbfm.9600499.

24. Van Dort CJ, Baghdoyan HA, Lydic R. Adenosine A(1) and A(2A) receptors in mouse prefrontal cortex modulate acetylcholine release and behavioral arousal. *J Neurosci*. 2009;29(3):871-881. doi:10.1523/JNEUROSCI.4111-08.2009.

25. Sperlágh B, Vizi ES. The role of extracellular adenosine in chemical neurotransmission in the hippocampus and Basal Ganglia: pharmacological and clinical aspects. *Curr Top Med Chem*. 2011;11(8):1034-1046. doi:10.2174/156802611795347564.

26. Huang Z-L, Urade Y, Hayaishi O. The role of adenosine in the regulation of sleep. *Curr Top Med Chem*. 2011;11(8):1047-1057. doi:10.2174/156802611795347654.

27. Pajski ML, Venton BJ. The mechanism of electrically stimulated adenosine release varies by brain region. *Purinergic Signal*. 2013;9(2):167-174. doi:10.1007/s11302-012-9343-2.

28. Kavalali ET. The mechanisms and functions of spontaneous neurotransmitter release. *Nat Rev Neurosci*. 2015;16(1):5-16. doi:10.1038/nrn3875.

Chapter 3: The role of CD73 and CD39 in the regulation of spontaneous and mechanically-stimulated adenosine formation

Abstract

Rapid adenosine release has been discovered in the brain *in vivo* and is important for local neuroprotection during ischemia and reperfusion injury. Rapid adenosine release can be observed spontaneously without a stimulation or can be evoked by mechanical perturbation, but the mechanism of formation is not fully known for these two modes of adenosine release. One mechanism of adenosine formation is through extracellular metabolism of ATP, which is first degraded by nucleoside triphosphate diphospholydrolase 1 (NTPDase1, CD39) that converts ATP or ADP to AMP and then by ecto-5′-nucleotidase (CD73) that converts AMP to adenosine. In this study, we used mice globally deficient in CD73 and CD39 to study the mechanisms of adenosine formation that contribute to spontaneous and mechanically-stimulated release using fast-scan cyclic voltammetry. CD73 knockout (KO) and CD39KO mice had fewer spontaneous adenosine release events but the concentration and duration of the events were the same. In addition, the concentration or duration of mechanically-stimulated adenosine was not different in CD39KO and CD73KO micecompared to the WT mice. Thus, CD73 and CD39 are not directly involved in the mechanisms of rapid adenosine formation but do regulate the frequency of spontaneous adenosine release. These findings show that that therapies targeting CD73 or CD39 would preferentially affect the frequency of spontaneous rather than mechanically-sensitive release, which could facilitate the future development of adenosine-based pharmacotherapies for specific adenosine modes.

3.1 Introduction

Adenosine is an endogenous nucleoside that plays an important role in physiological and pathological processes including sleep,[1] stroke,[2] vasodilation,[3] and inflammation[4]. Adenosine is formed at both intracellular and extracellular sites. The main source of intracellular formation of adenosine is intracellular breakdown via cytosolic 5′-nucleotidase, which dephosphorylates AMP.[5] Adenosine can be released via equilibrative adenosine transporters (ENTs) or by vesicular release, as adenosine is also presented in synaptic vesicles and released under physiological conditions by a Ca^{2+}-dependent secretion mechanism.[6] In most cases, extracellular formation of adenosine is through hydrolysis of released ATP, where nucleoside triphosphate diphosphohydrolases (NTPDases; e.g., CD39, NTPDase-1) degrade ATP or ADP to AMP and ecto-5′-nucleotidase (CD73) degrades AMP to adenosine.[7] ATP, which can also be released with other neurotransmitters by vesicular exocytosis and is a co-transmitter that rapidly and sequentially degrades to ADP, AMP and adenosine in the extracellular space.[8]

Previous studies using mice lacking either CD73 (CD73KO) or CD39 (CD39KO) have revealed the importance of extracellular adenosine formation for many physiological and pathological phenotypes. For example, deletion of CD73 leads to exaggerated inflammatory responses.[9] Extracellular adenosine produced by CD73 restrains tumor growth by suppressing NF-κB activity via A_{2B} signaling.[10] In addition, alteration of CD39 expression is correlated with reduced adenosine concentration,[11,12] while during stroke, CD39KO mice exhibit enlarged cerebral infarct volumes and decreased post-ischemic perfusion.[13] Administration of recombinant soluble human CD39 to CD39KO mice reduces cerebral infarct volume.[13] All of those functions are

relatively slow effects of adenosine, but adenosine also has rapid effects and therefore it is important to understand how CD73 and CD39 regulate physiological effects of rapid adenosine release.

Recently, rapid changes in adenosine have been discovered in brain *in vivo* and *ex vivo* using fast-scan cyclic voltammetry (FSCV).[14–17] One mode of rapid adenosine release is spontaneous release (without stimulation), which lasts only about 2-3 seconds and occurs randomly every 2-3 min.[16] Another mode of adenosine release is mechanical stimulation,[17] which occurs due to a mechanical perturbation of the tissue and lasts about 10 s (Chapter 2). Both modes of transient adenosine would be able to perform rapid neuromodulation without long-term depression of neuronal activity. Spontaneous adenosine release was also found in lamina II of sagittal spinal cord slices, where prostatic acid phosphatase (PAP) and CD73 KO decreased the concentration of adenosine release, implying it is due to breakdown of extracellular ATP.[14] Mechanically-stimulated adenosine release in the brain is activity-dependent and partially due to extracellular ATP metabolism, as it was decreased by an ATP inhibitor, POM-1, which targets NTPDase 1, 2 and 3.[17] However, it is currently unknown the extent to which rapid adenosine release is dependent on adenosine formed through sequential metabolism of extracellular ATP by CD39 and CD73.

Here, we investigated the contributions of CD73 and CD39 to the formation of pools of adenosine underlying spontaneous and mechanically-stimulated release using CD73KO and CD39KO mice. The number and frequency of spontaneous adenosine release events but not the event concentration or duration were decreased in CD73KO and CD39KO mice. In addition, deletion of CD73 or CD39 did not affect the

concentration or duration of mechanically-stimulated adenosine, suggesting this rapid adenosine release is independent of CD73 and CD39 activity. Thus, because the concentration does not change, these two modes do not seem to be dependent on adenosine formed by CD73 and CD39 metabolism of ATP, but there are other, redundant enzymes that could break down ATP; or release may be as adenosine per se. However, the dramatically different frequency of spontaneously released adenosine shows that spontaneous adenosine release is regulated by CD39 and CD73; thus, it may be possible to develop pharmacological treatments that differentially target modes of rapid adenosine release.

3.2 Materials and methods

3.2.1 Animals and surgery

Wild type C57BL/6J and CD73 deficient (CD73KO) males and females mice were purchased from Jackson Laboratory (Bar Harbor, ME). CD39 deficient (CD39KO) mice onto a C57BL/6 background were kindly provided by Dr. Bruce N. Cronstein and obtained from Taconic Biosciences (Rensselaer, NY).[34] All mice were used between 6-8 weeks of age. Animals were housed with food and water provided *ad libitum* on a standard 12:12-h light/dark cycle. Mice were anesthetized with 5% isoflurane in 100% oxygen in an anesthetic chamber and then transferred to a stereotaxic frame (Stoelting, Wood Dale, IL, USA). Anesthesia was maintained with 1.5-3% isoflurane in 100% oxygen delivered via a facemask (Stoelting, Wood Dale, IL, USA) and tail pinch to ensure that anesthesia is complete and substained. Isoflurane levels were adjusted until loss of righting reflex was observed. A homeothermic blanket system (Stoelting, Wood Dale, IL, USA) was used to maintain body temperature around 37 °C. Bupivacaine (0.20

mL) (Sensorcaine® MPF; APP Pharmaceuticals, LLC, Schaumburg, IL, USA) was administered under the skin for local anesthesia and the surgical site was shaved. Holes were drilled to allow the placement of the electrode in the hippocampus (AP -2.5 mm, ML + 2.4 mm, and DV -1.8 mm) based on the atlas of Paxinos and Frankline.[35] All experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia.

3.2.2 Chemicals

Phosphate-buffered saline (PBS) solution (all reagents were purchased from Fisher Scientific (Fair Lawn, NJ, USA)) was used for electrode calibration and consisted of 3.0 mM KCl, 10.0 mM NaH_2PO_4, 2.0 mM $MgCl_2$, 131.25 mM NaCl and 1.2 $CaCl_2$, with pH adjusted to 7.4. Adenosine was purchased from Sigma Aldrich (Milwaukee, WI, USA). A 10.0 mM stock solution of adenosine was prepared in 0.1 mM $HClO_4$ and stored at 4 ^{o}C. Adenosine was diluted daily in PBS solution to 1μM for calibration of the electrodes. All aqueous solutions were prepared using deionized water (Milli-Q Biocel; Millipore, Billerica, MA, USA).

3.2.3 Electrochemistry

Fabrication of carbon fiber microelectrode with T-650 carbon fiber was previously described.[36] Cylinder electrodes with diameters of 7 μm and lengths of 150-200 μm were used. Fast-scan cyclic voltammetry was used to detect adenosine as previously described.[36] For detection of adenosine, the electrode was scanned from -0.4 V to 1. 45 V and back to – 0.4 V every 100 ms at 400 V/s.

3.2.4 Data Analysis and Statistics

Transient adenosine events were first identified and analyzed using a new automated algorithm,[23] adenosine events were then confirmed by an analyst to exclude any signals that were not adenosine. The primary oxidation peaks of adenosine in Figure 1a-1c were filtered using a Fourier transform 1 Hz filter to reduce noise. All data were shown as mean ± SEM and all statistics were performed in GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). A one-way ANOVA with post hoc Tukey's test or a Student's unpaired *t*-test was performed on the number, concentration and duration of transient adenosine to assess differences between knockout and wile type mice. The distribution of inter-event time of adenosine events was analyzed using Kruskal-Wallis test with post-hoc Dunn's test or a Kolmogorov-Smirnov (KS) test. Some data points for adenosine inter-event time are not shown in order to better show the lower inter-event time where changes were more obvious. However, all data for inter-event time were used for statistical analyses. Statistical significance was designated at $p < 0.05$.

3.3 Results

The goal of this study was to test the extent to which extracellular metabolism of ATP by CD39 and CD73 is involved in formation of adenosine for spontaneous and mechanically-stimulated release. Adenosine was measured in the hippocampus, an area associated with memory and learning and also an important region in stroke studies, where hypoxia causes adenosine release to depress synaptic transmission.[18–22] We have recently shown that the mouse hippocampus has the highest frequency of spontaneous adenosine release and the highest concentration of mechanically-stimulated adenosine release (Chapter 2). Therefore, the hippocampus was chosen for this study.

Transient changes of adenosine were measured in anesthetized mice using FSCV at a carbon-fiber microelectrode. Adenosine is identified with an oxidation peaks at 1.3 V on the cathodic scan and 1.2 V on the anodic scan in the cyclic voltammograms (Fig. 3.1A-C, top) and 3-D color plots (Fig. 3.1A-C, bottom).[16] An automated algorithm is used to identify adenosine transients and these confirmed transients are starred on the concentration traces.[23] Spontaneous adenosine release events were continuously measured in the hippocampus of anesthetized mice for 4 hours. Mixed male and female mice were used in this study, as there was no significant difference between genders in frequency, concentration, and duration of adenosine (Fig. 3.2). Adenosine release events vary between wild type (WT), CD73KO, and CD39KO mice (Fig. 3.1). In WT mice, there are 3 transient adenosine events in this example 70 s window (Fig. 3.1A). However, there are only 2 events in CD73KO and only 1 event in CD39KO mice in a 70 s window (Fig. 3.1B and 3.1C). The average number of adenosine events per hour was 57 ± 7 in WT, 35 ± 6 in CD73KO, and 20 ± 2 in CD39KO mice. There was a significant effect of strain on number of adenosine events (Fig. 3.1D, one-way ANOVA, WT: n = 8 animals, 4M and 4F; CD73KO: n = 9 animals, 4M and 5F; CD39KO: n = 8 animals, 4M and 4F; $p = 0.0008$). The number of events in CD73KO and CD39KO mice was significantly lower than in WT mice (Tukey's test, $p = 0.026$ and $p = 0.0006$, respectively). To examine the spontaneous release frequency, the inter-event time between two consecutive adenosine events was calculated. The average inter-event time was 63 ± 2 s in WT, 92 ± 3 s in CD73KO, and 174 ± 7 s in CD39KO mice. This means that transients happen on average once a minute in WT while they occurred about 3 minutes apart in CD39KO mice. There were significant differences on the underlying distributions of inter-event time among all

three strains (Kruskal-Wallis test, $p < 0.0001$). Specifically, there was a significant difference between WT and CD73KO (Dunn's test, $p < 0.0001$), WT and CD39KO ($p < 0.0001$), as well as CD73KO and CD39KO ($p < 0.0001$). Spontaneous adenosine release occurs less frequently in CD73KO and least frequently in CD39KO compared to WT.

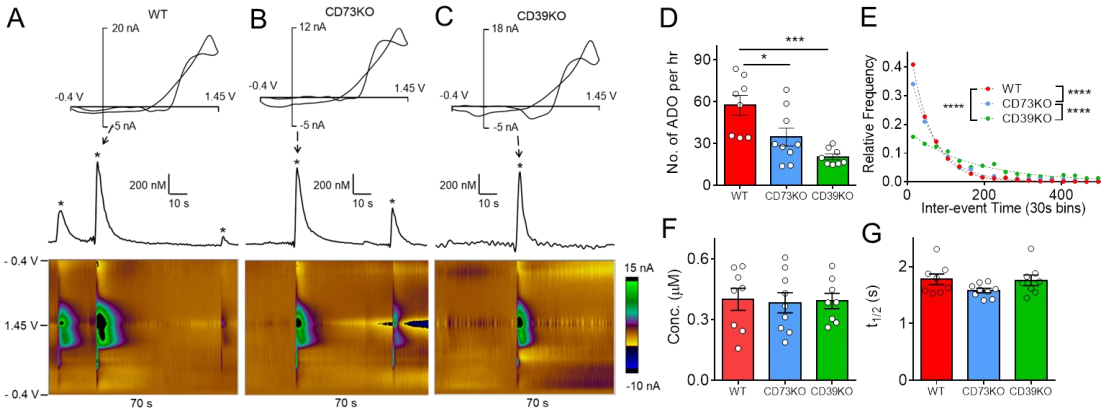

Figure 3.1 Spontaneous adenosine release in hippocampus. Example adenosine release in the (A) wild type (WT), (B) CD73KO, and (C) CD39KO mice. Adenosine can be identified in cyclic voltammograms (top) by its primary oxidation peak at 1.3 V on the cathodic scan and secondary oxidation peak at 1.2 V on the anodic scan. Concentration *vs.* time traces (middle) were derived from corresponding 3-D color plots (bottom). Adenosine oxidations are the green/purple area in the middle of the color plot. (D) Number of adenosine events per hour (main effect, one-way ANOVA, $p = 0.0008$). The number of adenosine events was significantly higher in WT than CD73KO and CD39KO (Tukey's test, $p = 0.026$ and $p = 0.0006$, respectively). (E) Inter-event time distributions. The underlying inter-event time distributions of adenosine were significantly different (Kruskal-Wallis test, $p < 0.0001$. Dunn's multiple comparisons test, WT vs. CD73KO: $p < 0.0001$; WT VS. CD39KO: $p < 0.0001$; CD73KO VS CD39KO: $p < 0.0001$). (F) Mean concentrations of adenosine events were not significantly different (one-way ANOVA, $p = 0.97$). (G) Mean $t_{1/2}$ did not significantly differ from each strain (one-way ANOVA, $p = 0.15$).

The mean concentration of adenosine events in each strain was similar with 0.40 ± 0.05 μM in WT, 0.38 ± 0.05 μM in CD73KO, and 0.39 ± 0.04 μM in CD39KO. There was no significant effect of strain on adenosine concentration (Fig. 3.1F, one-way ANOVA, $p = 0.97$). The duration, $t_{1/2}$, was calculated from the width at the half peak

height. The mean $t_{1/2}$ was 1.8 ± 0.1 s in WT, 1.6 ± 0.1 s in CD73KO, 1.7 ± 0.09 s in CD39KO, not significantly different (Fig. 3.1G, one-way ANOVA, p = 0.15). Thus, deletion of CD73 and CD39 did not affect the concentration or $t_{1/2}$, but significantly decreased the number and frequency of spontaneous adenosine release events.

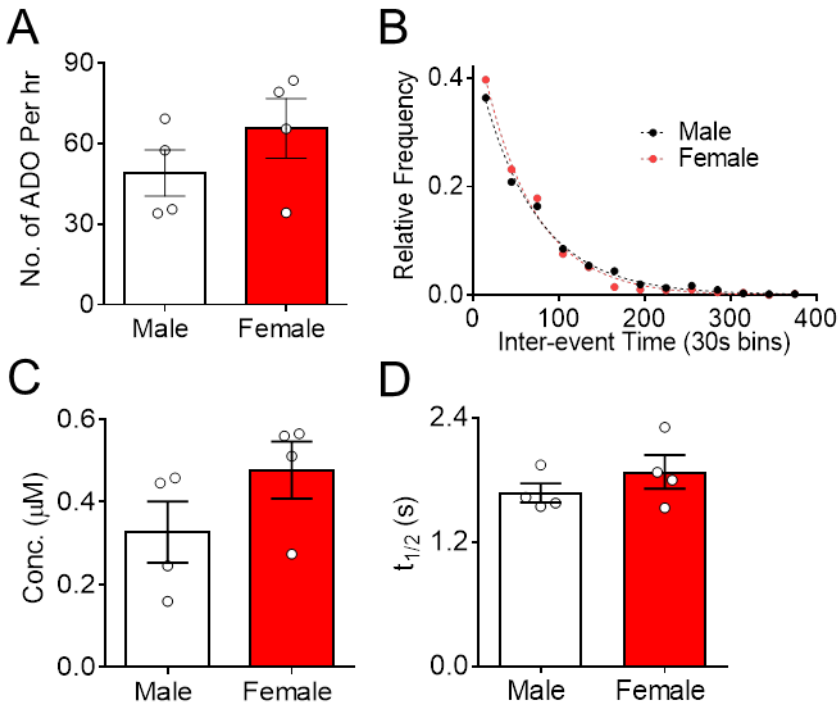

Figure 3.2 Spontaneous adenosine release in the hippocampus of male and female wild type mice. (A) The number of adenosine events was not significantly different between male and female mice (unpaired *t*-test, p = 0.28). (B) The underlying inter-event time distributions of adenosine were not significantly different (KS test, p = 0.99). (C) The mean concentrations of adenosine events between male and female mice were not significantly different (unpaired *t*-test, p = 0.19). (D) The mean $t_{1/2}$ of adenosine events were also not significantly different (unpaired *t*-test, p = 0.31).

Mechanically-stimulated adenosine release was performed by lowering the carbon-fiber microelectrode 0.1 mm in the same animal after 4 hours data collection of spontaneous adenosine release. The mechanical stimulation was repeated every 15 min for a total of 4 stimulations. Occasionally, a mechanical stimulation did not induce detectable adenosine release. In both WT and CD39KO, 4 out of 32 stimulations (n=8

animals each) did not evoke release while in CD73KO 2 out of 36 stimulations (n = 9 animals) did not evoke adenosine release. Thus, there is no difference in frequency of evoked release among the different strains.

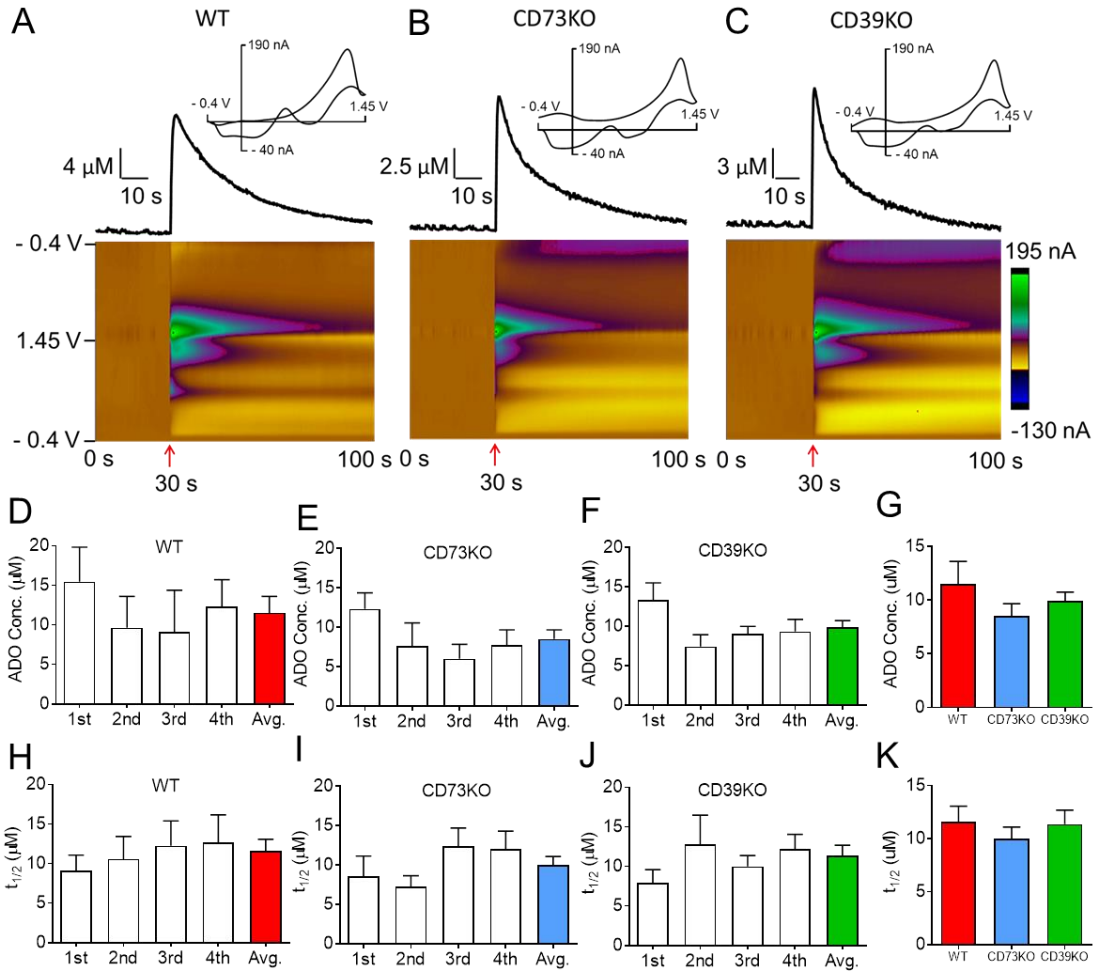

Figure 3.3 Mechanically-stimulated adenosine release in hippocampus. (A) Example mechanically-stimulated adenosine release in the hippocampus of (A) wild type, (B) CD73KO, and (C) CD39KO mice. Electrode was lowered 0.1 mm at 30 s (arrow). Concentration *vs.* time traces show the peak concentration of adenosine. Four consecutive stimulations were performed every 15 min for a total of 4 stimulations in (D) wild type, (E) CD73KO, and (F) CD39KO mice. There was no significant effect of stimulation number on mechanically-stimulated adenosine in any strain (one-way ANOVA, WT: n = 8 animals, 4M and 4F, p = 0.66; CD73KO: n = 9 animals, 4M and 5F, p = 0.24; CD39KO: n = 8 animals, 4M and 4F, p = 0.084). (G) Average concentrations of stimulated adenosine were not significantly different among all three strains (one-way ANOVA, p = 0.33). Average $t_{1/2}$ of adenosine did not differ by stimulation number in (H) WT, (I) CD73KO, and (J) CD39KO mice (one-way ANOVA, p = 0.42). (K) Mean $t_{1/2}$ of adenosine did not vary among three strains (one-way ANOVA, p = 0.63).

Figure 3.3A-C show examples of mechanically-stimulated adenosine release in WT, CD73KO and CD39KO mice after the electrode was lowered 0.1 mm at 30 s. The peak concentration of adenosine is 12 μM with a $t_{1/2}$ of 10 s in WT, 8 μM with a $t_{1/2}$ of 5.5 s in CD73KO and 9 μM with a $t_{1/2}$ of 6.2 s in CD39KO mice. Fig. 3.3D-K show the average concentration and $t_{1/2}$ of each successive mechanical stimulation as well as average for all stimulations. Overall, there was no significant effect of stimulation number on concentration (one-way ANOVA, WT: n = 8 animals, 4M and 4F, p = 0.66; CD73KO: n = 9 animals, 4M and 5F, p = 0.27; CD39KO: n = 8 animals, 4M and 4F, p = 0.42) or $t_{1/2}$ of mechanically-evoked adenosine (one-way ANOVA, WT: p = 0.75; CD73KO: p = 0.27; CD39KO: p = 0.42). Thus, the concentration and $t_{1/2}$ of adenosine are constant for multiple stimulations. The average concentrations of adenosine for all stimulations were slightly lower in CD73KO with a concentration of 9 ± 1 μM and CD39KO with a concentration of 10 ± 1 μM compared to a concentration of 12 ± 2 μM in WT, but there was no significant difference among all three strains (one-way ANOVA, p = 0.33). The average $t_{1/2}$ of mechanically-stimulated adenosine was 12 ± 2 s in WT, 10 ± 1 s in CD73KO, and 11 ± 1 s in CD39KO, and there was no significant effect of mice strains on $t_{1/2}$ (one-way ANOVA, p = 0.63). Overall, deletion of CD73 or CD39 did not affect the concentration or $t_{1/2}$ of mechanically-stimulated adenosine release.

3.4 Discussion

In this experiment, we tested the effect of CD39KO and CD73KO on the production of spontaneous and mechanically-stimulated adenosine release. With FSCV detection, carbon-fiber microelectrodes are up to 6-fold more sensitive for adenosine than ATP.[24] In addition, the secondary peak is more prominent for adenosine than ATP

detection;[25] thus, with FSCV we are only observing adenosine and not ATP. Our hypothesis was that in the CD73KO and CD39KO mice, the number and concentration of adenosine events would decrease if the mechanism of adenosine formation was due to extracellular ATP hydrolysis. Knocking out either CD39, which metabolizes ATP or ADP to AMP or CD73, which metabolizes AMP to adenosine, reduces the frequency of spontaneous adenosine release events, but does not change the concentration of each event. For mechanically-stimulated release, the concentration is also not affected by CD39KO or CD73KO, and the frequency is not affected either, as adenosine is seen with most of the mechanical stimulations. Thus, knocking out CD39 or CD73 has different effects on different modes of rapid adenosine release but does not affect the concentration of either event. Therefore, the events that are observed may not be due to breakdown of extracellular ATP. Here, we discuss the similarities and differences between the modes of adenosine release and how that could be important in the future for developing specific drug targets.

Adenosine is formed by two different mechanisms of formation, intracellular and extracellular. Intracellular formed adenosine is released from equilibrative adenosine transporters[5] or stored in synaptic vesicles and released by a Ca^{2+}-dependent excitation-secretion mechanism[6]. The intracellular mechanism of release has been identified during cortical seizure, where adenosine is released directly from neurons to activate A_1 receptors *in vivo*.[26] However, most studies have identified extracellular breakdown of ATP as the main source of extracellular adenosine. For example, rapid conversion of extracellular ATP, ADP and AMP to adenosine is observed in the hippocampus slice, where ATP is quickly degraded to adenosine in about 200 milliseconds.[27] Breakdown of

ATP is responsible for adenosine accumulation during high frequency stimulation, where application of ATP metabolism inhibitor, ARL 67156, decreases the amount of adenosine.[28]

The predominant enzyme responsible for the conversion of ATP or ADP to AMP is CD39 (NTPDase1) and for conversion of AMP to adenosine is CD73 (ecto-5'-nucleotidase).[29] CD39 hydrolyzes ATP and ADP about equally.[30] However, hydrolysis of ATP to adenosine is such an important pathway that there are redundant, alternative enzymes. ATP and ADP can also be hydrolyzed by NTPDase2 and 3; NTPDase2 has a strong preference for ATP and NTPDase3 is a functional intermediate that also preferentially hydrolyzes ATP.[30] Alkaline phosphatase in brain tissue can also dephosphorylate ATP, ADP and AMP.[31] Prostatic acid phosphatase (PAP) produces adenosine from AMP.[14] Thus, knocking out a major pathway of ATP metabolism may still result in adenosine formation by other enzymes such as acid or alkaline phosphatases. In our spontaneously released adenosine results, the frequency of adenosine events was reduced 40% in CD73KO and 65% in CD39KO. Thus, one explanation of these results is that the remaining events are due to other pathways of ATP breakdown, but that some events are not seen because the ATP cannot be rapidly broken down.

The Zylka group discovered rapid adenosine transients in lamina II of spinal cord slices and used KO mice to determine that the transients were due to the extracellular breakdown of ATP. Double knock-out of PAP and CD73 reduced both the frequency and concentration of adenosine transients by more than 50%, but did not fully eliminate the spontaneous events.[14] A later paper found that tissue non-specific alkaline

phosphatase (TNAP) also contributed to AMP breakdown and that applying a drug to block TNAP virtually eliminated AMP breakdown in the slices.[31] Our findings of reduced frequency of adenosine transients are in agreement with these studies; however we did not see a decrease in event concentration while they did. The decreased frequency could be due to ATP events, where ATP cannot be broken down to adenosine. However, if other mechanisms are available to break down some of the ATP, we would have expected smaller concentration events and this was not observed. Thus, the steady concentration after CD39KO and CD73KO suggests that adenosine transients may not be due to the breakdown of extracellular ATP. In the future, it would be interesting to examine spontaneous adenosine release in mouse hippocampus using PAP or PAP/CD73 double KO, but the PAP mice are no longer available and considerable resources would be needed to make them again.

The reduced frequency, but no change in concentration, for spontaneous adenosine release leads to another possible explanation for the formation of adenosine. It is possible that CD73 or CD39 deletion causes higher basal levels of ATP and this could act at ATP receptors to cause an inhibition of release. For example, ATP receptors are located presynaptically on glutamate terminals and modulate glutamate release;[27] if adenosine or ATP was being co-released with glutamate, this would affect the frequency of the events. Build-up of ATP concentration might cause changes in ATP circuits that could regulate the release events. Thus, we cannot rule out that CD73 or CD39 deletion indirectly affects the frequency or events. In this scenario, it is more likely that the released nucleotide is adenosine itself, as that would lead to the same concentration of released adenosine and not the diminished concentration observed by the Zylka group.

For mechanically-stimulated release, our findings reveal that CD73 and CD39 are not majorly involved in the mechanism of rapid adenosine release. The frequency of mechanical stimulation evoking adenosine transients was similar in all genotypes, with stimulation causing release 88% in WT, 94% in CD73KO and 88% in CD39KO. The concentration of adenosine released per event was also not different in CD73KO and CD39KO than WT mice. Therefore, other mechanisms of adenosine formation contribute to the signal. Adenosine can be formed intracellularly and released from synaptic vesicles.[6] Adenosine formed intracellularly is often released directly through equilibrative nucleoside transporters (ENTs), such as ENT1 and ENT2.[32] However, inhibiting ENTs with NBTI in rat brain slice did not affect the concentration of mechanically-stimulated adenosine release.[17] In previous brain slice studies, blocking ATP metabolism with POM-1, a high affinity inhibitor of nucleoside triphosphate diphospholydrolase 1,2 and 3, did reduce the concentration of mechanically-stimulated adenosine release,[17] indicating that NTPDase 2 and 3 may contribute to the formation of mechanically-stimulated adenosine release. In addition, application of tetrodotoxin to block sodium channels or chelating calcium with EDTA decreased the concentration of mechanically-sensitive adenosine release, suggesting mechanically-stimulated adenosine release is activity dependent and vesicular.[17] Thus, future research is required to identify if other ATP enzymes are sufficient to break down ATP to adenosine or if adenosine is released per se.

These data allow a direct investigation on the formation of spontaneous and mechanically-stimulated adenosine release, because measurements were made in the same animals. Deletion of CD73 or CD39 eliminates about half of spontaneous adenosine release events but do not affect the adenosine that resulting from mechanically-stimulated

release. For both mechanisms, the concentration of events is not different with CD73KO or CD39KO. Therefore, there are two possibilities that will need to be tested in the future: (1) that the mechanism of release is due to other enzymes that are responsible for ATP breakdown and (2) that adenosine is not a breakdown product of extracellular ATP but is released as intracellularly formed adenosine. Because the frequency of spontaneous release could be influenced by the basal level changes in ATP, either of these mechanisms could be responsible for spontaneous or mechanically-stimulated release. However, this study does show an interesting result that might be useful for developing future drug targets. The effect of deletion of CD73 or CD39 on spontaneous release frequency argues that these enzymes can be targeted to modulate spontaneous release, and they would not have any effect on mechanically-stimulated release. Spontaneous release is generally modulated by frequency and not concentration, and these enzymes are additional targets. Preferential targeting of one mode of adenosine release might be valuable for adenosine-based therapies, as many have failed due to the ubiquitous nature of adenosine. For example, our previous study showed that spontaneous adenosine release events increased during ischemia-reperfusion injury and provide fast, local neuroprotection.[33] Treatments to upregulate CD73 or CD39 might provide even more local neuroprotection. While most previous studies have found that extracellular ATP breakdown is the source of adenosine, our results show that the mechanism of rapid adenosine release is complicated and may not be due to extracellular adenosine breakdown. More studies are needed to understand the modes of release and how they can be manipulated to provide better neuroprotection.

3.5 References

1. Bjorness TE, Greene RW. Adenosine and sleep. *Curr Neuropharmacol*. 2009;7(3):238-245. doi:10.2174/157015909789152182.

2. Williams-Karnesky RL, Stenzel-Poore MP. Adenosine and stroke: maximizing the therapeutic potential of adenosine as a prophylactic and acute neuroprotectant. *Curr Neuropharmacol*. 2009;7(3):217-227. doi:10.2174/157015909789152209.

3. O'Regan M. Adenosine and the regulation of cerebral blood flow. *Neurol Res*. 2005;27(2):175-181. doi:10.1179/016164105X21931.

4. Haskó G, Cronstein B. Regulation of Inflammation by Adenosine. *Front Immunol*. 2013;4:85. doi:10.3389/fimmu.2013.00085.

5. dos Santos-Rodrigues A, Pereira MR, Brito R, de Oliveira NA, Paes-de-Carvalho R. Adenosine Transporters and Receptors: Key Elements for Retinal Function and Neuroprotection. *Vitam Horm*. 2015;98:487-523. doi:10.1016/BS.VH.2014.12.014.

6. Corti F, Cellai L, Melani A, Donati C, Bruni P, Pedata F. Adenosine is present in rat brain synaptic vesicles. *Neuroreport*. 2013;24(17):982-987. doi:10.1097/WNR.0000000000000033.

7. Latini S, Pedata F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem*. 2001;79(3):463-484. doi:10.1046/j.1471-4159.2001.00607.x.

8. Westfall DP, Todorov LD, Mihaylova-Todorova ST. ATP as a Cotransmitter in Sympathetic Nerves and Its Inactivation by Releasable Enzymes. *J Pharmacol Exp Ther*. 2002;303(2):439-444. doi:10.1124/jpet.102.035113.

9. Yegutkin GG. Enzymes involved in metabolism of extracellular nucleotides and nucleosides: Functional implications and measurement of activities. *Crit Rev Biochem Mol Biol*. 2014;49(6):473-497. doi:10.3109/10409238.2014.953627.

10. Leclerc BG, Charlebois R, Chouinard G, et al. CD73 Expression Is an Independent Prognostic Factor in Prostate Cancer. *Clin Cancer Res*. 2016;22(1):158-166. doi:10.1158/1078-0432.CCR-15-1181.

11. Enjyoji K, Sévigny J, Lin Y, et al. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nat Med*. 1999;5(9):1010-1017. doi:10.1038/12447.

12. Lanser AJ, Rezende RM, Rubino S, et al. Disruption of the ATP/adenosine balance in CD39 $^{-/-}$ mice is associated with handling-induced seizures. *Immunology*. 2017;152(4):589-601. doi:10.1111/imm.12798.

13. Pinsky DJ, Broekman MJ, Peschon JJ, et al. Elucidation of the thromboregulatory role of CD39/ectoapyrase in the ischemic brain. *J Clin Invest*. 2002;109(8):1031-1040. doi:10.1172/JCI10649.

14. Street SE, Walsh PL, Sowa NA, et al. PAP and NT5E inhibit nociceptive neurotransmission by rapidly hydrolyzing nucleotides to adenosine. *Mol Pain*. 2011;7:80. doi:10.1186/1744-8069-7-80.

15. Lee ST, Venton BJ. Regional Variations of Spontaneous, Transient Adenosine Release in Brain Slices. *ACS Chem Neurosci*. 2018;9(3):505-513. doi:10.1021/acschemneuro.7b00280.

16. Nguyen MD, Lee ST, Ross AE, Ryals M, Choudhry VI, Venton BJ. Characterization of spontaneous, transient adenosine release in the caudate-putamen and prefrontal cortex. *PLoS One*. 2014;9(1):e87165. doi:19326203.

17. Ross AE, Nguyen MD, Privman E, Venton BJ. Mechanical stimulation evokes rapid increases in extracellular adenosine concentration in the prefrontal cortex. *J Neurochem*. 2014;130(1):50-60. doi:10.1111/jnc.12711.

18. Fowler JC. Changes in extracellular adenosine levels and population spike amplitude during graded hypoxia in the rat hippocampal slice. *Naunyn Schmiedebergs Arch Pharmacol*. 1993;347(1):73-78. doi:10.1007/BF00168775.

19. Fredholm BB, Dunwiddie T V, Bergman B, Lindström K. Levels of adenosine and adenine nucleotides in slices of rat hippocampus. *Brain Res*. 1984;295(1):127-136.

20. Frenguelli BG, Llaudet E, Dale N. High-resolution real-time recording with microelectrode biosensors reveals novel aspects of adenosine release during hypoxia in rat hippocampal slices. *J Neurochem*. 2003;86(6):1506-1515. doi:10.1046/j.1471-4159.2003.01957.x.

21. Andiné P, Rudolphi KA, Fredholm BB, Hagberg H. Effect of propentofylline (HWA 285) on extracellular purines and excitatory amino acids in CA1 of rat hippocampus during transient ischaemia. *Br J Pharmacol*. 1990;100(4):814-818. doi:10.1111/j.1476-5381.1990.tb14097.x.

22. Frenguelli BG, Wigmore G, Llaudet E, Dale N. Temporal and mechanistic dissociation of ATP and adenosine release during ischaemia in the mammalian hippocampus. *J Neurochem*. 2007;101(5):1400-1413. doi:10.1111/j.1471-4159.2007.04425.x.

23. Borman RP, Wang Y, Nguyen MD, Ganesana M, Lee ST, Venton BJ. Automated

Algorithm for Detection of Transient Adenosine Release. *ACS Chem Neurosci*. 2017;8(2):386-393. doi:10.1021/acschemneuro.6b00262.

24. Swamy BEK, Venton BJ. Subsecond Detection of Physiological Adenosine Concentrations Using Fast-Scan Cyclic Voltammetry. *Anal Chem*. 2007;79:744-750. doi:10.1021/AC061820i.

25. Ross AE, Venton BJ. Sawhorse waveform voltammetry for selective detection of adenosine, ATP, and hydrogen peroxide. *Anal Chem*. 2014;86(15):7486-7493. doi:10.1021/ac501229c.

26. Lovatt D, Xu Q, Liu W, et al. Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity. *Proc Natl Acad Sci U S A*. 2012;109(16):6265-6270. doi:10.1073/pnas.1120997109.

27. Dunwiddie T V, Diao L, Proctor WR, Oliveira CR, Cunha RA. Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. *J Neurosci*. 1997;17(20):7673-7682. doi:20026618.

28. Bekar L, Libionka W, Tian G-F, et al. Adenosine is crucial for deep brain stimulation–mediated attenuation of tremor. *Nat Med*. 2008;14(1):75-80. doi:10.1038/nm1693.

29. Reutershan J, Vollmer I, Stark S, Wagner R, Ngamsri K-C, Eltzschig HK. Adenosine and inflammation: CD39 and CD73 are critical mediators in LPS-induced PMN trafficking into the lungs. *FASEB J*. 2009;23(2):473-482. doi:10.1096/fj.08-119701.

30. Zimmermann H. Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol*. 2000;362(4-5):299-309. doi:10.1007/s002100000309.

31. Street SE, Kramer NJ, Walsh PL, et al. Tissue-nonspecific alkaline phosphatase acts redundantly with PAP and NT5E to generate adenosine in the dorsal spinal cord. *J Neurosci*. 2013;33(27):11314-11322. doi:10.1523/JNEUROSCI.0133-13.2013.

32. Lovatt D, Xu Q, Liu W, et al. Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity. *Proc Natl Acad Sci*. 2012;109(16):6265-6270. doi:10.1073/pnas.1120997109.

33. Ganesana M, Venton BJ. Early changes in transient adenosine during cerebral ischemia and reperfusion injury. *PLoS One*. 2018;13(5):e0196932. doi:10.1371/journal.pone.0196932.

34. Fernández P, Perez-Aso M, Smith G, et al. Extracellular generation of adenosine

by the ectonucleotidases CD39 and CD73 promotes dermal fibrosis. *Am J Pathol*. 2013;183(6):1740-1746. doi:10.1016/j.ajpath.2013.08.024.

35. Watson C, Paxinos G. *Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, Fourth Edition*.; 2012.

36. Wang Y, Venton BJ. Correlation of transient adenosine release and oxygen changes in the caudate-putamen. *J Neurochem*. 2017;140(1):13-23. doi:10.1111/jnc.13705.

Chapter 4: Correlation of transient adenosine release and oxygen changes in the caudate-putamen

Abstract

Adenosine is an endogenous nucleoside that modulates important physiological processes, such as vasodilation, in the central nervous system. A rapid, 2-4 seconds, mode of adenosine release has been recently discovered, but the relationship between this type of adenosine and blood flow change has not been characterized. In this study, adenosine and oxygen changes were simultaneously measured using fast-scan cyclic voltammetry. Oxygen changes occur when there is an increase in local cerebral blood flow and thus are a measure of vasodilation. About 34% of adenosine transients in the rat caudate-putamen are correlated with a subsequent transient change in oxygen. The amount of oxygen was correlated with the concentration of adenosine release and larger adenosine transients (over 0.4 μM) always had subsequent oxygen changes. The average duration of adenosine and oxygen transients were 3.2 seconds and 3.5 seconds, respectively. On average, the adenosine release starts and peaks 0.2 seconds prior to the oxygen. The A_{2A} antagonist, SCH 442416, decreased the number of both adenosine and oxygen transient events by about 32%. However, the A_1 antagonist, DPCPX, did not significantly affect simultaneous adenosine and oxygen release. The nitric oxide (NO) synthase inhibitor L-NAME also did not affect the concentration or number of adenosine and oxygen release events. These results demonstrate that both adenosine and oxygen release are modulated via A_{2A} receptors. The correlation of transients concentrations, time delay between adenosine and oxygen peaks, and effect of A_{2A} receptors suggests adenosine modulates blood flow on a rapid, sub-second time scale.

4.1 Introduction

Adenosine is an endogenous nucleoside involved in many important biochemical processes throughout the body, including energy balance. Adenosine formation increases during times of accelerated neuronal activity,[1,2] and it can be released from cells, becoming an extracellular signal that more energy is being consumed. One way adenosine signals for more energy to be delivered is by acting as a vasodilator, increasing blood flow.[3,4] Tissue metabolism has a continuous need for oxygen, which usually is delivered by blood flow.[5] In stressful conditions, when energy consumption is high, vasodilation occurs and the amount of oxygen delivered to the tissue is actually higher than during normoxic conditions. Previous studies showed that the concentration of adenosine in extracellular space of the brain dramatically increases during conditions such as ischemia.[6–8] Endogenous adenosine acts as a vasodilator to increase cerebral blood flow,[9] an effect primarily mediated by A_{2A} receptors located on blood vessels;[10,11] however, the relationship between adenosine and oxygen and the extent to which A_{2A} receptors regulates both adenosine and blood flow changes are not fully known.

Local blood flow increases are measured in several ways. One is hydrogen clearance, [12,13] where platinum electrodes are used to measure the clearance of inhaled H_2. Another method for estimating blood flow is measuring changes in cerebrovascular resistance using laser Doppler spectroscopy.[14] Changes in vessel diameter are also measured to investigate cerebral vasodilation after pharmacological administration.[15] Blood flow changes can be estimated by monitoring oxygen level changes using blood-oxygen level dependent (BOLD) imaging.[16,17] The increase in oxygen can also be measured electrochemically, using either amperometry or fast-scan cyclic voltammetry

(FSCV).[5,18] FSCV is useful because it can measure both neurotransmission and oxygen levels. For example, FSCV has been used to measure dopamine or norepinephrine levels simultaneously with oxygen levels, using a modified waveform that scans down to -1.4 V to measure oxygen reduction.[19,20] To measure adenosine and oxygen levels, the FSCV waveform can be modified to scan up to 1.5 V and down to -1.4 V to both oxidize adenosine and reduce oxygen.[21]

Previous studies have shown that adenosine accumulates in times of stress, such as ischemia, and can induce vasodilation and change cerebral blood flow. Recently, a new type of rapid adenosine release has been characterized in the caudate-putamen and prefrontal cortex regions *in vivo*.[22] This spontaneous, transient adenosine release is fast, lasting only 2 – 5 seconds in the extracellular space.[23] Transient adenosine release can modulate dopamine neurotransmission on a rapid time scale.[24] However, it is not known if this rapid mode of adenosine release has an effect on cerebral blood flow.

The goal of this study was to determine the relationship between transient adenosine and changes in cerebral blood flow. We tested the hypothesis that spontaneous, transient adenosine is correlated with vasodilation in local areas of the brain. Adenosine and oxygen transients were measured simultaneously in caudate-putamen of anesthetized rats using FSCV. Oxygen release was detected after about 34% of adenosine transients. The concentrations of adenosine and oxygen changes were correlated and big adenosine transients were always followed by an oxygen transient. The A_{2A} antagonist, SCH 442416, decreased the number of adenosine and oxygen transients and increased the time interval between each transient release but A_1 antagonist, DPCPX, did not. Thus,

endogenous transient adenosine was correlated with transient local vasodilation and both were modulated through A_{2A} receptors.

4.2 Materials and Methods

4.2.1 Chemicals and Drugs

Phosphate buffered saline (PBS) solution (all components in mM: 3.0 KCl, 10.0 NaH_2PO_4, 2.0 Na_2SO_4, 1.2 $MgCl_2$, 131.25 NaCl and 1.2 $CaCl_2$, with pH adjusted to 7.4) were purchased from Fisher Scientific (Fair Lawn, NJ, USA)) was used for electrode calibration. Adenosine was purchased from Sigma-Aldrich (Milwaukee, WI, USA). A 10.0 mM stock solution of adenosine was prepared in 0.1 M perchloric acid ($HClO_4$) and stored in the refrigerator. A 1.0 μM adenosine solution was made in PBS on the day of use. All aqueous solutions were prepared using deionized water (Milli-Q Biocel; Millipore, Billerica, MA, USA).

8-Cyclopentyl-1, 3-dipropylxanthine (DPCPX, 6 mg/kg, Sigma-Aldrich) and 2-(2- Furanyl)-7-[3-(4-methoxyphenyl) propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5- amine (SCH 442416, 3 mg/kg, Tocris, UK) were dissolved in 300 μL dimethyl sulfoxide (DMSO, Amresco, Solon, OH, USA), whereas Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 mg/kg, Sigma-Aldrich) was dissolved in 1 mL heated saline and all drugs were administered intraperitoneally (*i.p*). These doses were selected based on previous experiments reported in the literature.[19,22,25]

4.2.2 Fabrication of Carbon-Fiber Microelectrodes and Fast Scan Cyclic Voltammetry

Carbon-fiber microelectrodes were fabricated by aspirating a 7 µm diameter T-650 carbon-fiber (Cytec Engineering Materials, West Patterson, NJ, USA) into a glass capillary (1.2 mm x 0.68 mm; A-M System, Inc., Seqium, WA, USA) and pulling it in a vertical micropipette puller (model PE-21; Narishige, Tokyo, Japan). The extended carbon fiber was cut with a scalpel to a length of around 100 µm. Prior to use, electrodes were soaked in 2-propanol for at least 10 minutes and then backfilled with 1 M KCl.

Fast-scan cyclic voltammetry (FSCV) was used to detect adenosine and oxygen with sub-second temporal resolution. The cyclic voltammetric waveform was generated and the resulting signals were computer controlled by Tar Heel CV or HDCV (from Mark Wightman, UNC at Chapel Hill), written in LabVIEW (National Instruments, Austin, TX, USA). A Dagan Chem Clamp potentiostat (Dagan Corporation; Minneapolis, MN, USA) was used to apply the potential and measure the current. For simultaneous detection of adenosine and oxygen, the electrode was scanned from 0 V to 1.45 V, then -1.4 V and back to 0 V every 100 milliseconds at 450 V/s. The reference electrode was a Ag/AgCl electrode. All color plots and cyclic voltammograms were background subtracted to remove any non-Faradic currents, by subtracting the average of 10 CVs collected no more than 10 seconds before the event.

4.2.3 Flow-Injection Apparatus and in vitro Calibrations

A flow-injection apparatus was used to calibrate the electrode in concentrations of adenosine and oxygen after *in vivo* experimentation [26]. Electrodes were calibrated with 1.0 µM adenosine in PBS buffer. To calibrate oxygen concentration, air- and nitrogen-

saturated PBS buffers were used. Oxygen concentrations were measured in various mixtures of nitrogen- and air-saturated PBS with volume ratios of 2:1, 5:1, 10:1, and 15:1 using D.O. 6+ dissolved oxygen meter (Eutech Instruments Pte Ltd. Singapore). The concentration of oxygen in each solution was compared to nitrogen-saturated PBS, which contains no oxygen. Peak reduction currents for oxygen were used for calibration, shown in Figure 4.1.

4.2.4 Animals and Surgery

Male Sprague-Dawley rats (250 – 350 g; Charles-River, Wilmington, MA, USA) were housed on a 12:12-h light/dark cycle with food and water provided ad libitum. Rats were initially anesthetized with isoflurane (1 mL/100 g rat weight) and then injected with urethane (1.5 g/kg, i. p.). The surgical site was shaved and the rat was placed in a stereotaxic frame. Bupivacaine (0.25 mL) (Sensorcaine® MPF, APP Pharmaceuticals, LLC; Schaumburg, IL, USA) was administered under the skin for local anesthesia. Holes were drilled using a stereotaxic drill for electrode placement after the skull was exposed. Electrodes were placed in the caudate-putamen (+ 1.2 mm anterior-posterior (AP), + 2.0 mm mediolateral (ML), and - 4.5 mm dorsoventral (DV). A Ag/AgCl reference electrode was inserted on the contralateral side of brain. The body temperature of the rat was maintained at 37 °C using an isothermal pad (FHC, Bowdoin, ME, USA). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia.

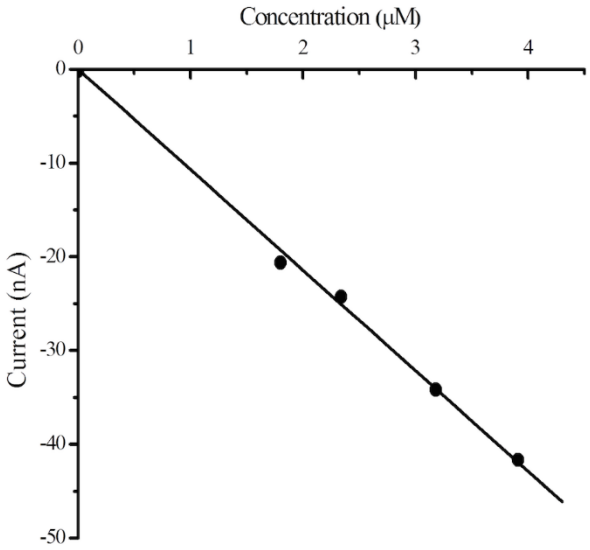

Figure 4.1 Calibration of oxygen. The oxygen concentration was calibrated using nitrogen- and air-saturated PBS buffer with volume ratio of 1:0, 2:1, 5:1, 10:1, and 15:1. Nitrogen-saturated PBS buffer without oxygen was used as running buffer. The linear equation is $y = -0.34x$ ($R^2 = 0.99$). This graph allows calculation of oxygen concentration.

4.2.5 Data Collection and Analysis

Electrodes were implanted and equilibrated for at least 30 minutes with the applied waveform prior to data collection. Data were excluded if less than 10 transients were observed within the initial 30 minutes. If robust transients were not found, a new electrode was inserted and up to five electrodes were tried for each animal. After transients were identified, the electrode placement was optimized and data were collected for four hours.

Principal component regression analysis was used to identify adenosine and oxygen transients. A training set was obtained using the five largest and most definitive adenosine transients for each rat in vivo.[22] For oxygen, a training set was compiled for the five different concentrations of oxygen that were measured *in vitro*. The oxidation

peak of adenosine and the reduction peak of oxygen were filtered using a Fourier transform 1 Hz filter to reduce noise.

4.2.6 Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA), or OriginPro 7.5 (Origin Lab Corporation, Northampton, MA, USA). A student's *t*-test was used to compare the effects of the drugs on pre- and post-drug response of the number and concentrations of adenosine and oxygen transients. The distribution of inter-event times of adenosine and oxygen transients were analyzed using a Kolmogorov-Smirnov (KS) test. Some data points for adenosine and oxygen inter-event times are not shown in order to better show the lower inter-event times where changes are more obvious after drug administration. However, all data for inter-event times were used for statistical analyses. Statistical significance was designated at $p < 0.05$ and all data are presented as mean ± standard error of the mean (SEM).

4.3 Results

4.3.1 Adenosine and oxygen cyclic voltammetry *in vivo*

Fast-scan cyclic voltammetry at a carbon-fiber microelectrode was used to simultaneously detect fast changes in adenosine and oxygen with sub-second temporal resolution. The potential was scanned from 0 V to 1.45 V, then to -1.4 V and back to 0 V at 450 V/s every 100 milliseconds as shown in Figure 4.2A. The double layer charging at the electrode produces a large but stable background current and can be subtracted out to obtain a background-subtracted cyclic voltammogram (Fig. 4.2B and 4.2D). Oxidation of adenosine results in two oxidation peaks in the background-

subtracted cyclic voltammogram. With this waveform, the primary oxidation of adenosine occurs right after the switching potential at 1.27 V on the cathodic scan because of slow electron transfer. A secondary oxidation occurs at 1.26 V on the anodic scan. The reduction of oxygen occurs at -1.3 V on the cathodic scan. A representative color plot is shown in Figure 4.2C. In the color plot, time is plotted on the abscissa, electrode potential is plotted on the ordinate, and the current is depicted in false color. The green/purple ovals represent the adenosine oxidation currents. The top oval is

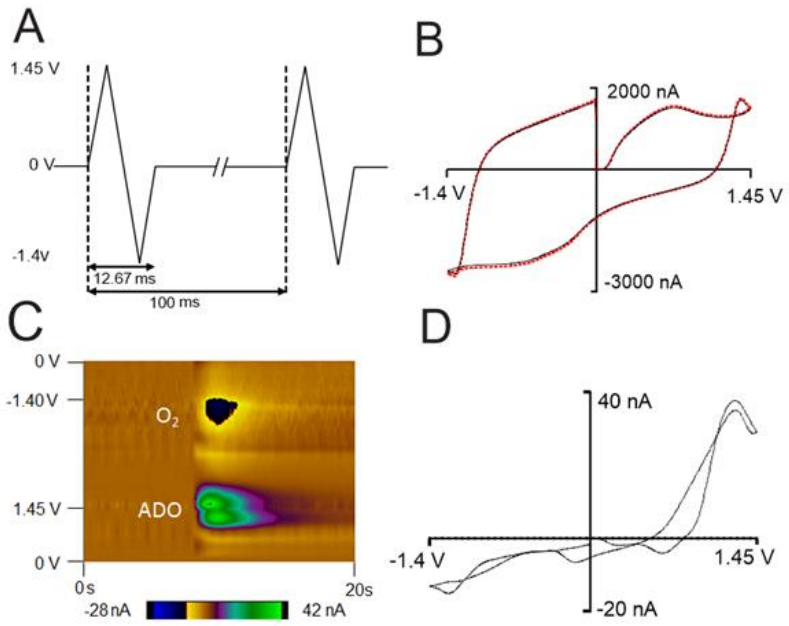

Figure 4.2 Fast-scan cyclic voltammetry for detection of adenosine and oxygen. (A) The potential applied to the carbon fiber microelectrode is ramped from 0 V to 1.45 V, then -1.4 V and back to 0 V at a scan rate of 450 V/s. (B) The potential sweep produces a large background current. The solid black line is the background current, and the dashed red line is the current observed in the presence of adenosine and oxygen. (C) A color plot of an adenosine transient that is 0.75 μM and an oxygen transient of 58 μM *in vivo*, where the x-axis is time in seconds, the y-axis is the applied potential, and z-axis is current (nA) depicted in false color. (D) A cyclic voltammogram for adenosine and oxygen. The primary and secondary oxidations of adenosine occur at 1.27 V on the back scan and 1.26 V on the front scan, respectively. The reduction of oxygen occurs at -1.3 V.

the primary oxidation and the bottom oval is the secondary oxidation of adenosine. The secondary oxidation is slightly delayed in time from the primary oxidation because it is due to the oxidative byproduct formed after the primary oxidation. For oxygen, the dark blue area around -1.3 V is the reduction of oxygen. Because the oxidation of adenosine and reduction of oxygen occur at different potentials, adenosine and oxygen release can be detected simultaneously.

4.3.2 Correlation of adenosine and oxygen

To determine whether the oxygen signal was delayed from the adenosine signal, the starting points of adenosine and oxygen peaks were measured and compared (Fig. 4.3). Current versus time traces are plotted at the primary oxidation potential of adenosine and the reduction potential of oxygen, which help identify the timing of the events. In this example, adenosine starts to rise about 0.1 second before oxygen starts to change. The cyclic voltammogram (CV) shows that the first CV that has a clear primary oxidation peak of adenosine and does not have an oxygen peak that is discernible from the noise. On average, adenosine started to increase 0.2 ± 0.03 seconds before oxygen, indicating the adenosine transient occurs before the release of oxygen.

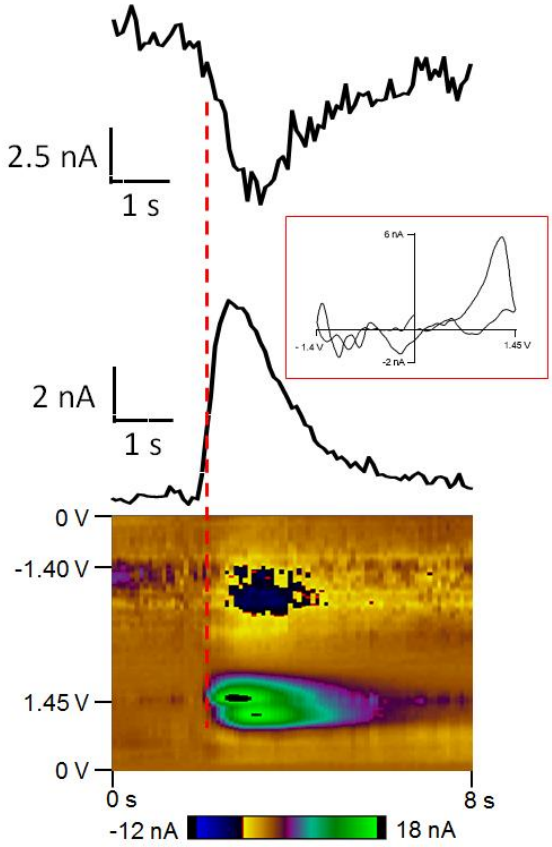

Figure 4.3 Timing of adenosine and oxygen transients in vivo. Color plot, CV, and current versus time traces depicting adenosine and oxygen dynamics in caudate-putamen. The color plot and current versus time traces shows that the change in adenosine occurs before the change in oxygen concentration. The inset CV is the very first CV where adenosine is clearly present (taken from dashed red line) and there is no discernible oxygen peak above the noise.

Additionally, the time difference between the peak adenosine and oxygen concentration was calculated for each set of transients and plotted in 0.1 second bins (Fig. 4.4A). Further supporting that adenosine comes first, the mean and median of delay time for the oxygen peak were 0.4 seconds and 0.2 seconds, respectively, which means if an oxygen transient was observed, the oxygen usually peaked 0.2 seconds after the adenosine peak.

Figure 4.4B shows a correlation plot of the concentration of spontaneously released adenosine vs. the oxygen concentration. Data are only plotted for adenosine transients that occurred with an oxygen transient. There is a linear relationship between the transient adenosine concentration and the oxygen concentration. The correlation coefficient is 0.63, indicating that 63 percent of the variance in oxygen concentration is due to the variance in spontaneous transient adenosine concentration.

To further understand how often adenosine was released with an oxygen event, a plot was made of binned adenosine concentration vs. percentage of transient adenosine events with an accompanying oxygen event (Fig. 4.4C). The minimum concentration of adenosine that was ever released with an oxygen event was 0.02 μM, which is higher than 0.01 μM, the lowest concentration of adenosine that we can detect. When the concentration of adenosine was 0.15 μM, about 50% of adenosine events were released with oxygen, and when the concentration of adenosine was 0.4 μM or higher, all adenosine events had oxygen transients that followed. Overall, because most adenosine release events were low concentration, 34% of measured adenosine events were accompanied by an oxygen transient.

The duration of both adenosine and oxygen changes were short, demonstrating that that adenosine and oxygen are available to mediate responses in the extracellular space for only a few seconds. The durations of adenosine and oxygen release events were 3.2 ± 1.9 seconds and 3.5 ± 1.9 seconds, respectively. High concentration release events of adenosine or oxygen had a longer duration because it takes a longer time to clear higher concentrations.[27]

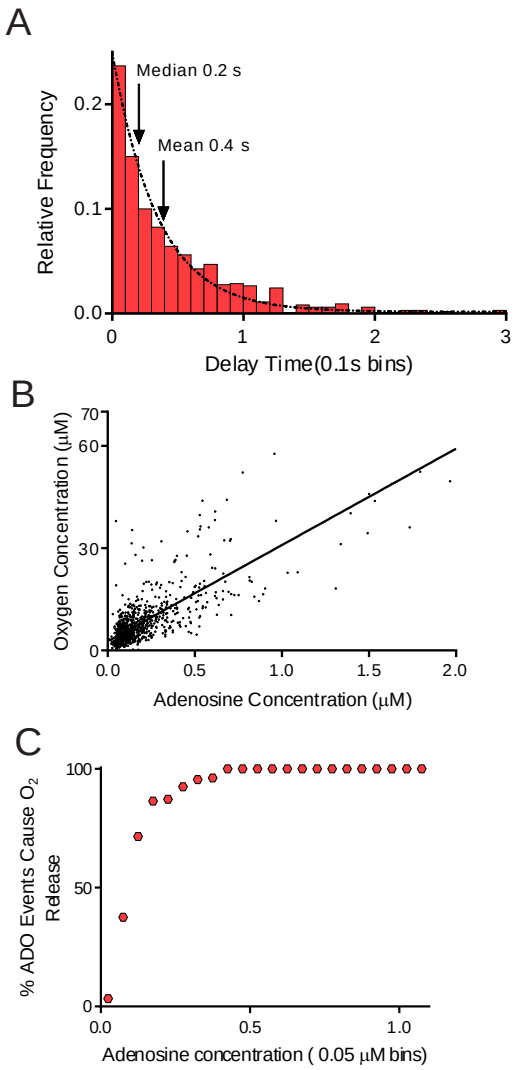

Figure 4.4 Correlation of adenosine and oxygen. (a) Time delay between peak adenosine and peak oxygen (in 0.1 s bins). The exponential fit (dashed line) is $y = 0.25e^{-2.9x}$ (R^2= 0.98) (n = 24 animals). (b) Correlation plot of adenosine concentration versus oxygen concentration. There is a linear relationship with a correlation coefficient of 0.63 (n = 983 adenosine/oxygen transients pairs from 24 animals). (c) Percent of transient adenosine events that cause oxygen release (adenosine transients binned in 0.05 μM bins on x-axis, n = 24 animals).

4.3.3 Adenosine and Oxygen Transients Over Time

Spontaneous adenosine and oxygen release events were continuously measured for four hours and comparison made between the first two hours and second two hours as a control for pharmacological experiments. The number of transients was averaged for each animal, and the plot shows box and whisker plots with the median and quartiles in the box and the range marked by the whiskers (Fig. 4.5A-B). The variance is large due to differences from animal to animal and there were no significant changes in the number of adenosine or oxygen transients between first two hour and second two-hour time periods (n = 6, paired *t*-test, $p = 0.13$ and $p = 0.89$, respectively). To examine the frequency, the time between consecutive transients was plotted as a histogram (Fig. 4.5C-D). The adenosine transients occur closer together than the oxygen transients because not all adenosine transients were followed by oxygen release. The distribution of inter-event times for the first two hours and second two hours were compared using the Kolmogorov- Smirnov (KS) test and were not significantly different (n = 6 animals, $p = 0.51$ and $p = 0.46$, respectively). The average cumulative concentration of released adenosine was plotted by adding the peak concentrations of adenosine over time and then the concentrations were divided by the number of animals. While the average cumulative concentration traces of both all adenosine and oxygen transients show higher concentration in the second two hours (Figure 4.5E-F), the maximum cumulative concentrations, which is maximum cumulative concentration from each animal, were not significant for adenosine or oxygen transients (student's paired *t*-test, n = 6 animals, $p = 0.064$ and $p = 0.087$,

respectively). The median event concentration was calculated per animal and box and whisker plots are plotted in Fig. 4.5G-H for all adenosine, adenosine transients with oxygen (Ad w/O_2) and oxygen concentrations. There were no significant effects of time on the median concentrations of transients (paired *t*-test, n = 6, $p = 0.099$, $p = 0.22$, and $p = 0.43$, respectively). Thus, the number of transients, inter-event time, or concentrations of adenosine and oxygen transients do not change over four hours of data collection. In addition, a four-hour control experiment was also performed by injecting the vehicle, DMSO, after 2 hours. The administration of DMSO did not affect the number of transients, inter-event time, or concentration of adenosine or oxygen transients (Fig. 4.6).

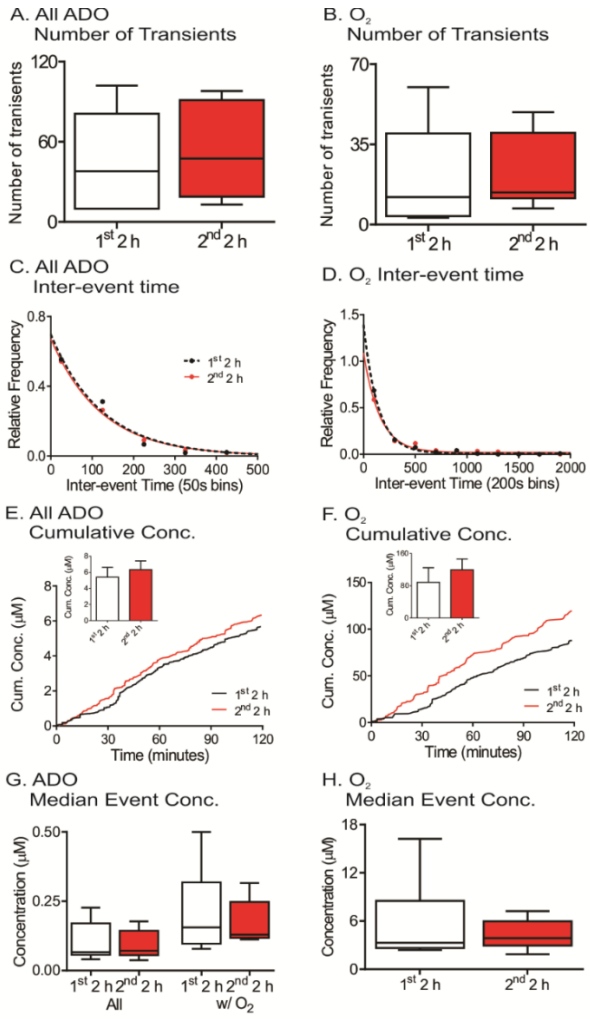

Figure 4.5 Spontaneous adenosine and oxygen release in the caudate-putamen. Spontaneous adenosine and oxygen release in the caudate putamen. (A) Number of adenosine transients did not change between the first two hours and second two hours blocks (paired t-test, n=6, *p*=0,13). (B) Number of oxygen transients also did not change (p = 0.89. (C) Inter-event time of all adenosine transients. The exponential fit (dashed black line) in the first two hours is $y = 0.70e^{-0.0081x}$ (R^2 = 0.98) and in the second two hours (solid red line) is $y = 0.67e^{-0.0082x}$ (R^2 =0.99). There was no significant difference between the underlying distributions in the first two hours and second two hours (KS-test, n=6 animals, p = 0.51). (D) Inter-event time of oxygen. The exponential fit in the first two hours (dashed black line) is $y = 1.4e^{-0.0072x}$ (R^2 = 0.99) and in the second two hours (solid red line) is $y = 1.1e^{-0.0064x}$ (R^2 = 0.98). The distributions were not significantly different (KS-test, p = 0.46). (E) Cumulative concentration of adenosine for first two hours (black) and second two hours (red). Insert shows the maximum cumulative concentration which was not significantly different (paired *t*-test, n = 6, p = 0.064). (F) Cumulative concentration traces of oxygen also did not significantly change for the first two hours and second two hours (p = 0.087). (G) The median event concentration of all adenosine transients and adenosine with oxygen (Ad w/O_2) were not significantly different (paired *t*-test, p = 0.098 and p = 0.22). (H) The median event concentration of each oxygen transient also did not differ with time (p = 0.43).

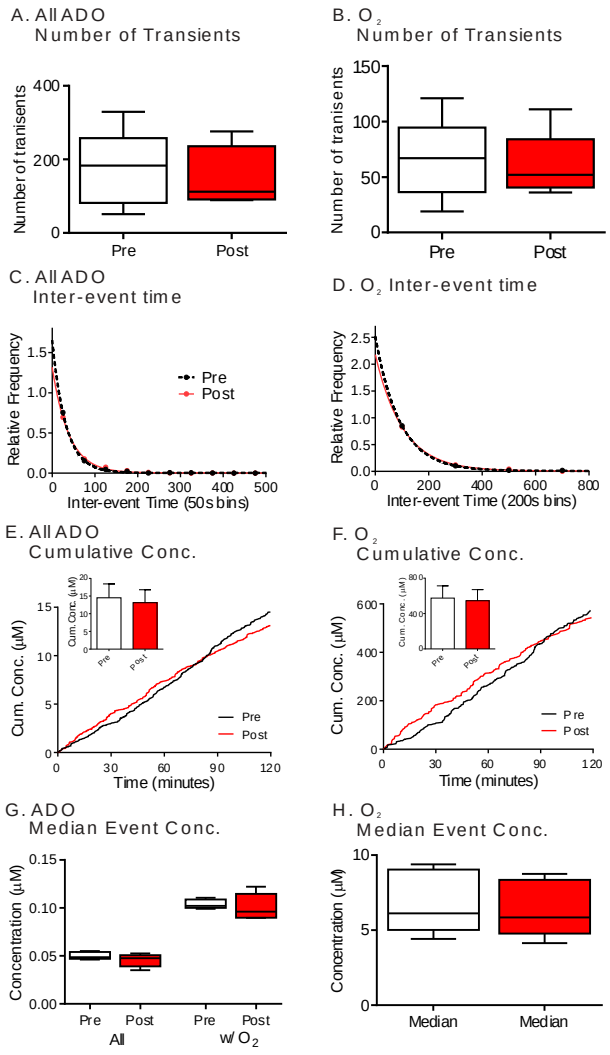

Figure 4.6 Effect of DMSO (300 µL. *i.p.*) on adenosine and oxygen transients. All data and statistics are for n = 5 animals. (A) Number of adenosine transients did not significantly change after DMSO (student's paired *t*-test, p = 0.75). (B) Number of oxygen transients did not significantly change after DMSO (p = 0.62). (C) Inter-event time of all adenosine transients. The exponential fit (dashed black line) before DMSO is $y = 1.6e^{-0.0031x}$ (R^2 = 0.99) and after DMSO (solid red line) is $y = 1.3e^{-0.0030x}$ (R2 =0.99). There was no significant difference between the underlying distributions before and after DMSO (KS-test, p = 0.99). (D) Inter-event time of oxygen. The exponential fit before DMSO (dashed black line) is $y = 2.5^{e-0.010x}$ (R^2 = 0.99) and after DMSO (solid red line) is $y = 2.2e^{-0.0075x}$ (R^2 = 0.99). The underlying distributions before and after DMSO were not significantly different (KS-test, p = 0.99). (E) Cumulative concentration traces for adenosine, with an inset plotting the maximum cumulative concentrations. The maximum cumulative adenosine concentration did not change after DMSO (paired *t*-test, p = 0.48). (F) Maximum cumulative oxygen concentration did not change after DMSO (p = 0.74). (G) Median event concentration of adenosine transients. The median event concentrations of all adenosine and Ad w/O_2 were not significantly change after DMSO (student's paired *t*-test, p = 0.13 and p = 0.56, respectively). (H) The median event concentration of oxygen transients was not significantly change after DMSO (p = 0.22).

4.3.4 A_1, A_{2A}, and NO Modulation of Oxygen Response

To investigate the receptor subtype specificity involved in the relationship between adenosine and blood flow changes, we tested the effects of A_1 and A_{2A} receptor drugs on simultaneous adenosine and oxygen transients. For all pharmacological experiments, two hours of baseline data were collected and then the drug was administered and data were collected for an additional two hours.

The A_1 receptor antagonist, DPCPX, was administered at 6 mg/kg, *i.p*. The number of adenosine and oxygen transients did not significantly change after DPCPX, although there was a trend towards more oxygen transients (Fig. 4.7A-B, student's paired *t*-test, n = 8 animals, $p = 0.47$ and $p = 0.055$, respectively). Administration of DPCPX did not significantly change the underlying inter-event time distributions for either transient adenosine or oxygen release (Fig. 4.7C-D, KS test, n= 8 animals, $p = 0.69$ and $p = 0.87$, respectively). The average cumulative concentrations of both adenosine and oxygen transients were lower after DPCPX administration (Fig. 4.7E-F), but the differences of maximum cumulative concentrations were not significant (student's paired *t*-test, n = 8 animals, $p = 0.31$ and $p = 0.46$, respectively). There was a significant decrease in median event concentration for all spontaneous adenosine transients (student's paired *t*-test, n = 8 animals, $p = 0.016$) (Fig. 4.7G), but DPCPX did not significantly affect the median event concentration of Ad w/O_2 or oxygen transients (student's paired *t*-test, n = 8 animals, $p = 0.093$ and $p = 0.086$, respectively) (Fig. 4.7G-H). Thus, A_1 receptor blockade with DPCPX did not change the number, concentration or inter-event time of simultaneous adenosine and oxygen release but only decreased the median concentration of all adenosine transients.

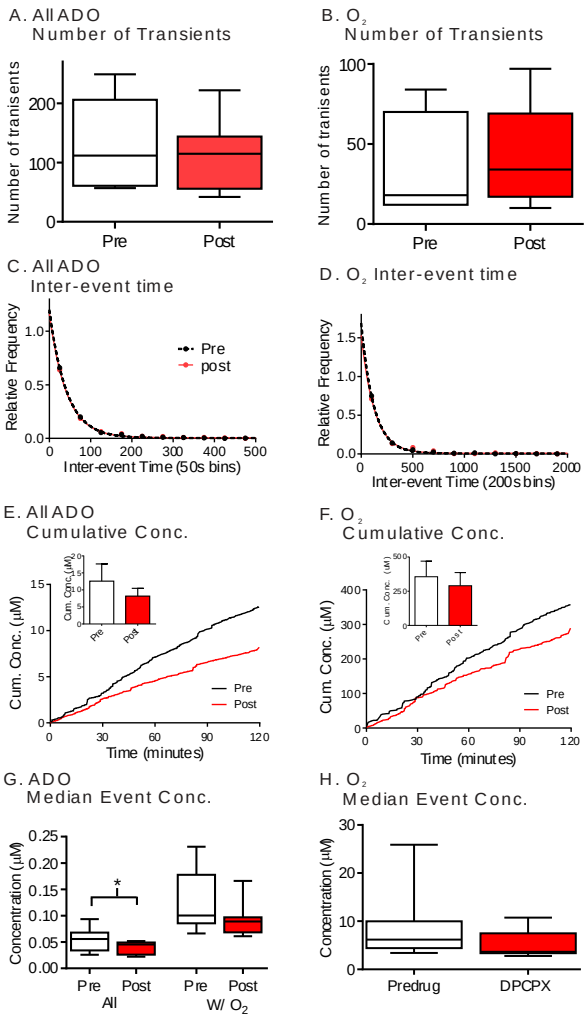

Figure 4.7 Effect of the A_1 antagonist, DPCPX (6 mg/kg. *i.p.*), on adenosine and oxygen transients. All data and statistics are for n=8 animals. (A) Number of adenosine transients did not significantly change after DPCPX (student's paired *t*-test, $p = 0.47$). (B) Number of oxygen transients did not significantly change after DPCPX ($p = 0.055$). (C) Inter-event time of all adenosine transients. The exponential fit (dashed black line) before DPCPX is $y = 1.2e^{-0.024x}$ ($R^2 = 0.99$) and after DPCPX (solid red line) is $y = 1.2e^{-0.024x}$ ($R^2 = 0.99$). There was no significant difference between the distributions before and after DPCPX (KS-test, $p = 0.69$). (D) Inter-event time of oxygen. The exponential fit before DPCPX (dashed black line) is $y = 1.7e^{-0.0082x}$ ($R^2 = 0.99$) and after DPCPX (solid red line) is $y = 1.5e^{-0.0078x}$ ($R^2 = 0.99$). The distributions before and after DPCPX were not significantly different (KS-test, $p = 0.87$). (E) Cumulative concentration traces for adenosine, with an inset plotting the maximum cumulative concentrations. The cumulative adenosine concentration did not change after DPCPX (paired *t*-test, $p = 0.31$). (F) Maximum cumulative oxygen concentration did not change after DPCPX ($p = 0.46$). (G) Median event concentration of adenosine transients. The median event concentration of all adenosine significantly decreased after DPCPX (paired *t*-test, $p = 0.016$), but the concentration of Ad w/O_2 was not significantly different ($p = 0.093$). (H) The median event concentration of oxygen transients was not significantly different after DPCPX ($p = 0.086$).

SCH 442416 (3 mg/kg, i.p.), an A_{2A} receptor antagonist, was administered to test the extent to which A_{2A} receptors mediated the oxygen transients. SCH 442416 significantly decreased the average number of adenosine transients from 132 to 90 (Fig. 4.8A, paired *t*- test, n = 7 animals, $p = 0.023$) and oxygen transients from 49 to 33 (Fig. 4.8B, paired *t*-test, n = 7 animals, $p = 0.030$). In addition, SCH 442416 increased the mean inter-event time for adenosine transients from 53 to 77 seconds and for oxygen from 129 to 216 seconds. The underlying inter-event time distributions of both adenosine and oxygen transients were significantly different than pre-drug (n = 7 animals, KS test, $p = 0.0016$ and $p = 0.044$, respectively) (Fig. 4.8C-D). The average cumulative concentration of both adenosine and oxygen transients were lower after SCH 442416 administration, but only the maximum cumulative concentrations of adenosine transient significantly decreased (paired *t*-test, n = 7 animals, $p = 0.030$); There is no significant change in maximum cumulative concentrations of oxygen (paired *t*-test, n = 7 animals, $p = 0.17$). SCH 442416 significantly decreased the median adenosine event concentration of all adenosine transients, but not the median concentration of Ad w/O_2 and oxygen (paired *t*-test, n = 7, $p = 0.041$, $p = 0.73$, and $p = 0.66$, respectively). Therefore, blocking A_{2A} receptors did not change the concentration of released oxygen, but both adenosine and oxygen were released less frequently.

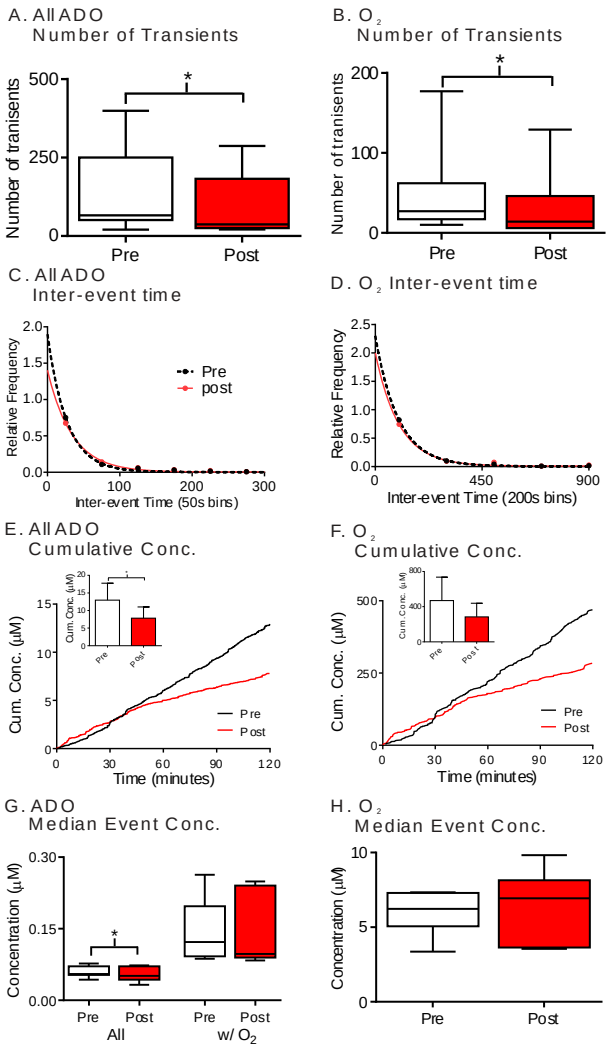

Figure 4.8 Effect of the A_{2A} antagonist, SCH 442416 (3 mg/kg. *i.p.*), on adenosine and oxygen transients. Effect of the A_{2A} antagonist, SCH 442416 (3 mg/kg, *i.p*), on adenosine and oxygen transients. All data and statistics are for n=7 animals. (A) Number of adenosine transients decreased after SCH 442416 (n = 7 animals, p = 0.023). (B) Number of oxygen transients also decreased after A_{2A} antagonist (p = 0.030). (C) Inter-event time of all adenosine transients. The interevent time was significantly longer after SCH 442416 (KS-test, p = 0.0016). The exponential fit (dashed black line) before SCH 442416 is $y = 1.9e^{-0.037x}$ ($R^2 = 0.99$) and after SCH 442416 (solid red line) is $y = 1.4e^{-0.029x}$ (R^2 =0.99). (D) Inter-event time of oxygen. The distributions after SCH 442416 were significantly different (KS-test, p = 0.044). The exponential fit before SCH 442416 (dashed black line) is $y = 2.3e^{-0.010x}$ ($R^2 = 0.99$) and after SCH 442416 (solid red line) is $y = 2.0e^{-0.010x}$ (R^2 = 0.99). (E) Cumulative concentration traces for adenosine were significantly lower after SCH 442416 (paired *t*-test, p = 0.030) (F) Cumulative concentration for oxygen was not significantly different postdrug (p = 0.17). (G) The median concentration of all adenosine transients significantly decreased (paired *t*-test, p = 0.041), but the median event concentration of Ad w/O_2 was not significantly different (p = 0.73). (H) The median event concentration of oxygen transients was also not significantly different after SCH 442416 (paired *t*-test, p = 0.66).

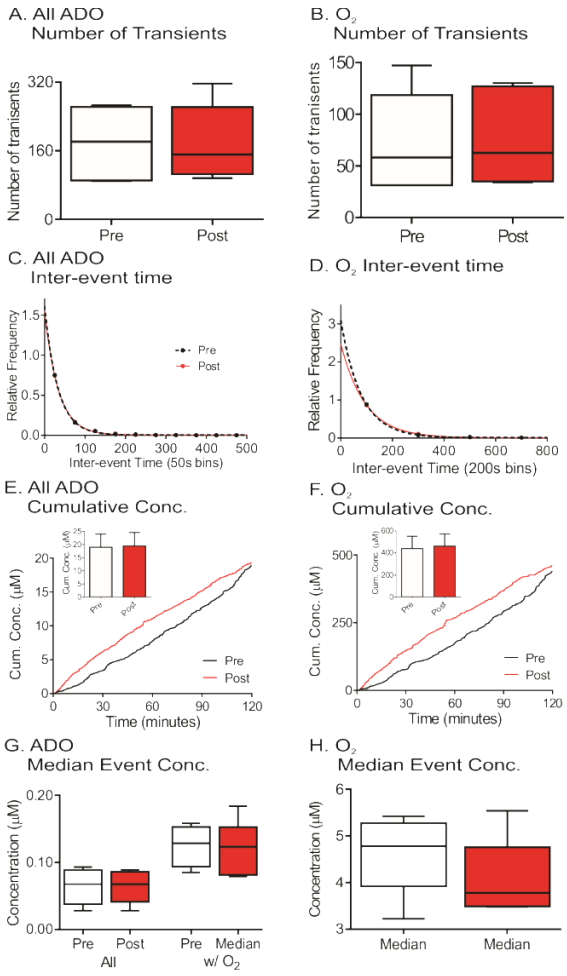

Figure 4.9 Effect of the L-NAME (100 mg/kg. *i.p.*) on adenosine and oxygen transients. All data and statistics are for n = 6 animals. (A) Number of adenosine transients did not significantly change after L-NAME (paired *t*-test, p = 0.98). (B) Number of oxygen transients did not significantly change after L-NAME (p = 0.75). (C) Inter-event time of all adenosine transients. The exponential fit (dashed black line) before L-NAME is $y = 1.6e^{-0.0037x}$ (R^2 = 0.99) and after L-NAME (solid red line) is $y = 1.5e^{-0.0033x}$ (R^2 =0.99). There was no significant difference between the underlying distributions before and after L-NAME (KS-test, p = 0.50). (D) Inter-event time of oxygen. The exponential fit before L-NAME (dashed black line) is $y = 3.1e^{0.011x}$ (R^2 = 0.99) and after L-NAME (solid red line) is $y = 2.4e^{-0.0050x}$ (R^2 = 0.99). The underlying distributions before and after L-NAME were not significantly different (KStest, p = 0.98). (E) Cumulative concentration trances for adenosine, with an inset plotting the maximum cumulative concentrations. The cumulative adenosine concentration did not change after L-NAME (Student's paired *t*-test, p = 0.66). (F)Maximum cumulative oxygen concentration did not change after L-NAME (p = 0.32). (G) Median event concentration of adenosine transients. The median concentrations of all adenosine and Ad w/O_2 were not significantly change after L-NAME (and oxygen transients had no significant differences between before and after L-NAME (unpaired *t*-test, p = 0.95 and p = 0.84, respectively). (H) The median event concentration of oxygen transients was not significantly difference after L-NAME (p =0.32).

To test the extent to which the oxygen signals were dependent on nitric oxide (NO), nitric oxide (NO) synthase was inhibited using L-NAME(100 mg/kg, i.p).[19,28] There were no significant differences between before and after L-NAME in terms of number of transients, inter-event time, or concentration of adenosine or oxygen transients (Fig. 4.9). These results confirm that NO does not play a role in simultaneous adenosine and oxygen release and therefore, this transient vasodilation does not rely on NO.

4.4 Discussion

Here we demonstrate that spontaneous, transient changes in adenosine and oxygen are correlated in the caudate-putamen. Transient changes in oxygen release are an indicator of increased blood flow. Adenosine transients were followed by oxygen release about one-third of the time, and the concentration of adenosine and evoked oxygen changes are correlated. Typically, the oxygen release started and peaked about 0.2 seconds after the adenosine transient. A_1 receptors do not have an effect on simultaneous adenosine and oxygen release while A_{2A} receptors modulated the number of adenosine and oxygen transients. Thus, spontaneous adenosine release are correlated with oxygen release, particularly when the adenosine release is large, suggesting adenosine modulates blood flow on a rapid, sub-second time scale.

4.4.1 Transient adenosine and blood flow changes on a rapid time scale

Transient release of adenosine has recently been characterized, showing that adenosine can signal on a rapid time scale.[22,23,29] Transient adenosine modulates phasic dopamine release, but the modulation only occurs when adenosine is present.[24] However,

the extent to which this rapid mode of adenosine release can transiently mediate vasodilation was unknown. Most previous studies of adenosine mediating blood flow correlated changes that lasted for minutes. For example, cerebral blood flow increased at 4th minute of intravenous infusion of adenosine.[30] One of the faster studies of adenosine build up found that cerebral adenosine was elevated within 5 seconds after the onset of ischemia, and they hypothesized that this rapid rise of adenosine caused vasodilation associated with ischemia.[31]

Our method is unique in that both adenosine and oxygen changes can be measured simultaneously with sub-second temporal resolution. The results clearly demonstrate that transient adenosine and oxygen release are temporally correlated. The increase in adenosine always precedes the oxygen increase, with a median delay time between the peak adenosine and oxygen concentrations of 0.2 seconds. The appearance and clearance of both adenosine and oxygen are fast, with an average duration of only 3 seconds. Other physiological stimuli, such as a tail pinch, cause vasodilation that lasts for minutes.[32] Changes in oxygen due to electrical stimulations have been measured, including a first peak that is not mediated by adenosine, which occurs within 3 - 4 seconds after stimulation, and a second peak mediated by adenosine that occurs 10 - 30 seconds after the stimulation and lasts for 20 seconds.[19] Therefore, our data here is the fastest reported correlation between adenosine release and shifts in blood flow and the duration of the oxygen changes are much shorter than with other stimuli.

The concentration of spontaneous adenosine and oxygen changes were also correlated. Previous studies also found a correlation between the concentration of adenosine and the dilation magnitude of pial artery.[33,34] While about one-third of

adenosine transients were accompanied by changes in oxygen, high concentration adenosine transients (over 0.4 μM) were always followed by oxygen release. Low concentrations of adenosine were not always correlated with oxygen release, likely because the event did not cause sufficient receptor activation. However, some smaller adenosine transients did cause oxygen release. Our electrodes only measure in one discrete location, and it is possible that for some smaller amounts of adenosine, the electrode was not at the center of the release, making the event look smaller. Also, there may be differences in global blood flow that would affect local blood flow and consequently modulate delivery of oxygen.[35]

4.4.2 Role of Adenosine Receptors in Mediating Adenosine and Blood Flow Changes

The effects of adenosine are mediated by four G-protein coupled receptors, A_1, A_{2A}, A_{2B}, and A_3.[36] A_1 and A_{2A} receptors are highly expressed in the caudate-putamen of the brain.[37,38] A_1 receptors mediate the inhibitory modulation of phasic dopamine release by transient adenosine in the caudate-putamen.[24] Both stimulated and spontaneous adenosine release are self-regulated by A_1 receptors, which have autoreceptor characteristics.[22,39] Here, our study showed that blocking A_1 receptors decreased the concentration of adenosine transients but did not significantly affect the number of transients or inter-event times. In contrast, our previous study found a significant decrease in the inter-event time of transient adenosine release but no change in concentration after DPCPX.[22] That study used a different FSCV waveform that did not sweep to the low potentials necessary for oxygen reduction. While the effects were slightly different here, they are consistent with A_1 receptors being inhibitory and self-regulating transient adenosine. The concentration of oxygen did not decrease because the

concentration of adenosine that correlated with oxygen did not decrease; i.e. the large adenosine transients were still correlated with oxygen release. The number of oxygen transients was the same, indicating that A_1 receptors do not mediate vasodilation.

A_{2A} receptors are highly expressed on cerebral blood vessels,[40–42] and are located on endothelial cells.[43] In addition, A_{2A} receptors play a protective role in hypoxia/ischemia, as A_{2A} antagonists protect against brain damage after hypoxia.[3,44,45] In the brain, the density of A_{2A} receptors in the basal ganglia is about 20 times greater than other brain regions.[46,47] The number of both adenosine and oxygen transients were reduced by the A_{2A} antagonist, SCH 442416, while the concentration of adenosine transients that correlated with oxygen and oxygen transients stayed the same. These results suggest that A_{2A} antagonist acts remotely through more complex pathways to modulate the activity of the neuron that is releasing adenosine. While the link between A_{2A} receptors on endothelial cells regulating blood flow had been previously established, this work shows that the A_{2A} antagonist can function by lowering the amount of adenosine transients and therefore oxygen transients.

Our results definitely show that adenosine and oxygen transients in the brain are correlated, especially for large adenosine transients which are always accompanied by oxygen transients. This leads to the question of whether the adenosine transients cause the oxygen changes. The delay time of 0.2 seconds between the start of the adenosine transient and the oxygen transient demonstrates that the adenosine transients come first. The distance between blood vessels in the brain is about 50 μm, and diffusion of adenosine to a blood vessel, 25 μm at most, would take less than 0.5 seconds. Thus, the time delay is of the correct order of magnitude for adenosine diffusion. The proposed

mechanism of adenosine causing vasodilation and oxygen increases is by acting at A_{2A} receptors on endothelial cells, and the expected result that would confirm this is a decrease in the number of oxygen transients compared to adenosine transients. Instead, the observed result is that the A_{2A} antagonist causes adenosine and oxygen transients to decrease proportionally, a 32% decrease for both. This proportional decrease is harder to interpret; it does not definitively show that the adenosine transients were working at the A_{2A} receptor, but it does confirm that the receptor is important for regulating adenosine transients and thus oxygen transients. Therefore, there are two possible explanations for the data: (1) adenosine transients cause the oxygen transients or (2) a large metabolic event causes the release of both adenosine and other vasodilatory molecules that lead to the oxygen increases. We tested L-NAME to block NO production to test the hypothesis that NO might also play a role in these oxygen changes.[19] No effect was seen, indicating that these oxygen changes were not due to inducible nitric oxygen synthase made NO. However, there are other sources of NO and other vasodilatory signals such as pH and CO_2 that have not been ruled out.[19,49,50] Nevertheless, the bulk of the evidence points towards adenosine causing the oxygen changes although the exact mechanism has not been delineated. A more definitive approach in the future would be to try these experiments in mice and test the effects of global A_{2A} knock outs or even specific A_{2A} KO just on the blood vessels to test causation.

4.5 Conclusion

In conclusion, this study provides evidence that spontaneous, adenosine transients are correlated with transient oxygen changes. The subsequent oxygen changes occur quickly, usually within about 0.2 seconds, and the clearance is fast, lasting only about 3.5

seconds. Our study also shows that A_{2A} receptors regulate this transient vasodilation, by modulating the number of adenosine transients that are correlated with oxygen transients. Because adenosine is rapidly metabolized,[51] adenosine release is likely local rather than systemic and could transiently affect local blood flow.[23] Future research will focus on whether transient adenosine causes local vasodilation and increases oxygen release.

4.6 References

1. Benington JH, Heller HC. Restoration of brain energy metabolism as the function of sleep. *Prog Neurobiol*. 1995;45(4):347-360. doi:10.1016/0301-0082(94)00057-O.

2. Obal F, Krueger JM. Biochemical regulation of non-rapid-eye-movement sleep. *Front Biosci*. 2003;8:d520-50. doi:10.2741/1033.

3. Phillis JW. Adenosine in the control of the cerebral circulation. *Cerebrovasc Brain Metab Rev*. 1989;1(1):26-54.

4. Pelligrino DA, Vetri F, Xu H-L. Purinergic mechanisms in gliovascular coupling. *Semin Cell Dev Biol*. 2011;22(2):229-236. doi:10.1016/j.semcdb.2011.02.010.

5. Lowry JP, Boutelle MG, Fillenz M. Measurement of brain tissue oxygen at a carbon paste electrode can serve as an index of increases in regional cerebral blood flow. *J Neurosci Methods*. 1997;71(2):177-182. doi:10.1016/S0165-0270(96)00140-9.

6. Winn HR, Rubio R, Berne RM. Brain adenosine concentration during hypoxia in rats. *Am J Physiol*. 1981;241(2):H235-42. doi:10.1152/ajpheart.1981.241.2.H235.

7. Van Wylen DG, Park TS, Rubio R, Berne RM. Increases in cerebral interstitial fluid adenosine concentration during hypoxia, local potassium infusion, and ischemia. *J Cereb Blood Flow Metab*. 1986;6:522-528. doi:10.1038/jcbfm.1986.97.

8. Paul S, Elsinga PH, Ishiwata K, Dierckx R a JO, van Waarde a. Adenosine A(1) receptors in the central nervous system: their functions in health and disease, and possible elucidation by PET imaging. *Curr Med Chem*. 2011;18:4820-4835. doi:10.2174/092986711797535335.

9. Winn HR, Rubio GR, Berne RM. The role of adenosine in the regulation of cerebral blood flow. *J Cereb Blood Flow Metab*. 1981;1(3):239-244. doi:10.1038/jcbfm.1981.29.

10. Belardinelli L, Shryock JC, Snowdy S, et al. The A2A adenosine receptor mediates coronary vasodilation. *J Pharmacol Exp Ther*. 1998;284(3):1066-1073. doi:10.1161/01.CIR.98.7.711.

11. Shryock JC, Belardinelli L. Adenosine and Adenosine Receptors in the Cardiovascular System: Biochemistry, Physiology, and Pharmacology. *Am J Cardiol*. 1997;79(12):2-10. doi:10.1016/S0002-9149(97)00256-7.

12. Demchenko IT, Boso AE, Natoli MJ, et al. Measurement of cerebral blood flow in rats and mice by hydrogen clearance during hyperbaric oxygen exposure. *Undersea Hyperb Med*. 1998;25(3):147-152.

13. Barbu A, Jansson L, Sandberg M, Quach M, Palm F. The use of hydrogen gas clearance for blood flow measurements in single endogenous and transplanted pancreatic islets. *Microvasc Res*. 2015;97:124-129. doi:10.1016/j.mvr.2014.10.002.

14. Kusano Y, Echeverry G, Miekisiak G, et al. Role of adenosine A2 receptors in regulation of cerebral blood flow during induced hypotension. *J Cereb Blood Flow Metab*. 2010;30(4):808-815. doi:10.1038/jcbfm.2009.244.

15. Ngai AC, Coyne EF, Meno JR, West GA, Winn HR. Receptor subtypes mediating adenosine-induced dilation of cerebral arterioles. *Am J Physiol Heart Circ Physiol*. 2001;280:H2329-H2335. doi:10.1152/ajpheart.2001.280.5.H2329.

16. Mulderink TA, Gitelman DR, Mesulam M-M, Parrish TB. On the use of caffeine as a contrast booster for BOLD fMRI studies. *Neuroimage*. 2002;15(1):37-44. doi:10.1006/nimg.2001.0973.

17. Morton DW, Maravilla KR, Meno JR, Winn HR. Systemic Theophylline Augments the Blood Oxygen Level--Dependent Response to Forepaw Stimulation in Rats. *AJNR Am J Neuroradiol*. 2002;23(4):588-593.

18. Zimmerman JB, Kennedy RT, Wightman RM. Evoked neuronal activity accompanied by transmitter release increases oxygen concentration in rat striatum in vivo but not in vitro. *J Cereb Blood Flow Metab*. 1992;12(4):629-637. doi:10.1038/jcbfm.1992.87.

19. Venton BJ, Michael DJ, Wightman RM. Correlation of local changes in extracellular oxygen and pH that accompany dopaminergic terminal activity in the rat caudate-putamen. *J Neurochem*. 2003;84(2):373-381. doi:10.1046/j.1471-4159.2003.01527.x.

20. Bucher ES, Fox ME, Kim L, et al. Medullary norepinephrine neurons modulate local oxygen concentrations in the bed nucleus of the stria terminalis. *J Cereb Blood Flow Metab*. 2014;34(7):1128-1137. doi:10.1038/jcbfm.2014.60.

21. Cechova S, Venton BJ. Transient adenosine efflux in the rat caudate-putamen. *J Neurochem*. 2008;105(4):1253-1263. doi:10.1111/j.1471-4159.2008.05223.x.

22. Nguyen MD, Lee ST, Ross AE, Ryals M, Choudhry VI, Venton BJ. Characterization of spontaneous, transient adenosine release in the caudate-putamen and prefrontal cortex. *PLoS One*. 2014;9(1):e87165. doi:19326203.

23. Nguyen MD, Ross AE, Ryals M, Lee ST, Venton BJ. Clearance of rapid adenosine release is regulated by nucleoside transporters and metabolism. *Pharmacol Res Perspect*. 2015;3(6). doi:10.1002/prp2.189.

24. Ross AE, Venton BJ. Adenosine transiently modulates stimulated dopamine release in the caudate-putamen via A1 receptors. *J Neurochem*. 2015;132(1):51-60. doi:10.1111/jnc.12946.

25. Orrú M, Quiroz C, Guitart X, Ferré S. Pharmacological evidence for different populations of postsynaptic adenosine A2A receptors in the rat striatum. *Neuropharmacology*. 2011;61(5-6):967-974. doi:10.1016/j.neuropharm.2011.06.025.

26. Strand AM, Venton BJ. Flame etching enhances the sensitivity of carbon-fiber microelectrodes. *Anal Chem*. 2008;80:3708-3715. doi:10.1021/ac8001275.

27. Ross AE, Nguyen MD, Privman E, Venton BJ. Mechanical stimulation evokes rapid increases in extracellular adenosine concentration in the prefrontal cortex. *J Neurochem*. 2014;130(1):50-60. doi:10.1111/jnc.12711.

28. Iadecola C, Pelligrino DA, Moskowitz MA, Lassen NA. Nitric oxide synthase inhibition and cerebrovascular regulation. *J Cereb Blood Flow Metab*. 1994;14(2):175-192. doi:10.1038/jcbfm.1994.25.

29. Nguyen MD, Venton BJ. Fast-scan Cyclic Voltammetry for the Characterization of Rapid Adenosine Release. *Comput Struct Biotechnol J*. 2015;13:47-54. doi:10.1016/j.csbj.2014.12.006.

30. Soricelli A, Postiglione A, Cuocolo A, et al. Effect of Adenosine on Cerebral Blood Flow as Evaluated by Single-Photon Emission Computed Tomography in Normal Subjects and in Patients With Occlusive Carotid Disease : A Comparison With Acetazolamide. *Stroke*. 1995;26(9):1572-1576. doi:10.1161/01.STR.26.9.1572.

31. Winn HR, Rubio R, Berne RM. Brain adenosine production in the rat during 60 seconds of ischemia. *Circ Res*. 1979;45(4):486-492. doi:10.1161/01.RES.45.4.486.

32. Lowry JP, Boutelle MG, Fillenz M. Measurement of brain tissue oxygen at a carbon past electrode can serve as an index of increases in regional cerebral blood flow. *J Neurosci Methods*. 1997;71(2):177-182. doi:0.1016/S0165-0270(96)00140-9.

33. Shin HK, Shin YW, Hong KW. Role of adenosine A2B receptors in vasodilation of rat pial artery and cerebral blood flow autoregulation. *Am J Physiol Hear Circ Physiol*. 2000;278(2):H339-344. doi:10.1152/ajpheart.2000.278.2.H339.

34. Hein TW, Xu W, Ren Y, Kuo L. Cellular signalling pathways mediating dilation of porcine pial arterioles to adenosine A2A receptor activation. *Cardiovasc Res*. 2013;99(1):156-163. doi:10.1093/cvr/cvt072.

35. Shimosegawa E, Kanno I, Hatazawa J, et al. Photic stimulation study of changing the arterial partial pressure level of carbon dioxide. *J Cereb Blood Flow Metab*. 1995;15(1):111-114. doi:10.1038/jcbfm.1995.12.

36. Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev*. 2001;53(4):527-552.

37. Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W. Structure and function of adenosine receptors and their genes. *Naunyn Schmiedebergs Arch Pharmacol*. 2000;362(4-5):364-374. doi:10.1007/s002100000313.

38. Cieślak M, Komoszyński M, Wojtczak A. Adenosine A(2A) receptors in Parkinson's disease treatment. *Purinergic Signal*. 2008;4(4):305-312. doi:10.1007/s11302-008-9100-8.

39. Cechova S, Elsobky AM, Venton BJ. A1 receptors self-regulate adenosine release in the striatum: evidence of autoreceptor characteristics. *Neuroscience*. 2010;171(4):1006-1015. doi:10.1016/j.neuroscience.2010.09.063.

40. Mills JH, Alabanza L, Weksler BB, Couraud P-O, Romero IA, Bynoe MS. Human brain endothelial cells are responsive to adenosine receptor activation. *Purinergic Signal*. 2011;7(2):265-273. doi:10.1007/s11302-011-9222-2.

41. O'Regan M. Adenosine and the regulation of cerebral blood flow. *Neurol Res*. 2005;27(2):175-181. doi:10.1179/016164105X21931.

42. Pelligrino DA, Xu H-L, Vetri F. Caffeine and the control of cerebral hemodynamics. *J Alzheimers Dis*. 2010;20 Suppl 1:S51-62. doi:10.3233/JAD-2010-091261.

43. Coney AM, Marshall JM. Role of adenosine and its receptors in the vasodilatation induced in the cerebral cortex of the rat by systemic hypoxia. *J Physiol*. 1998;509 (Pt 2:507-518. doi:10.1111/j.1469-7793.1998.507bn.x.

44. Monopoli A, Lozza G, Forlani A, Mattavelli A, Ongini E. Blockade of adenosine A2A receptors by SCH 58261 results in neuroprotective effects in cerebral ischaemia in rats. *Neuroreport*. 1998;9(17):3955-3959.

45. Melani A, Pantoni L, Bordoni F, et al. The selective A2A receptor antagonist SCH 58261 reduces striatal transmitter outflow, turning behavior and ischemic brain

damage induced by permanent focal ischemia in the rat. *Brain Res*. 2003;959(2):243-250. doi:10.1016/S0006-8993(02)03753-8.

46. Svenningsson P. Distribution, biochemistry and function of striatal adenosine A2A receptors. *Prog Neurobiol*. 1999;59(4):355-396. doi:10.1016/S0301-0082(99)00011-8.

47. Lopes L V, Halldner L, Rebola N, et al. Binding of the prototypical adenosine A(2A) receptor agonist CGS 21680 to the cerebral cortex of adenosine A(1) and A(2A) receptor knockout mice. *Br J Pharmacol*. 2004;141(6):1006-1014. doi:10.1038/sj.bjp.0705692.

48. Venton BJ, Michael DJ, Wightman RM. Correlation of local changes in extracellular oxygen and pH that accompany dopaminergic terminal activity in the rat caudate-putamen. *J Neurochem*. 2003;84:373-381. doi:10.1046/j.1471-4159.2003.01527.x.

49. Yoon S, Zuccarello M, Rapoport RM. pCO(2) and pH regulation of cerebral blood flow. *Front Physiol*. 2012;3:365. doi:10.3389/fphys.2012.00365.

50. Hylland P, Nilsson GE, Lutz PL. Role of nitric oxide in the elevation of cerebral blood flow induced by acetylcholine and anoxia in the turtle. *J Cereb Blood Flow Metab*. 1996;16(2):290-295. doi:10.1097/00004647-199603000-00014.

51. Haskó G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov*. 2008;7(9):759-770. doi:10.1038/nrd2638.

Chapter 5: Spontaneous adenosine release correlates with oxygen increases during ischemia and reperfusion injury

Abstract

Adenosine is an endogenous neuroprotectant that modulates vasodilation in the central nervous system. Oxygen changes occur when there is an increase in local cerebral blood flow and thus are a measure of vasodilation. Oxygen transients correlated with rapid adenosine release have been recently discovered, but the relationship between adenosine and blood flow change during ischemia/reperfusion (I/R) injury has not been characterized. Caffeine is a nonselective adenosine receptor antagonist that can modulate the effects of adenosine in the brain, but how it affects adenosine and correlated oxygen levels during I/R is also unknown. In this study, extracellular changes in adenosine and oxygen were simultaneously monitored using fast-scan cyclic voltammetry during a mild ischemic event, bilateral common carotid artery occlusion (BCCAO), and the effects of a specific A_{2A} antagonist, SCH 442416, or general antagonist, caffeine, were studied. Measurements were made in the caudate-putamen for one hour of normoxia, followed by 30 min of BCCAO and 30 min of reperfusion. The frequency and number of both adenosine and oxygen transient events significantly increased during I/R injury. The specific A_{2A} antagonist, SCH 442416 (3 mg/kg, *i.p.*), eliminated the increase in adenosine and oxygen transients caused by I/R injury. The general adenosine receptor antagonist, caffeine (100 mg/kg, *i.p.*), decreased the frequency of adenosine and oxygen transient events during I/R injury. These results demonstrate that during BCCAO, there are more rapid release events of the neuromodulator adenosine and correlated local oxygen changes, and these rapid, local effects are dampened by caffeine and other A_{2A} antagonists.

5.1 Introduction

Ischemic brain injury is one of the leading causes of death and disability.[1] It occurs when cerebral blood flow is reduced, either transiently or permanently, which causes decreased delivery of oxygen and nutrients to the brain. Reestablishment of blood flow will deliver nutrients, but this reperfusion process also generates oxygen-derived free radicals that can actually increase ischemic tissue damage.[2] The brain is particularly sensitive to ischemia as complete blockade of blood flow to the brain for only 5 minutes can cause the death of vulnerable neurons.[3]

Adenosine is a major neuromodulator with neuroprotective properties in the central nervous system that plays an important role during ischemic injury. Adenosine has a cerebral vasodilatory effect,[4–6] causing increased blood flow and oxygen supply. Adenosine levels in plasma acutely rise during transient ischemic attack and stroke in human.[7] In the rat brain, intracerebral infusion of adenosine into the ischemic striatum using microdialysis significantly improves the neurological outcome and reduces the infarct volume after transient focal cerebral ischemia.[8] The protective effects are attributed to the activation of A_{2A} receptors, and systematic administration of adenosine A_{2A} agonist, CGS 21680, protects against neuronal loss during ischemia.[9] Our lab has recently identified transient increases in adenosine,[10] which increase in frequency during mild ischemia[11]. These adenosine transients are correlated with transient oxygen changes,[12] a measure of local blood flow, and the time-course of both adenosine and oxygen transients are fast, lasting only about 3 seconds. A_{2A} receptors mediate oxygen release; thus, transient adenosine likely causes local vasodilation and increases oxygen

release. However, the extent to which adenosine can cause oxygen changes during ischemia, when blood flow is occluded, is unknown.

Caffeine is a nonselective adenosine receptor antagonist that can act at A_{2A} receptors to affect cerebral blood flow. For example, a high dose of caffeine (50 μM) significantly attenuates the dose-dependent vasodilation caused by extraluminal application of adenosine.[13] Magnetic resonance imaging (MRI) studies show that caffeine consumption (184 mg daily) globally reduces cerebral blood flow in humans.[14] Field et al. suggest a linear correlation between daily caffeine intake and cerebral blood flow response.[15] Caffeine also reduces the intensity and duration of the cerebral hyperemia during anoxia.[16] In addition, caffeine regulates adenosine levels as it increases plasma adenosine in a dose dependent manner.[17] However, the effect of caffeine on transient adenosine release has not been studied and there are still many open questions about how caffeine regulates adenosine and blood flow changes. These questions are important to address so that we understand how caffeine might modulate potential neuroprotective effects of adenosine during I/R.

Here, we investigated the extent to which adenosine transients are correlated with oxygen transients during cerebral ischemia/reperfusion (I/R) injury *in vivo* and the effect of an A_{2A} antagonist or caffeine on transient adenosine and oxygen changes. Fast-scan cyclic voltammetry at carbon-fiber microelectrodes was used to simultaneously detect extracellular changes in adenosine and oxygen during normoxia or mild ischemic injury with bilateral common carotid artery occlusion (BCCAO). The frequency of spontaneous adenosine transients increased during I/R injury and that increase was accompanied by an increase in the frequency of oxygen transients. A large dose of the adenosine A_{2A}

antagonist, SCH 442416, eliminated the increase in adenosine and oxygen transients during I/R injury while a large dose of caffeine significantly decreased the frequency of adenosine and oxygen transients during I/R. This study shows that even when major arteries are occluded, adenosine can act to transiently increase local blood flow during ischemia and that large doses of caffeine can dampen that effect. Thus, caffeine may hinder the neuroprotective effects of adenosine during I/R, particularly by limiting transient adenosine release, which signals for increase in local blood flow.

5.2 Materials and Methods

5.2.1 Animals Care

Male Sprague-Dawley rats (250-350 g; Charles River, Wilmington, MA, USA) were pair-housed on a 12:12 light-dark cycle and were given food and water *ad libitum*. All experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Virginia.

Rats were initially anesthetized with isoflurane (1 mL/100g rat weight) in a desiccator and then injected with urethane (1.0 g/kg, *i.p.*). Additional doses of urethane (< 3/10 of the induction dose) were administered as necessary in order to maintain deep anesthesia. The surgical site was shaved and a ventral midline incision was made to expose the common carotid arteries; the right and left common carotid arteries were isolated. Vascular occluder cuffs (DOCXS, Ukiah, CA, USA) were wrapped around the exposed common carotid arteries and secured in place using suture material passed through the eyelets. Liquid was injected into the actuating tube by a syringe to inflate the diaphragm and compress the vessel into full occlusion to induce ischemia.

For adenosine and oxygen measurement, rats were placed in a stereotaxic frame (David Kopf instruments, Tujunga, CA, USA). Bupivacaine (0.25 mL) (Sensorcaine® MPF; APP Pharmaceuticals, LLC, Schaumburg, IL, USA) was administered under the skin for local anesthesia. The surgical site was shaved and holes were drilled above the caudate-putamen and the electrode was placed (AP +1.2 mm, ML +2.0 mm and DV -4.5 mm) based on the atlas of Paxinos and Watson.[18] A Ag/AgCl reference electrode was inserted on the contralateral side of brain. The body temperature of the rat was maintained at 37 °C using an isothermal pad and a rectal probe (FHC, Bowdoin, ME, USA).

For blood flow measurement, cerebral blood flow was monitored by laser Doppler flow meter with a 1.5 mm diameter probe (Model DRT4, Moor Instruments, Delaware, DE, USA). Rat was placed in a stereotaxic frame, anesthetized, and holes drilled in similar places as for the electrochemical experiments. The Doppler probe was placed over the hole in the skull and blood flow measured every 5 minutes under normoxia and I/R, and with pharmacological agents.

5.2.2 Chemicals and Drugs

Phosphate-buffered saline (PBS) solution (all components in mM: 3.0 KCl, 10.0 NaH_2PO_4, 2.0 Na_2SO_4, 1.2 MgCl2, 131. 25 NaCl and 1.2 $CaCl_2$, with pH adjust to 7.4 using concentrated HCl or NaOH were purchased from Fisher Scientific (Fair Lawn, NJ, USA)) was used for electrode calibration. A 10.0 mM stock solution of adenosine (Fisher Scientific, Fair Lawn, NJ, USA) was prepared in 0.1 M $HClO_4$ once a month and stored at 4^oC. A 1.0 μM adenosine solution for electrode calibration was prepared daily in PBS buffer. All drugs were administered intraperitoneally (*i.p.*). Caffeine (100

mg/kg, Sigma Aldrich, St. Louis, MO, USA) was dissolved in 1 mL warm saline. SCH 442416 (3 mg/kg; Tocirs, Bristol, UK) was dissolved in 300 μL dimethyl sulfoxide (DMSO; Amresco, Solon, OH, USA). Doses were selected based on previous experiments in the literature.[12,19]

5.2.3 Voltammetric Adenosine and Oxygen Measurements

For adenosine and oxygen measurement, electrodes were implanted and equilibrated for 1 hour with the applied waveform prior to data collection. Data were excluded if fewer than 20 transients were observed in the initial 1 hour of equilibration and a new electrode was inserted. After equilibration, one hour of normoxia data were collected, BCCAO was induced for 30 min by inflating the occluders, and then 30 min of reperfusion data were collected after the occulders were deflated. For pharmacology experiments, the drug was injected directly before BCCAO was induced.

Electrodes were calibrated with 1.0 μM adenosine solution. To calibrate oxygen concentration, various mixtures of nitrogen- and air-saturated PBS buffer with volume ratios of 1:0, 2:1, 5:1, 10:1 and 15:1 were prepared and oxygen concentration measured using a D.O. 6+ dissolved oxygen meter (Eutech Instruments Pte Ltd., Singapore). The microelectrode response was calibrated by running the flow cell analysis of different concentrations, with nitrogen-saturated PBS buffer as the comparison for background subtraction. This calibration curve was used to calculate oxygen concentration *in vivo*.

Principal component regression (PCR) analysis was used to identify adenosine and oxygen transients. A training set was obtained for each animal using the five largest and most definitive adenosine transients with clear secondary peaks *in vivo*.[10] For

oxygen, a training set was compiled from the five different concentrations of oxygen that were measured *in vitro*.[12]

5.2.4 Statistical Analysis

All statistics were performed in GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). All data were presented as mean ± SEM. The distributions of inter-event times of adenosine and oxygen transients were analyzed using a Kolmogorov-Smirnov (KS) test. For inter-event time graphs, a few data points with long times were not shown in order highlight the changes at lower inter-event times. However, all data for inter-event times were used for statistical analyses. Averages were analyzed with two-way ANOVA, one-way ANOVA, or paired t-tests, depending on the number of variables in the data sets. p values < 0.05 were considered significant.

5.3 Results

5.3.1 Adenosine and Oxygen Detection with Fast-scan Cyclic Voltammetry *in vivo*

Fast-scan cyclic voltammetry at a carbon-fiber microelectrode was used to monitor rapid fluctuations of adenosine and oxygen in the caudate-putamen of anesthetized rats *in vivo*.[12] The carbon-fiber microelectrode was ramped from 0 V to 1.45 V, then to -1.4 V and back to 0 V every 100 ms at a scan rate of 450 V/s. Concentration *vs* time traces were extracted from the primary oxidation potential for adenosine and reduction potential for oxygen (Fig. 5.1A) in the color plot (Fig. 5.1B). The green/purple ovals in the color plot are adenosine oxidation currents; the top oval is the primary

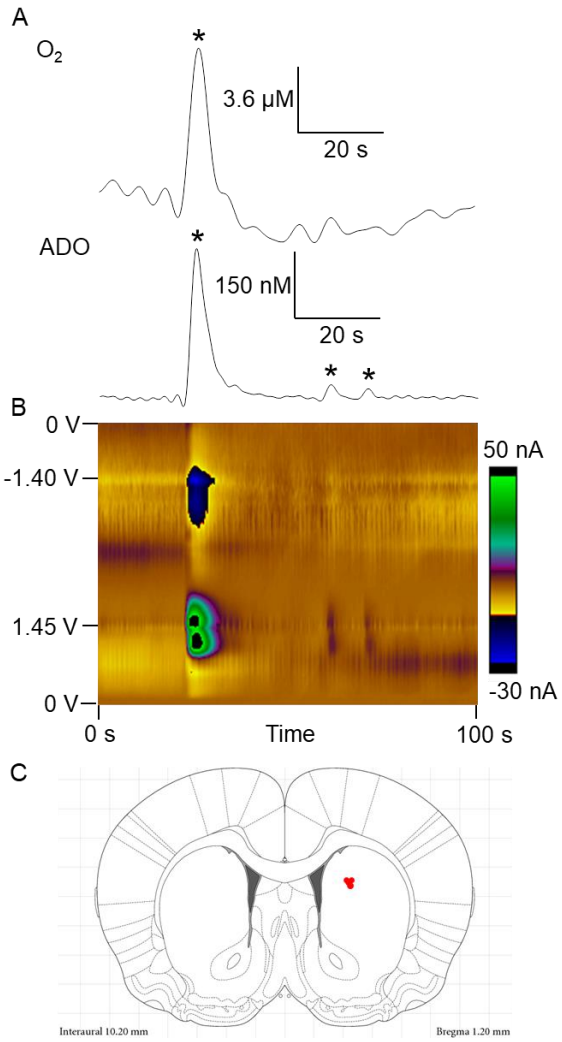

Figure 5.1 Spontaneous adenosine and oxygen release in the caudate-putamen. (A) Concentration *vs* time traces of adenosine (bottom) and oxygen (top) transients. (B) Color plot of adenosine and oxygen transients *in vivo*. One oxygen and three adenosine release events are starred, where one adenosine event is correlated with an oxygen release event. Adenosine oxidations are the green/purple in the middle of the plot while reduction of oxygen is dark blue at the top. (C) Microelectrode recording locations (red dots) in the caudate putamen. Drawing from the stereotaxic atlas of Paxinos and Watson.[18]

oxidation and the peak oxidation occurs at 1.27 V on the cathodic scan. The bottom oval directly below is the secondary oxidation of adenosine and peak oxidation occurs at 1.26 V on the anodic scan. The dark blue area around -1.3 V is the reduction of oxygen. Three spontaneous adenosine events and one oxygen release event are identified and marked by

a star. Only some adenosine transients are correlated with an oxygen transient and both adenosine and oxygen transients are fast, lasting about 3 seconds in the extracellular space. Figure 5.1C shows the placement of electrodes in the caudate-putamen.

5.3.2 Adenosine and oxygen release are correlated during ischemia/reperfusion injury

To investigate simultaneous adenosine and oxygen release during ischemia and reperfusion, bilateral common carotid artery occlusion (BCCAO) was performed to induce cerebral ischemia/reperfusion (I/R) in anesthetized rats. To confirm that BCCAO and reperfusion were successful in the caudate-putamen, blood flow was measured by a laser Doppler probe during normoxia and I/R. BCCAO resulted in immediate cerebral blood flow reduction of about 50% (Fig. 5.2A). Releasing the occluders to allow reperfusion led to an increase in blood flow, but the blood flow levels did not return to normal levels (Fig. 5.2A). The blood flow measurements for normoxia and BCCAO were significantly different (Fig. 5.2E, paired *t*-test, n = 3 animals, $p = 0.027$).

Spontaneous adenosine and oxygen events were continuously monitored for 1 hr normoxia followed by 30 minutes of BCCAO and 30 minutes of reperfusion. Example data show the difference between normoxia (Fig. 5.3A) and ischemia (Fig. 5.3B). During normoxia, three adenosine transients were observed during a 100 s window, one of which was followed by an oxygen transient, indicating local blood flow was transiently increased.

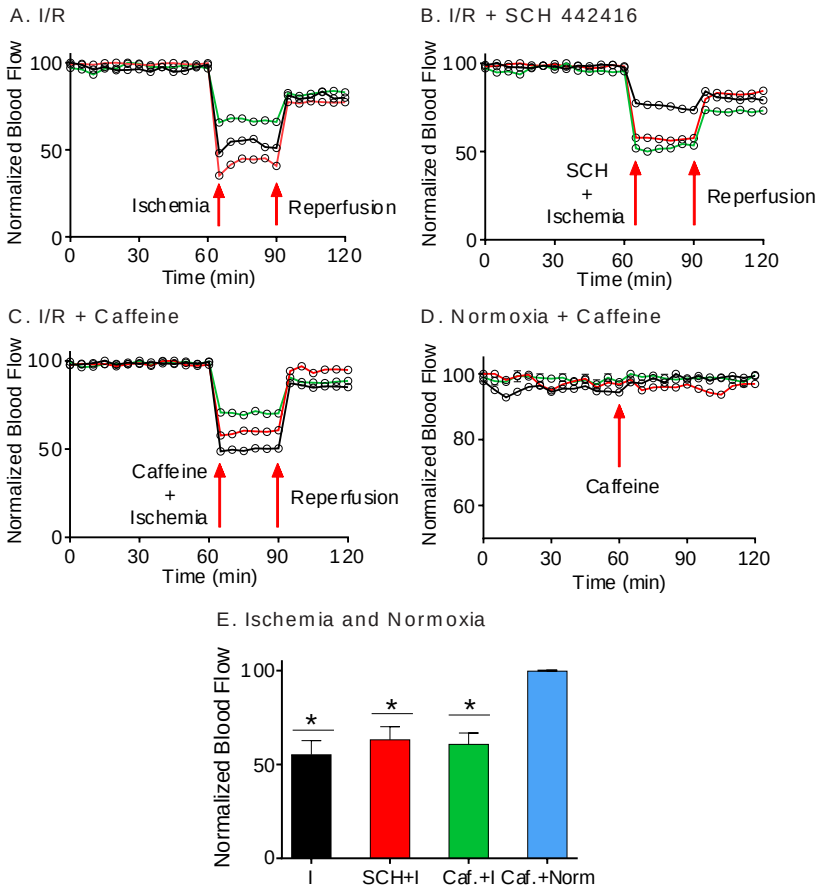

Figure 5.2 Cerebral blood flow changes in rat brain. Each color line represents averaged blood flow for one animal (% baseline). (A) Blood flow change during normoxia and I/R. Blood flow decreased during BCCAO and was partilly restored, but not to baseline, during reperfusion. (B) Blood flow change during I/R with SCH 442416 (3 mg/kg) administration is similar to plot without drug. (C) Blood flow change during I/R with caffeine (100 mg/kg) administration is similar to that without caffeine. (D) Caffeine control. Blood flow was measured before and after caffeine (100 mg/kg) during normoxia. Blood flow did not change. (E) Average changes in blood flow. Blood flow significantly decreased during 30 min of ischemia relative to normoxia (I) (p = 0.027), SCH+ ischemia (p = 0.034) and caffeine + ischemia (p = 0.023) groups compared to 30 min of normoxia (Norm). Caffeine administration itself did not affect blood flow under normoxia (p = 0.94). All paired t-test, values are mean ± SEM for n = 3 animals.

However, during ischemia, five adenosine transients were observed in the same time frame, two of which had corresponding oxygen transients. The number of transients was measured continuously and averaged in 6 min bins for each animal to understand the effects over time (Fig. 5.3C-D). A two-way ANOVA (time x I/R) revealed no main effect

of time on the number of adenosine and oxygen release events [adenosine: $F_{(9, 54)} = 0.96$, $p = 0.48$ and oxygen: $F_{(9, 54)} = 1.19$, $p = 0.32$], but a main effect of I/R injury [adenosine: $F_{(1, 6)} = 17.24$, $p = 0.01$ and oxygen: $F_{(1, 6)} = 11.54$, $p = 0.01$]. There was no interaction between time and I/R injury [adenosine: $F_{(9, 54)} = 0.82$, $p = 0.60$ and oxygen: $F_{(9, 54)} = 0.76$, $p = 0.65$]. The average number of transients per hour significantly increased for both adenosine and oxygen during I/R injury compared to normoxia (Fig. 5.3C, D inset, paired *t*-test, n = 7 animals, adenosine: $p = 0.0066$ and oxygen: $p = 0.022$). To examine the frequency, the time interval between consecutive transients, termed the inter-event time, was plotted as a histogram. I/R decreased the mean inter-event time for adenosine transients from 77 to 48 s and for oxygen from 177 to 96 s. The distributions of inter-event times were significantly different for normoxia and I/R for both adenosine and oxygen transients (Fig. 5.3E-F, KS test, n = 7 animals, adenosine: $p < 0.0001$ and oxygen: $p < 0.0001$).

The average event concentration, which was calculated by averaging the mean concentration from each animal, is plotted in Figures 5.3G-H for all adenosine events (All ADO), adenosine transients correlated with an oxygen transient (ADO w/O_2), and oxygen concentrations. I/R injury did not significantly change the concentration of transients (paired *t*-test, n = 7 animals, All ADO: $p = 0.62$, ADO w/O_2: $p = 0.42$ and oxygen: $p = 0.92$). Thus, I/R injury did not affect the concentration of spontaneously released adenosine and oxygen but both adenosine and oxygen were released more frequently during I/R injury, even when blood flow was decreased during ischemia.

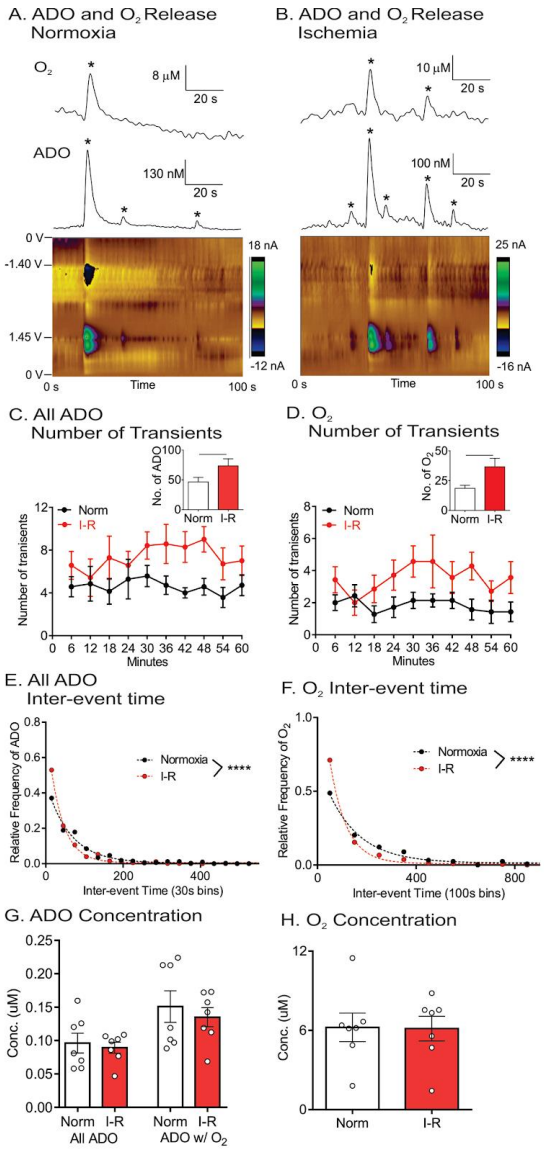

Figure 5.3 Spontaneous adenosine and oxygen release in the caudate-putamen during I/R injury. (n = 7 animals) (A) Example adenosine and oxygen release events during normoxia. Three adenosine and one oxygen transients (starred) were observed. (B) Example adenosine and oxygen release during ischemia, where five adenosine and two oxygen transients were observed. (C) Number of adenosine transients, in 6 min bins, during normoxia and I/R injury. Inset: Average number of adenosine transients increases during I/R (paired *t*-test, $p = 0.0066 < 0.01$). (D) Number of oxygen transients in 6 min bins. Inset: Oxygen events increased during I/R injury (paired *t*-test, $p = 0.022 < 0.01$). (E) Inter-event time histogram for adenosine. Underlying distributions were significantly different between normoxia and I/R injury (KS test, $p < 0.0001$). (F) Oxygen inter-event time distributions were significantly different for I/R injury (KS test, $p < 0.0001$). (G) Mean event concentration of adenosine transients was not different during I/R injury for all adenosine transients or adenosine transients with oxygen release (ADOw/O_2)(paired *t*-test, $p = 0.62$ and $p = 0.42$, respectively). (H) The mean concentration of each oxygen transient was not significantly different during I/R injury (paired *t*-test, $p = 0.92$).

5.3.3 A_{2A} receptor modulation of adenosine and oxygen release during ischemia/reperfusion injury

To evaluate the effects of A_{2A} receptors on rapid adenosine and oxygen dynamics during I/R injury, we tested a specific A_{2A} receptor antagonist, SCH 442416 (SCH) (3 mg/kg, *i.p.*). The drug was administered right before I/R, after 1 hour of normoxia. Blood flow was measured to investigate if SCH 442416 affects cerebral blood flow under I/R (Fig. 5.2B), but the pattern of blood flow with SCH 442416 was similar to that of I/R without drug administration. The blood flow measurement during normoxia and after BCCAO + SCH 442416 administration were significantly different (Fig. 5.2E, paired *t*-test, n = 3 animals, $p = 0.034$), just like I/R. Thus, administration of the A_{2A} antagonist, SCH 442416, did not affect global cerebral blood flow during I/R.

Figures 5.4A-B show the average number of adenosine and oxygen transients in 6-minute bins. A two-way ANOVA (time x I/R with SCH treatment) revealed no main effect of time on number of adenosine transients compared to normoxia [$F_{(9, 45)} = 0.73$, $p = 0.68$] nor effect of SCH + I/R [$F_{(1, 5)} = 0.22$, $p = 0.66$]. However, there was a main effect of SCH + I/R on number of oxygen transients [$F_{(1, 5)} = 1.87$, $p = 0.023$] but no main effect of time [$F_{(9, 45)} = 0.67$, $p = 0.73$]. There was no interaction between time and SCH + I/R for either number of adenosine or oxygen transients [adenosine: $F_{(9, 45)} = 0.79$, $p = 0.63$ and oxygen: $F_{(9, 45)} = 0.98$, $p = 0.47$]. The average number of adenosine transients did not significantly change after SCH + I/R (Fig. 5.4A inset, paired *t*-test, n = 6 animals, $p = 0.64$) and the number of oxygen transients also did not significantly increase (Fig. 5.4B inset, paired *t*-test, n = 6 animals, $p = 0.24$). The mean inter-event time decreased slightly from 48 to 44 s for adenosine and 135 to 103 s for oxygen, but there was no significant

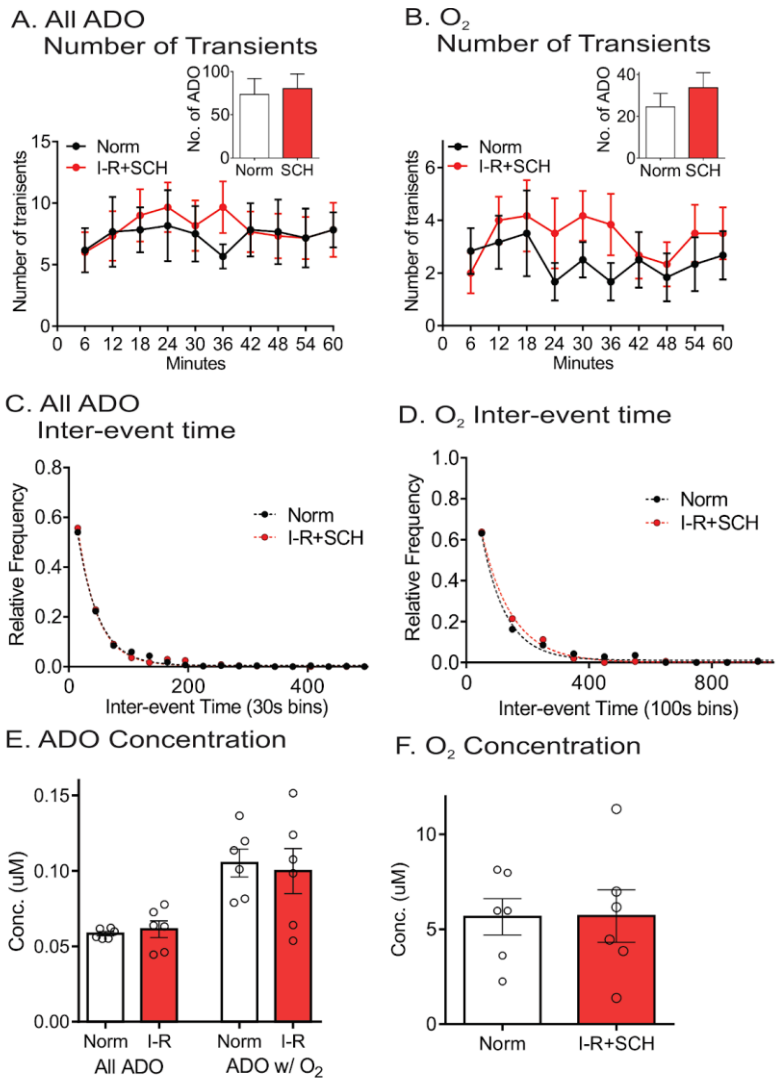

Figure 5.4 Effect of the A_{2A} antagonist, SCH 442416 (3 mg/kg, *i.p.*), on adenosine and oxygen release during I/R injury. (A) Number of adenosine transient release traces during every 6 min during normoxia and I/R injury. Inset: No change in number of adenosine transients between normoxia and I/R injury (paired *t*-test, n = 6 animals, p = 0.64). (B) Number of oxygen transients in 6 min. bins. Inset: Number of oxygen release events did not change during I/R (paired *t*-test, n = 6 animals, p = 0.24). (C) For adenosine, there are no differences in inter-event times between normoxia and I/R injury (KS test, n = 6 animals, p = 0.63). (D) For oxygen, there were no changes in inter-event times during I/R (KS test, n = 6 animals, p = 0.46). (E) Mean event concentration of all adenosine (ADO) and ADO w/O_2 were not significantly different during I/R injury (paired *t*-test, n = 6 animals, p = 0.55 and p = 0.63, respectively). (F) The mean event concentration of oxygen was not significantly different during I/R injury (paired *t*-test, n = 6 animals, p = 0.96).

change in frequency distributions for either adenosine or oxygen transients with SCH under I/R (Fig. 5.4C and 4D, KS test, n = 6 animals, adenosine: $p = 0.63$ and oxygen: $p = 0.46$). In addition, the concentration of all ADO, ADO w/O_2 and oxygen transients did not differ (Fig. 5.4E-F, paired *t*-test, n = 6 animals, All ADO: $p = 0.55$, ADO w/O_2: $p = 0.63$ and oxygen: $p = 0.96$). Therefore, with the A_{2A} antagonist SCH 442416, the number of adenosine and oxygen transients no longer increases during I/R injury.

5.3.4 Caffeine modulation of adenosine and oxygen release during ischemia/reperfusion injury

Caffeine has been proposed as a treatment to reduce the risk of stroke and is a nonselective antagonist of adenosine receptors.[20,21] Previous studies suggested that low doses of caffeine are stimulatory but high doses are inhibitory on neuronal activity.[22] We employed a large dose of caffeine (100 mg/kg, *i.p.*) to understand how it affects transient adenosine release during I/R. Blood flow during normoxia was compared to I/R with caffeine administration (Fig. 5.2C, n = 3 animals). Blood flow changes during I/R with caffeine treatment were similar to those during I/R without drug administration (Fig. 5.2E, paired *t*-test, n = 3 animals, $p = 0.023$). We also tested the same dose of caffeine (100 mg/kg *i.p.)* during normoxia and caffeine did not affect global blood flow (Fig. 5.2D, paired *t*-test, n = 3 animals, $p = 0.94$). Thus, administration of caffeine did not affect global blood flow during normoxia or I/R.

Figure 5.5A shows example data for normoxia and after caffeine during ischemia, where the number of adenosine transients decreases. There are 4 adenosine transients during normoxia, two of which are correlated with oxygen release (Fig. 5.5A), while during ischemia with caffeine, there are only two adenosine and one oxygen transient

(Fig. 5.5B). The number of transients for both adenosine and oxygen decreased during I/R injury following caffeine injection (Fig. 5.5C-D). A two-way ANOVA (time x I/R with caffeine treatment) revealed a significant effect of time for number of adenosine and oxygen transients [adenosine: $F_{(9, 45)} = 3.93$, $p = 0.001$ and oxygen: $F_{(9, 45)} = 2.61$, $p = 0.02$] and a main effect on caffeine + I/R injury [adenosine: $F_{(1, 5)} = 12.04$, $p = 0.02$ and oxygen: $F_{(1, 5)} = 20.24$, $p = 0.01$]. There was no interaction between time and caffeine + I/R [adenosine: $F_{(9, 45)} = 1.52$, $p = 0.17$ and oxygen: $F_{(9, 45)} = 1.34$, $p = 0.24$]. The number of both adenosine and oxygen transients significantly decreased compared to normoxia (Fig. 5.5C-D insets, paired *t*-test, n = 6 animals, adenosine: $p = 0.022$ and oxygen: $p = 0.0069$). In addition, caffeine increased the mean inter-event time for adenosine transients from 34 to 63 s and for oxygen from 83 to 177 s during I/R and the underlying inter-event time distributions were significantly different than normoxia (Fig. 5.5E-F, KS test, n = 6 animals, adenosine: $p < 0.0001$ and oxygen: $p < 0.0001$). However, caffeine + I/R did not affect the concentration of either adenosine or oxygen (Fig. 5.5G-H, paired *t*-test, n = 6 animals, All ADO: $p = 0.75$, ADO w/O_2: $p = 0.56$ and oxygen: $p = 0.96$). Therefore, while caffeine treatment did not affect global blood flow, it markedly reduced the frequency of transient adenosine and oxygen release during I/R.

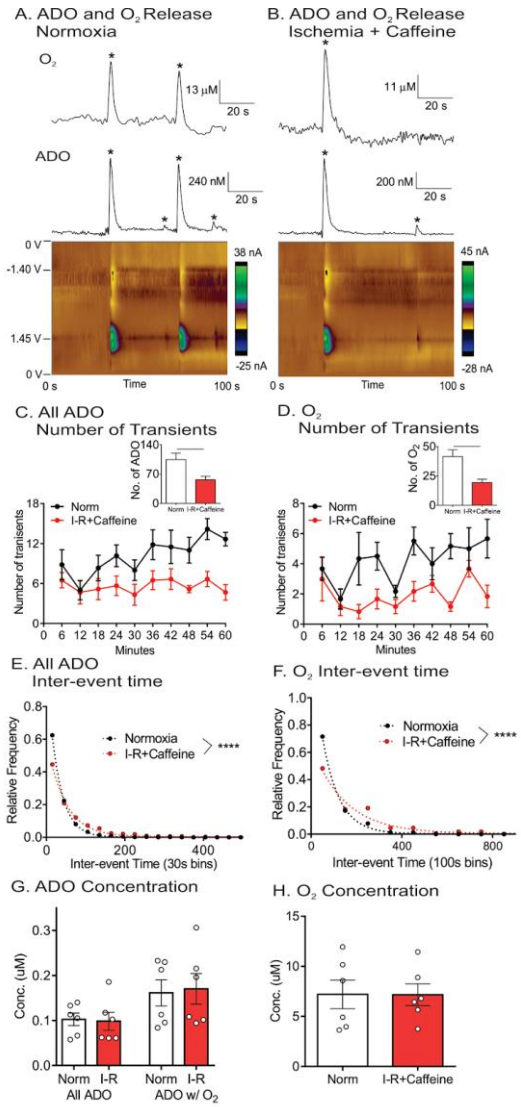

Figure 5.5 Effect of caffeine (100 mg/kg, *i.p.*) on adenosine and oxygen release during I/R injury. (n = 6 animals) (A) Example adenosine and oxygen release during normoxia, where four adenosine and two oxygen transients (starred) were observed. (B) Example adenosine and oxygen release during I/R injury with caffeine treatment, where two adenosine and one oxygen transients were observed. (C) Number of adenosine transient release traces during every 6 min during normoxia and I/R injury. Inset: Average number of adenosine release events decreased during caffeine+I/R (paired *t*-test, $p = 0.022$). (D) Number of oxygen events in 6 min bins. Inset: Average number of oxygen release events decreased during caffeine+ I/R injury (paired *t*-test, $p = <0.01$). (E) For adenosine, underlying distributions of inter-event times were significantly different between normoxia and caffeine + I/R injury (KS test, $p < 0.0001$). (F) For oxygen, the distributions of inter-event times were significantly different for caffeine+I/R (KS test, $p < 0.0001$). (G) Average event concentration of adenosine transients was not significantly different (paired *t*-test, all ADO: $p = 0.75$ and ADO w/O_2: $p = 0.56$). (H) Average oxygen transients concentration was not significantly different (paired *t*-test, $p = 0.96$).

5.4 Discussion

In this study, we demonstrate that transient adenosine release is correlated with transient oxygen increase, and that there are more transient adenosine and oxygen events during BCAAO and reperfusion, periods of reduced global blood flow. Thus, adenosine can act as a signal to transiently increase local blood flow even when some major arteries are blocked, as happens during BCCAO. The increases in oxygen and adenosine transients during I/R are mediated by A_{2A} receptors. The specific A_{2A} receptor antagonist, SCH 442416, blocks the increase in the number of adenosine and oxygen transients detected with I/R, while a high dose of caffeine actually decreases the number of adenosine and oxygen transients during I/R. Thus, adenosine is a rapid signal that transiently increases local blood flow, especially during I/R injury, and caffeine dampens that rapid neuromodulatory response of adenosine.

5.4.1 Adenosine and oxygen release are correlated during I/R

We performed ischemia with a BCCAO model in this study, which blocks the common carotid arties of the rat bilaterally and causes cerebral hypoperfusion. BCCAO and subsequent reperfusion causes significant microvascular alterations in rat,[23,24] and our previous study showed 30 minutes of BCCAO caused shrinkage in the cell nucleus and swollen mitochondria structure, but not cell death[11]. As both the carotid and vertebral arteries of rat provide the blood supply to the brain, blocking the carotid arties does not completely occlude blood flow to the brain;[25–29] therefore, this is a mild ischemic insult. BCCAO is also used to model ischemic preconditioning, where a small stroke can induce brain tolerance to larger ischemic injury.[30] Other models of stroke could be used in the future to provide global ischemia, either 4-vessel occlusion, with occlusion of the

vertebral and carotid arteries,[31] or BCCAO with hypotension, where the mean arterial blood pressure is reduced below 50 mmHg.[32,33] However, BCCAO is a good model of a partial, transient ischemic attack.[26]

During BCCAO and early reperfusion, adenosine and oxygen transients were still correlated and oxygen transients occurred more frequently during ischemia and reperfusion compared to normoxia. Thus, even when major arteries were occluded, local oxygen can still increase, which is a sign of greater local blood flow. The likely cause of the transient increases in oxygen is capillaries dilating to provide local increases in blood flow. Capillary dilation produces 84% of the blood flow increase in response to neural activity,[34] which is generated by a relaxation of pericytes. Pericytes constrict capillaries and often die during ischemia,[35] which explains why blood flow was not completely restored during reperfusion (Fig. 5.2A-C).

The number of adenosine transients increased similarly during both ischemia and reperfusion compared to normoxia (Fig. 5.3A). During normoxia, 43% of adenosine transients are correlated with an oxygen release event and during I/R, 49% of adenosine transients were followed by oxygen transients, but this increase is not significant (Fig. 5.6, paired *t*-test, n = 7 animals, $p = 0.34$). The similar percentage of adenosine transients causing oxygen is likely because the concentration of each adenosine event did not change during I/R, and a high concentration of adenosine is most correlated with an oxygen release event.[12] Therefore, the increase in local blood flow during I/R was primarily caused by an increase in adenosine transient frequency, and more adenosine and oxygen events occurred. These results demonstrate that adenosine could be a stress signal that causes local blood flow and oxygen increases to help mitigate against I/R

injury. The duration of adenosine and oxygen transients was only last about 3 s, so adenosine promotes transient, local vasodilation even when global blood flow is reduced.

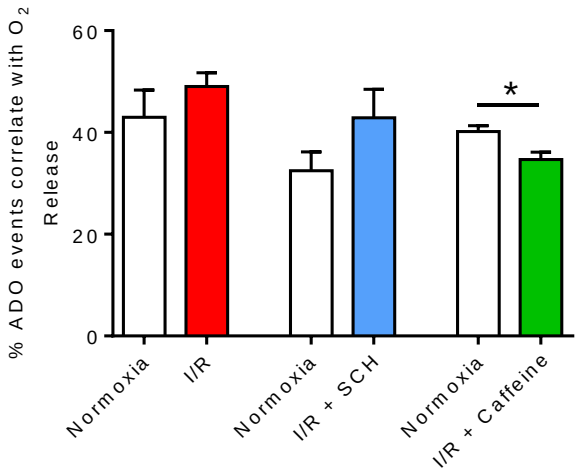

Figure 5.6 Percent of transient adenosine events that correlate with an oxygen event. Each animal was used as its own control to compare percentage of correlated adenosine and oxygen release events during normoxia and I/R with/without drug treatment. The percent of transient adenosine events that correlates with an oxygen release event did not significantly change under I/R injury (Paired *t*-test, n = 7 animals, $p = 0.34$) and I/R injury with SCH 442416 administration (Paired *t*-test, n = 6 animals, $p = 0.059$). However, the percent of adenosine events that correlates with an oxygen release significantly decreased under I/R injury with caffeine administration (Paired *t*-test, n = 6 animals, $p = 0.043$).

5.4.2 A_{2A} receptors diminish adenosine and oxygen release events during I/R

Extracellular adenosine plays an important role during cerebral ischemia, and drugs that target the adenosine system have been proposed as stroke treatments.[36] A_{2A} antagonists are neuroprotective during stroke, although their effects can vary by dose, with lower doses sometimes being more effective.[37,38] However, the high dose of SCH 442416 administered here is protective against cell swelling and death.[11] A_{2A} receptors are highly expressed in the cerebral vasculature[39–41] and are located on endothelial as well as smooth muscle cells.[42] Adenosine regulates vasodilation through A_{2A} receptors, which are coupled to excitatory G proteins.[43,44] Treatment with an A_{2A} antagonist, ZM-241385,

reduces the vasodilation caused by neuronal activation[45] and A_{2A} receptor knockout mice have a significantly reduced capacity for vasodilation of cerebral blood vessels during short periods of hypotension.[45] The objective of this study was to determine whether an A_{2A} antagonist affects adenosine release for oxygen increases and our hypothesis was that there would be fewer oxygen transients after an A_{2A} antagonist.

While I/R injury increased the number of adenosine and oxygen release events in controls, that increase was not observed when the A_{2A} antagonist SCH 442416 was administered. These results are also similar to our previous finding on non-ischemic rats, where blocking A_{2A} receptors with SCH 442416 decreased the number of both adenosine and oxygen transients but did not change the concentration.[12] There was not a significant change in the percentage of adenosine events with a subsequent oxygen transient under I/R + SCH (Fig. 5.6, paired *t*-test, n = 6 animals, $p = 0.059$). Thus, the main driver for the lower number of oxygen transients with the A_{2A} antagonist is that there are fewer adenosine transients to cause them. However, oxygen transients were not completely eliminated by the A_{2A} antagonist, and thus there may also be other molecules co-released that stimulate endothelial second messengers systems, such as prostaglandins or nitric oxide.[46] Future studies could clarify the role of A_{2A} receptors on adenosine-induced transient blood flow change by using A_{2A} knockout mice. Even though blockage of A_{2A} receptors by SCH 442416 did not suppress global cerebral blood flow (i.e Fig. 5.2), it reduced the extent of local, transient vasodilation. Therefore, the A_{2A} antagonist suppressed the release of rapid adenosine and the subsequent oxygen transients so there was not as much delivery of local blood oxygen to the brain.

5.4.3 Caffeine diminishes adenosine and oxygen release events during I/R

Caffeine is a nonselective adenosine receptor antagonist that can reduce the physical, cellular, and molecular damage caused by a stroke.[21] Vasoconstrictive effects of caffeine were investigated,[47,48] and dietary caffeine with a mean dose of 250 mg decreased 30% cerebral blood flow in humans.[49] Another study demonstrated that high caffeine users (950 mg/day) had less cerebral blood flow than the low (45 mg/day) or moderate users (405 mg/day).[50] In rats, caffeine produces a dose-dependent reduction in cerebral blood flow during neuronal activation[51] and attenuates exogenous adenosine-induced vasodilation during somatosensory stimulation.[13] Previous experiments suggest that various caffeine dosages could either increase or attenuate ischemia induced brain damage depending on the treatment paradigm.[52,53] Here, we examined the effects of caffeine on local blood flow changes caused by transient adenosine release during I/R.

A large dose of caffeine 100 mg/kg (*i.p.*) before I/R injury significantly reduced the number and frequency of adenosine and oxygen transients. In addition, the percentage of adenosine transients that were followed by an oxygen transient significantly decreased during caffeine + I/R (Fig. 5.6, paired *t*-test, n = 6 animals, $p = 0.043$). The average spontaneous adenosine transient concentration was a couple hundred nM,[54] which was sufficient to activate high affinity A_1 (approximately 70 nM) and A_{2A} receptors (150 nM) receptors, but not A_{2B} (5100 nM) and A_3 receptors (6500 nM).[55,56] The potency of caffeine for adenosine receptors is similar (A_1, $K_D = 20$ μM and A_{2A}, $K_D = 8.1$ μM)[57] and we used a high dose that would bind to both receptors. However, the data are in line with caffeine acting primarily to block the excitatory A_{2A} receptors, and it had even more of an effect of blocking adenosine transients than the A_{2A} antagonist. The blood flow data

indicate that global blood flow is still attenuated during I/R with a large dose of caffeine, but the decrease in local adenosine and oxygen transients indicate that this caffeine dose is likely not locally neuroprotective and could exacerbate the effects of ischemia. Future work is needed to link the transient changes in adenosine and blood flow to ischemic damage, but a high dose of caffeine depresses the local increase in oxygen normally observed during I/R.

5.5 Conclusions

Our results demonstrate that adenosine transiently signals for more oxygen release and that this effect is more pronounced during I/R injury because of an increase in the number of transient adenosine events. The specific A_{2A} antagonist SCH 442416 and non-selective antagonist caffeine decrease the amount of adenosine and oxygen transients during I/R. This rapid mode of adenosine release likely functions as a local neuromodulator, which produces a local, rapid increase in blood flow in response to I/R injury, and this local mode of blood flow is decreased with caffeine, despite the fact that global blood flow is unaffected. Therefore, correlated adenosine and oxygen transients may play an important role during ischemic preconditioning. During mild stroke, our work indicates that adenosine continues to cause transient, local oxygen increases and that this release is dampened by an A_{2A} antagonist or caffeine. Future studies of more severe stroke will reveal if adenosine could still signal for more blood flow when more arteries are blocked or blood pressure is low, and how local actions of adenosine contribute to its neuroprotective effects.

5.6 References

1. Heron M. Deaths: Leading Causes for 2011. *Natl Vital Stat Rep*. 2015;64(7):1-96.

2. Williams-Karnesky RL, Stenzel-Poore MP. Adenosine and stroke: maximizing the therapeutic potential of adenosine as a prophylactic and acute neuroprotectant. *Curr Neuropharmacol*. 2009;7(3):217-227. doi:10.2174/157015909789152209.

3. Lee JM, Grabb MC, Zipfel GJ, Choi DW. Brain tissue responses to ischemia. *J Clin Invest*. 2000;106(6):723-731. doi:10.1172/JCI11003.

4. Heistad DD, Marcus ML, Gourley JK, Busija DW. Effect of adenosine and dipyridamole on cerebral blood flow. *Am J Physiol*. 1981;240(5):H775-80. doi:10.1152/ajpheart.1981.240.5.H775.

5. Phillis JW. Adenosine in the control of the cerebral circulation. *Cerebrovasc Brain Metab Rev*. 1989;1(1):26-54.

6. Pelligrino DA, Vetri F, Xu H-L. Purinergic mechanisms in gliovascular coupling. *Semin Cell Dev Biol*. 2011;22(2):229-236. doi:10.1016/j.semcdb.2011.02.010.

7. Laghi Pasini F, Guideri F, Picano E, et al. Increase in plasma adenosine during brain ischemia in man: A study during transient ischemic attacks, and stroke. *Brain Res Bull*. 2000;51(4):327-330. doi:10.1016/S0361-9230(99)00240-3.

8. Kitagawa H, Mori A, Shimada J, Mitsumoto Y, Kikuchi T. Intracerebral adenosine infusion improves neurological outcome after transient focal ischemia in rats. *Neurol Res*. 2002;24(3):317-323. doi:10.1179/016164102101199819.

9. Sheardown MJ, Knutsen LJS. Unexpected neuroprotection observed with the adenosine A2A receptor agonist CGS 21680. *Drug Dev Res*. 1996;39(1):108-114. doi:10.1002/(SICI)1098-2299(19960901)39:1<108::AID-DDR8>3.0.CO;2-J.

10. Nguyen MD, Lee ST, Ross AE, Ryals M, Choudhry VI, Venton BJ. Characterization of spontaneous, transient adenosine release in the caudate-putamen and prefrontal cortex. *PLoS One*. 2014;9(1):e87165. doi:19326203.

11. Ganesana M, Venton BJ. Early changes in transient adenosine during cerebral ischemia and reperfusion injury. *PLoS One*. 2018;13(5):e0196932. doi:10.1371/journal.pone.0196932.

12. Wang Y, Venton BJ. Correlation of transient adenosine release and oxygen changes in the caudate-putamen. *J Neurochem*. 2017;140(1):13-23. doi:10.1111/jnc.13705.

13. Meno JR, Nguyen TK, Jensen EM, et al. Effect of Caffeine on Cerebral Blood Flow Response to Somatosensory Stimulation. *J Cereb Blood Flow Metab*. 2005;25(6):775-784. doi:10.1038/sj.jcbfm.9600075.

14. Vidyasagar R, Greyling A, Draijer R, Corfield DR, Parkes LM. The effect of black tea and caffeine on regional cerebral blood flow measured with arterial spin labeling. *J Cereb Blood Flow Metab*. 2013;33(6):963-968. doi:10.1038/jcbfm.2013.40.

15. Field AS, Laurienti PJ, Yen Y-F, Burdette JH, Moody DM. Dietary Caffeine Consumption and Withdrawal: Confounding Variables in Quantitative Cerebral Perfusion Studies? *Radiology*. 2003;227(1):129-135. doi:10.1148/radiol.2271012173.

16. Phillis JW, Preston G, DeLong RE. Effects of Anoxia on Cerebral Blood Flow in the Rat Brain: Evidence for a Role of Adenosine in Autoregulation. *J Cereb Blood Flow Metab*. 1984;4(4):586-592. doi:10.1038/jcbfm.1984.83.

17. Conlay LA, Conant JA, deBros F, Wurtman R. Caffeine alters plasma adenosine levels. *Nature*. 1997;389(6647):136-136. doi:10.1038/38160.

18. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. Elsevier; 2007.

19. Ohta A, Lukashev D, Jackson EK, Fredholm BB, Sitkovsky M 1,3,7-Trimethylxanthine (Caffeine) May Exacerbate Acute Inflammatory Liver Injury by Weakening the Physiological Immunosuppressive Mechanism. *J Immunol*. 2007;179(11):7431-7438. doi:10.4049/jimmunol.179.11.7431.

20. Larsson SC, Männistö S, Virtanen MJ, Kontto J, Albanes D, Virtamo J. Coffee and Tea Consumption and Risk of Stroke Subtypes in Male Smokers. *Stroke*. 2008;39(6). doi:10.1161/STROKEAHA.107.504183.

21. Rivera-Oliver M, Díaz-Ríos M. Using caffeine and other adenosine receptor antagonists and agonists as therapeutic tools against neurodegenerative diseases: a review. *Life Sci*. 2014;101(1-2):1-9. doi:10.1016/j.lfs.2014.01.083.

22. Svenningsson P, Nomikos GG, Fredholm BB. The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. *J Neurosci*. 1999;19(10):4011-4022. doi:10.1523/JNEUROSCI.19-10-04011.1999.

23. Lapi D, Vagnani S, Pignataro G, et al. Protective effects of quercetin on rat pial microvascular changes during transient bilateral common carotid artery occlusion and reperfusion. 2012. doi:10.3389/fphys.2012.00032.

24. Lapi D, Vagnani S, Pignataro G, Esposito E, Paterni M, Colantuoni A. Rat Pial

Microvascular Responses to Transient Bilateral Common Carotid Artery Occlusion and Reperfusion: Quercetin's Mechanism of Action. *Front Physiol*. 2012;3:99. doi:10.3389/fphys.2012.00099.

25. Grotta JC, Lo EH. Stroke: pathophysiology, diagnosis, and management. *Elsevier Health Sciences*, 2015.

26. Vanella A, Sorrenti V, Castorina C, et al. Lipid peroxidation in rat cerebral cortex during post-ischemic reperfusion: Effect of exogenous antioxidants and Ca++-antagonist drugs. *Int J Dev Neurosci*. 1992;10(1):75-80. doi:10.1016/0736-5748(92)90008-N.

27. Shaheen AA, Abd-el-Fattah AA, Seif-el-Nasr M. Influence of verapamil on the efficacy of vitamin E in preventing the ischemia-reperfusion-induced biochemical dearrangement in cerebral cortex of rat. *Arzneimittelforschung*. 1996;46(7):670-673.

28. Aabdallah DM, Eid NI. Possible neuroprotective effects of lecithin and α-tocopherol alone or in combination against ischemia/reperfusion insult in rat brain. *J Biochem Mol Toxicol*. 2004;18(5):273-278. doi:10.1002/jbt.20037.

29. Ghoneim, A. I., Abdel-Naim, A. B., Khalifa, A. E., & El-Denshary ES. Protective effects of curcumin against ischaemia/reperfusion insult in rat forebrain. *Pharmacol Res*. 2002;46(3):273-279. doi:10.1016/S1043-6618(02)00123-8.

30. Speetzen LJ, Endres M, Kunz A. Bilateral common carotid artery occlusion as an adequate preconditioning stimulus to induce early ischemic tolerance to focal cerebral ischemia. *J Vis Exp*. 2013;(75):e4387. doi:10.3791/4387.

31. Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann Neurol*. 1982;11(5):491-498. doi:10.1002/ana.410110509.

32. Plaschke K, Bardenheuer HJ, Martin E, Sartor K, Heiland S. Evolution of apparent diffusion coefficient and transverse relaxation time (T2) in the subchronic stage of global cerebral oligemia in different rat models. *Exp Brain Res*. 2006;169(3):361-368. doi:10.1007/s00221-005-0146-3.

33. Smith M-L, Bendek G, Dahlgren N, Rosén I, Wieloch T, Siesjö BK. Models for studying long-term recovery following forebrain ischemia in the rat. 2. A 2-vessel occlusion model. *Acta Neurol Scand*. 1984;69(6):385-401. doi:10.1111/j.1600-0404.1984.tb07822.x.

34. Hall CN, Reynell C, Gesslein B, et al. Capillary pericytes regulate cerebral blood flow in health and disease. *Nature*. 2014;508(7494):55-60. doi:10.1038/nature13165

35. Attwell D, Mishra A, Hall CN, O 'farrell FM, Dalkara T. What is a pericyte? *J Cereb Blood Flow Metab*. 2016;36(2):451-455. doi:10.1177/0271678X15610340.

36. Pedata F, Corsi C, Melani A, Bordoni F, Latini S. Adenosine extracellular brain concentrations and role of A2A receptors in ischemia. *Ann N Y Acad Sci*. 2001;939:74-84. doi:10.1111/j.1749-6632.2001.tb03614.x.

37. Pintor A, Quarta D, Pèzzola A, Reggio R, Popoli P. SCH 58261 (an adenosine A(2A) receptor antagonist) reduces, only at low doses, K(+)-evoked glutamate release in the striatum. *Eur J Pharmacol*. 2001;421(3):177-180. doi:10.1016/S0014-2999(01)01058-5.

38. Popoli P, Pintor A, Domenici MR, et al. Blockade of Striatal Adenosine A2A Receptor Reduces, through a Presynaptic Mechanism, Quinolinic Acid-Induced Excitotoxicity: Possible Relevance to Neuroprotective Interventions in Neurodegenerative Diseases of the Striatum. *J Neurosci*. 2002;22(5):1967-1975. doi:10.1523/JNEUROSCI.22-05-01967.2002.

39. O'Regan M. Adenosine and the regulation of cerebral blood flow. *Neurol Res*. 2005;27(2):175-181. doi:10.1179/016164105X21931.

40. Pelligrino DA, Xu H-L, Vetri F. Caffeine and the control of cerebral hemodynamics. *J Alzheimers Dis*. 2010;20 Suppl 1:S51-62. doi:10.3233/JAD-2010-091261.

41. Mills JH, Alabanza L, Weksler BB, Couraud P-O, Romero IA, Bynoe MS. Human brain endothelial cells are responsive to adenosine receptor activation. *Purinergic Signal*. 2011;7(2):265-273. doi:10.1007/s11302-011-9222-2.

42. Coney AM, Marshall JM. Role of adenosine and its receptors in the vasodilatation induced in the cerebral cortex of the rat by systemic hypoxia. *J Physiol*. 1998;509 (Pt 2:507-518. doi:10.1111/j.1469-7793.1998.507bn.x.

43. Phillis JW. Adenosine and adenine nucleotides as regulators of cerebral blood flow: roles of acidosis, cell swelling, and KATP channels. *Crit Rev Neurobiol*. 2004;16(4):237-270. doi:10.1615/CritRevNeurobiol.v16.i4.20.

44. Miekisiak G, Kulik T, Kusano Y, Kung D, Chen J-F, Winn HR. Cerebral Blood Flow Response in Adenosine 2a Receptor Knockout Mice during Transient Hypoxic Hypoxia. *J Cereb Blood Flow Metab*. 2008;28(10):1656-1664. doi:10.1038/jcbfm.2008.57.

45. Kusano Y, Echeverry G, Miekisiak G, et al. Role of adenosine A2 receptors in regulation of cerebral blood flow during induced hypotension. *J Cereb Blood Flow Metab*. 2010;30(4):808-815. doi:10.1038/jcbfm.2009.244.

46. Mortensen SP, Nyberg M, Thaning P, Saltin B, Hellsten Y. Adenosine Contributes to Blood Flow Regulation in the Exercising Human Leg by Increasing Prostaglandin and Nitric Oxide Formation. *Hypertension*. 2009;53(6):993-999. doi:10.1161/HYPERTENSIONAHA.109.130880.

47. Terai N, Spoerl E, Pillunat LE, Stodtmeister R. The effect of caffeine on retinal vessel diameter in young healthy subjects. *Acta Ophthalmol*. 2012;90(7):e524-e528. doi:10.1111/j.1755-3768.2012.02486.x.

48. Addicott MA, Yang LL, Peiffer AM, et al. The effect of daily caffeine use on cerebral blood flow: How much caffeine can we tolerate? *Hum Brain Mapp*. 2009;30(10):3102-3114. doi:10.1002/hbm.20732.

49. Cameron OG, Modell JG, Hariharan M. Caffeine and human cerebral blood flow: A positron emission tomography study. *Life Sci*. 1990;47(13):1141-1146. doi:10.1016/0024-3205(90)90174-P.

50. Addicott MA, Yang LL, Peiffer AM, et al. The Effect of Daily Caffeine Use on Cerebral Blood Flow: How Much Caffeine Can We Tolerate? *Hum Brain Mapp*. 2009;10(10):3102-3114. doi:10.1002/hbm.20732.

51. Gotoh J, Kuang TY, Nakao Y, et al. Regional differences in mechanisms of cerebral circulatory response to neuronal activation. *Am J Physiol Heart Circ Physiol*. 2001;280(2):H821-9. doi:10.1152/ajpheart.2001.280.2.H821.

52. Jacobson KA, von Lubitz DK, Daly JW, Fredholm BB. Adenosine receptor ligands: differences with acute versus chronic treatment. *Trends Pharmacol Sci*. 1996;17(3):108-113. doi:10.1016/0165-6147(96)10002-X.

53. Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB. Neuroprotective role of adenosine in cerebral ischaemia. *Trends PharmacolSci*. 1992;13:439-445. doi:10.1016/0165-6147(92)90141-R.

54. Nguyen MD, Lee ST, Ross AE, Ryals M, Choudhry VI, Venton BJ. Characterization of spontaneous, transient adenosine release in the caudate-putamen and prefrontal cortex. *PLoS One*. 2014;9(1):e87165. doi:19326203.

55. Svenningsson P. Distribution, biochemistry and function of striatal adenosine A2A receptors. *Prog Neurobiol*. 1999;59(4):355-396. doi:10.1016/S0301-0082(99)00011-8.

56. Dunwiddie T V, Masino SA. The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci*. 2001;24:31-55. doi:10.1146/annurev.neuro.24.1.31.

57. Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE. Actions of Caffeine in the Brain with Special Reference to Factors That Contribute to Its Widespread Use. *Pharmacol Rev*. 1999;51(1). doi:0031-6997/99/5101-0083.

Chapter 6: Conclusions and Future Directions

Chapter 6 Conclusions and Future Directions

Rapid adenosine signaling detection in the brain using FSCV at an implanted carbon- fiber microelectrode allows a better understanding of the dynamics of adenosine signaling and neuromodulation in real time. In this thesis, I examined the mechanism of formation and modulation by receptors as well as function of rapid adenosine signaling. In the last chapter, I will briefly summarize the main achievements of my work and outline possible directions for future research.

6.1 Rapid adenosine signaling in the brain

Two modes of rapid adenosine signaling have been discovered in the rat brain using FSCV: (1) spontaneous release, without stimulation and (2) mechanically-stimulated release. Even though the mechanisms of rapid adenosine release have been previous studied, there are still many gaps in our understanding on the mechanisms of adenosine formation that need to be filled. Spontaneous and mechanically-stimulated adenosine release in mice were characterized for the first time in **Chapter 2**. The frequency of spontaneous adenosine and concentration of mechanically-stimulated adenosine varied by region. The average concentration of spontaneous adenosine events was sufficient to activate A_1 and A_{2A} receptors while the average concentration of mechanically-stimulated adenosine was 10-fold higher and was able to activate all adenosine receptors (A_1, A_{2A}, A_{2B} and A_3). The short duration of spontaneous adenosine release would provide frequent, local, neuroprotective effects while mechanically-stimulated adenosine release, with longer duration, could extend this effect. Thus, adenosine signaling as well as its neuromodulatory effects varied by brain region, which is important for designing adenosine-based treatments for neurological disorders. For

example, regions with low numbers of adenosine transients might benefit from drugs that increase the frequency. **Chapter 3** focused on the mechanism of rapid adenosine formation. Mice globally deficient in nucleoside triphosphate diphosphohydrolase (CD39), which converts ATP or ADP to AMP, as well as ecto-5′-nucleotidase (CD73), which converts AMP to adenosine were used to investigate if rapid adenosine signaling is from metabolism of extracellular ATP. The concentration of adenosine release was independent of CD73 and CD39, suggesting spontaneous and mechanically-stimulated adenosine release was likely formed by intracellular release of adenosine. However, CD39 and CD73KO decreased the frequency of spontaneous transients, so targeting these enzymes would specifically affect spontaneous but not mechanically stimulated release. The finding that enzymes responsible for ATP metabolism differentially affect different modes of adenosine release will help in the design of drugs that can exploit or avoid specific pathways or modes of release.

Adenosine functions as a vasodilator to provide improved oxygen delivery to tissue by increasing blood flow. However, it remains unclear whether rapid adenosine signaling could induce vasodilation and the duration of rapid adenosine signaling acts at its receptors to modulate blood flow. Thus, in **Chapter 4**, the relationship between spontaneous adenosine and blood flow was investigated by simultaneously detecting adenosine and oxygen events. Transient oxygen changes are a measure of the change of local blood flow. Adenosine and oxygen only lasted in the extracellular space for a few seconds and 34% of adenosine events were correlated with an oxygen event. The frequency of correlated adenosine and oxygen release is regulated by adenosine A_{2A} receptors but is independent of A_1 receptors. Thus, spontaneous adenosine transiently

increases oxygen due to modulation of blood flow, providing more oxygen and nutrients during increased tissue metabolic activity. The correlated adenosine and oxygen events during ischemia-reperfusion (I/R) injury was further characterized in **Chapter 5**. The frequency of correlated adenosine and oxygen events increased under ischemia and reperfusion, indicating the local blood flow is transiently increased even when a major artery is occluded. Adenosine A_{2A} receptors eliminated the increase of adenosine and oxygen under I/R and blocking adenosine receptors with caffeine decreased the frequency of adenosine and oxygen events. This study provides an understanding of the amount of spontaneous adenosine that is available to act at receptors to affect transient changes in local blood flow. This study provides valuable information that spontaneous adenosine could locally modulate oxygen and therefore blood flow changes, even when global blood flow is decreased. The local effect of spontaneous adenosine could be crucial to maintain brain functions under mild ischemic reperfusion condition.

The discovery of rapid adenosine signaling using FSCV provides a better understanding on the time course of adenosine related neuromodulator effects. Information on rapid adenosine formation and regional variation of rapid release is critical to manipulate the signaling. Adenosine receptors are involved in a variety of diseases, including ischemia. Future therapeutic strategies against diseases could target on the generation of adenosine through specific pathway as well as adenosine receptor by applying specific agonists or antagonists. For example, administration of an adenosine agonist to increase the frequency of spontaneous adenosine release during ischemia and reperfusion could provide more frequent and locally oxygen and nutrients, which may be beneficial to attenuate stroke. Better manipulation of rapid adenosine signaling need to be

developed in the future, which will significantly accelerate important therapeutic applications.

6.2 Future Research

The purpose of this work was to gain a better understanding of rapid adenosine signaling in the brain. As shown in this work, rapid adenosine release varies in brain region, but the mechanism of adenosine formation is still not fully known. Therefore, more studies are needed to further determine whether rapid adenosine signaling is neuroprotective during different diseases. Future research on functions and mechanisms of adenosine formation by combining FSCV with other techniques will provide more fundamental information to advance our understanding on rapid adenosine signaling.

6.2.1 Mechanism of release and formation of rapid adenosine

The mechanisms of release and formation of rapid adenosine have been studied but the main pathway of release and formation is not fully known. There are two sources of extracellular adenosine: (1) formed intracellularly and released to extracellular space by adenosine transporters or directly released through exocytosis; (2) formed by metabolism of extracellular adenine nucleotides (ATP, ADP, and AMP) through various enzymes.

6.2.1.1 Adenosine transporters

Intracellularly-formed adenosine can be released to extracellular space by adenosine transporters. There are two types of adenosine transporters: equilibrative nucleoside transporters (ENTs), which are diffusion-limited channels and for passive transport, and concentrative nucleoside transporters (CNTs), which are sodium-dependent

transporters for active transport.[1] There are four types of ENTs: ENT1, ENT2, ENT3, and ENT4. ENT1 and ENT2 are expressed in the central nervous system.[2,3] ENT3 is expressed outside of central nervous system and located intracellularly,[4] therefore, it is not involved in the mechanisms of rapid adenosine release in the brain. ENT4 mainly transports adenosine but its activity is low at neutral pH and greatly increased at acidic pH.[5] Thus, the ENT4 is not active at normal physiological condition and is unlikely to be a major contributor to adenosine formation. There are three types of sodium dependent CNTs: CNT1, CNT2, and CNT3, that transport both naturally occurring nucleosides or synthetic nucleoside analogs.[6] CNT1 preferentially transports pyrimidine nucleosides using a 1:1 sodium-to-nucleoside ratio. CNT2 preferentially transports purine nucleosides by 1:1 sodium-to-nucleoside ratio. CNT3 transports both pyrimidine and purine nucleosides.[7] CNT1 and CNT2 transcripts are expressed at low levels in the brain but CNT3 is not detected in the brain.[7] The affinity of adenosine is greater for CNT1 and CNT2 than ENT1, but the transport rate of adenosine is much slower with CNT1 and CNT2.[1,8] Therefore, ENT1 and ENT2 function predominantly in the brain, and the contribution of CNT1 and CNT2 is minimal.

Past studies have looked at the effects of transporters on rapid adenosine release. A specific inhibitor of ENT1, NBTI (1 mg/kg, *i.p.*), did not affect the concentration of spontaneously released adenosine or mechanically-stimulated adenosine.[9,10] In addition, inhibiting ENT1 and ENT2 with dipyridamole (10 mg/kg, *i.p.*) did not affect the concentration of adenosine resulting from spontaneous release. Therefore, ENT1 and ENT2 are ruled out as one of the mechanisms of spontaneously released adenosine and ENT1 is ruled out for mechanically-stimulated adenosine release. As CNT1 and CNT2

have slow transport rate of adenosine, these two transporters are unlikely involved in the mechanism of spontaneously released adenosine but future research is needed to investigate whether CNT1 and CNT2 participate in mechanically-stimulated adenosine release. A CNT inhibitor, such as phloridzin,[11] could be used in brain slices. In addition, ENT2 knockout mice can be used to further investigate the mechanisms of mechanically-stimulated adenosine. However, there is little evidence supporting adenosine transporters as the main mechanism of adenosine release.

6.2.1.2 Exocytosis

Exocytotic process constitutes various molecules, including soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins (SNAP-25, synaptobrevin and syntaxin), synaptotagmins, and Rab proteins that drive vesicle docking, activation and fusion at the active zone.[12,13] Exocytosis can be triggered by action potentials, where an increase in the concentration of intracellular calcium ion through voltage-gated calcium channels. Exocytosis also happens spontaneously, where vesicles are released spontaneously and independent on action potential.[14] Previous studies have demonstrated that the frequency of spontaneously released adenosine increased during ischemia and reperfusion injury but the concentration did not change compared to normoxia condition (Chapter 5),[15] suggesting the release of adenosine may be regulated through exocytosis. A previous study on mechanically-stimulated adenosine release showed that the release is activity-dependent.[10] Chelating calcium with 1mM EDTA or preventing action potential by inhibiting sodium channels with tetrodotoxin (TTX, 0.5 μM) in rat brain slice significantly reduced the concentration resulting from mechanically-stimulated adenosine, indicating the release of adenosine is calcium

dependent and activity dependent.[10] However, TTX (0.2 μM) did not affect the concentration or frequency of spontaneously released adenosine,[16] indicating spontaneously release adenosine is not due to activity-dependent exocytosis. One possible mechanism for spontaneously released adenosine could be spontaneous, action potential-independent exocytosis. Future research could knock down vesicular nucleotide transporter or apply botulinum neurotoxin types A and B to selectively block exocytosis by cleavage of SNAP-25 and synaptobrevin, respectively, in brain slices to further investigate if spontaneously adenosine and mechanically-stimulated adenosine are directly released by excytosis.[17,18]

6.2.1.3 ATP channels

There are multiple types of ATP release channels, where ATP can be released from the cytosol into the extracellular space and rapidly broken down to adenosine. Pannexins are channel proteins that mediate ATP release under physiological and pathological conditions. There are three types of pannexins(1-3). Pannexin1 and Pannexin2 are widely expressed and co-expressed in mouse tissues, such as brain and kidney in human and rodent tissues.[19] Low levels of pannexin3 were detected in human hippocampus extracts but mouse brain tissue has little or no detectable pannexin3.[19,20] Thus, pannexin3 is unlikely involved here in the mechanism of rapid adenosine release in the brain. Pannexin1 channels can be activated by membrane depolarization and cytoplasmic calcium ion.[19] They are mechanosensitive and thus can also be activated by mechanical stimulation.[21] A previous study on spontaneously release adenosine showed that the pannexin1 specific inhibitor, spironolactone (20 μM), or pannexin1 knockout mice did not affect the concentration or frequency of adenosine events in mice brain

slices.[16] When applied 10 μM of a non-specific inhibitor, carbenoxolone, which blocks pannexin channels, connexin channel, postsynaptic NMDA receptors and calcium channels, the concentration and frequency of release events of spontaneously released adenosine decreased, indicating ATP is released into extracellular space through channels or exocytosis and broken down to adenosine. It also possible that adenosine is directly released by exocytosis. However, high concentration of carbenoxolone (100 μM) had no effect on spontaneously released adneosine.[16] Low concentration of carbenoxolone is a relatively specific inhibitor of pannexin channels over connexin channels while high concentration of carbenoxolone is a more general inhibitor. Deletion of pannexin1 did not affect spontaneously released adenosine, suggesting that pannexin1 is not responsible for spontaneously release adenosine. Future research could use pannexin1 knockout mice to further study the mechanism of mechanically-stimulated adenosine release. Whether pannexin2 is involved in rapid adenosine release could also be investigated.

In addition, connexin hemichannels can also release ATP, which are the subunits of vertebrate gap junction channels. There are 43 types of connexin hemichannels and about 21 types of connexin hemichannels are expressed in the brain in astrocytes, oligodendrocytes, microglia, neurons.[22,23] There are other channels, such as calcium homeostasis modulator1, volume-regulated anion channels and maxi-anion channel that are also ATP-release channels.[24] Future research on the mechanism of rapid adenosine release could further investigate if ATP is release through these channels and break down to adenosine.

6.2.1.4 Possible formation of adenosine

ATP can be released through ATP release channels and break down to adenosine in the extracellular space. ATP and ADP can also be released by exocytosis and rapidly broken down to adenosine in the extracellular space, thus, inhibiting release of ATP and ADP by exocytosis would subsequently affect rapid adenosine release. Adenosine may be coreleased with ATP or ADP by exocytosis, therefore, inhibiting enzymes that break down ATP, ADP, and AMP will partially decrease the concentration of adenosine; however, the concentration of adenosine that directly released by exocytosis is unaffected.

There are 5 types of enzymes that are involved in hydrolysis of adenine nucleotides: NTPDases, alkaline phosphatases, prostatic acid phosphatase, CD73, and ectonucleotide pryophosphatase/phosphodiesterase (NPPs). For NTPDases, there are 8 types of NTPDases (1-8), where NTPDase 1, 2, 3 and 8 are located at the surface of the plasma membrane and hydrolyze extracellular nucleotides.[25] NTPDase 4 is allocated to the Golgi apparatus and does not hydrolyze ATP and ADP.[26] NTPDase 5 and 6 are located in the intracellular organelles but can be trafficked to the cell surface and secreted by a proteolytically cleavage.[25] NTPDase 7 is entirely located in the intracellular organelles,[27] which might hydrolyze extracellular nucleotides when released from cells. Thus, NTPDase 4 is not involved in mechanism of rapid adenosine release and NTPDase 5-7 are unlikely the major mechanism of rapid adenosine release. In chapter 3, deletion of CD39 (NTPDase 1) does not change the concentration of spontaneously released adenosine and mechanically-stimulated adenosine, indicating CD39 is not directly involved in the mechanism of rapid adenosine release. For mechanically-stimulated

adenosine release, blocking extracellular ATP breakdown by NTPDase 1, 2 and 3 inhibitor, POM-1 (100 μM), significantly reduced the concentration of adenosine resulting from mechanical stimulation.[10] Therefore, NTPDase 1 is ruled out from the mechanism of rapid adenosine release. Future research could investigate if NTPDase 2,3 and 8 are involved in the mechanism of rapid adenosine release.

In addition, ecto-nucleotide pyrophosphatease/phosphidesterase (NPPs) are other ectonuleotidases widely expressed in the brain and are capable of hydrolyzing ATP and ADP but not AMP. There are 5 types of NPPs (1-5), where NPP1 and NPP3 are type II membrane proteins and NPP2 is secreted.[28] NPP4 and NPP5 has been identified as additional types of NPPs but little is known about their catalytic acivity.[29] NPP1 is expressed on capillary walls and NPP3 is located in ependymal and choroid. NPP2 is not found in neurons and astrocytes but is associated with early oligodendrocyte lineage cells.[28] Thus, NPPs are unlikely the dominant ectonucleotidases for rapid adenosine release.

Alkaline phosphatase is an extracellularly active protein of nonspecific ecto-phosphomonoesterase that hydrolyzes ATP, ADP, and AMP to adenosine and is widely expressed in the brain. Alkaline phosphatase can hydrolyze ATP, ADP, and AMP at neutral and basic but not acidic pH.[30,31] Alkaline phosphatase can be redundantly a potential source of extracellular adenosine when CD73 is absent.[32] Prostatic acid phosphatase (PAP) and CD73 are enzymes that hydrolyze AMP to adenosine. PAP is widely expressed in various tissues, including prostate, nonpeptidergic dorsal root ganglia neurons, brain, kidney and the prostate is the richest source in human and mouse.[33,34] Previous studies showed PAP is the main acid phosphatase with CD73 activity in the

male mouse saliva but not in female animals, suggesting that PAP activity has sex-specific requirements.[35] Spontaneously released adenosine events decreased by more than 50% in dorsal root ganglia and spinal lamina II of PAP/CD73 double knockout mice and virtually eliminated the hydrolysis of AMP to adenosine by blocking alkaline phosphatase with MLS-0038949 (50 μM). In our study, deletion of CD73 does not affect the concentration of both spontaneous and mechanically-stimulated adenosine. However, deletion of CD73 may increase PAP and alkaline phosphatase activities. Further research should use qPCR to quantify gene expression of PAP and alkaline phosphatase in WT and knockout mice. In addition, enzyme histochemistry could be used to localize the PAP and alkaline phosphatase activities. Overall, ATP and ADP can be hydrolyzed by NTPDase and NPPs. AMP can be hydrolyzed by PAP, CD73. However, alkaline phosphatase is the only phosphatase in brain that can hydrolyze ATP, ADP and AMP. Future research could investigate if NTPDase 2,3 and 8 are involved in the mechanism of rapid adenosine release. The mechanism of rapid adenosine release can be further studied by blocking alkaline phosphatase in brain slice or using alkaline phosphatase knockout mice. In addition, male and female mice should be separately studied using PAP knockout mice as the activity of PAP varies in different sexes in brain. In addition, further research could combine transgenic animals and pharmacological studies to investigate the mechanism of rapid adenosine release.

Overall, the mechanisms of release and formation of rapid adenosine are complicated (Figure 6.1). Adenosine can be formed intracellularly and released into extracellular space via adenosine transporters or directly released by exocytosis, but adenosine transporters are unlikely the main sources of rapid adenosine release.

Adenosine can also be formed extracellularly by breakdown of ATP, ADP and AMP, where ATP and ADP break down to AMP by NTPDases, NPPs, and alkaline phosphatase and AMP breaks down to adenosine by PAP, CD73 and alkaline phosphatase. Breakdown of adenine nucleotide could contribute to the formation of adenosine and subsequent, the release of adenosine. Future research is needed to fully address the

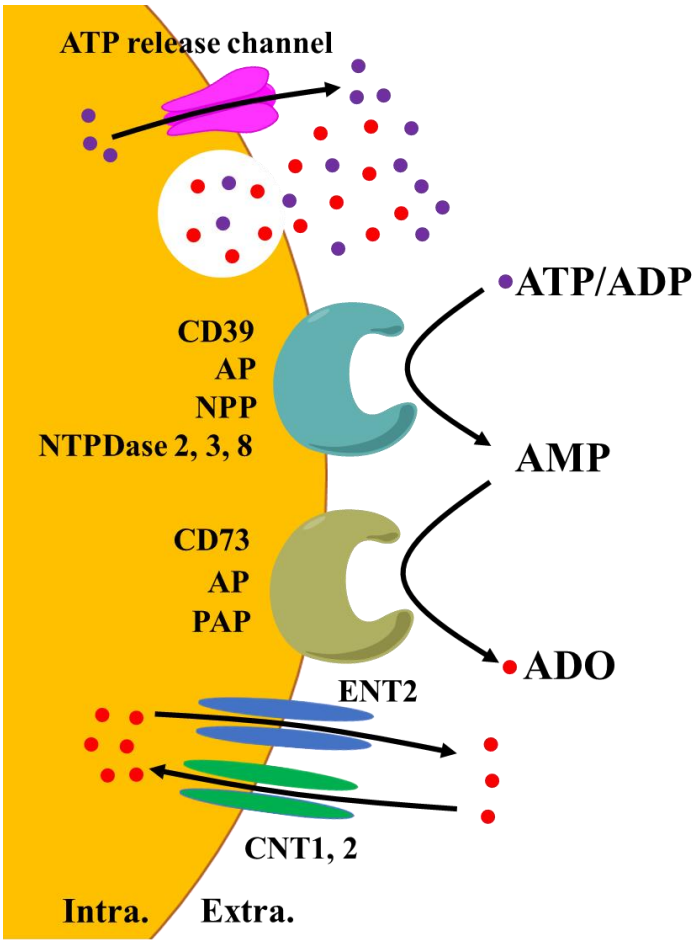

Figure 6.1 Possible mechanism of rapid adenosine release. Rapid adenosine (ADO) release can be formed in intracellular space and release into extracellular space through adenosine transporters (CNT1, CNT2, ENT2) or by exocytosis. Rapid adenosine release can also be formed by metabolism of ATP, where ATP can be released through different channels or exocytosis and break down to AMP by NTPDases, NPPs, or alkaline phosphatase (AP). AMP can break down to adenosine by CD73, prostatic acid phosphatase (PAP) or AP.

following questions: Is rapid adenosine directly released by spontaneous exocytosis or from breakdown of adenine nucleotides? If adenosine is formed by breakdown of adenine nucleotides, which ATP channel and enzyme dominate the formation of rapid adenosine? Does blocking ATP release channel or enzyme activity cause other known ATP release channels or enzymes upregulated? Answering these questions will provide a better understanding on the mechanism of rapid adenosine release, which is crucial to be able to selectively manipulate it for future therapeutic benefit.

6.2.1 Spontaneous adenosine release in freely moving rats.

Measurements of rapid changes in adenosine are usually performed in anesthetized animals or in brain slices. However, whether rapid adenosine signaling can be observed in freely moving animals is still unclear. Adenosine plays an important role in sleep control. The concentration of adenosine increases during wakefulness and decreases during sleep.[36] Measurements of adenosine concentration during the sleep-wake cycle have been widely investigated *in vivo* by microdialysis. However, microdialysis is limited for detecting neurochemical change in real time and can only measure the basal concentration of chemicals.

Electroencephalography (EEG) is an electrophysiological method that measures the electrical activities of the brain and determines sleep and waking state. While the combination of EEG and microdialysis is able to detect electrical activities and measure the basal concentration of neurotransmitter change, how neurotransmitters change on a fast time scale is unknown during sleep. Thus, FSCV and EEG could be combined to investigate how rapid adenosine changes during sleep-wake cycle. For example, sleep deprivation experiments could be performed to investigate the role of rapid adenosine

signaling. Acute and chronic treatment of caffeine, an adenosine nonselective antagonist, could also be used to study how it affects spontaneous adenosine release during sleep-wake cycle.

In addition, FSCV combined with video-EEG can also be used to study the role of adenosine in seizures. Epilepsy is a neurological disorder that is caused by abnormal electrical activity in the brain and is characterized by spontaneous recurrent seizures.[37] Pilocarpine is a muscarinic cholinergic agonist, which is commonly used to induce status epilepticus in animals. The pilocarpine model is highly isomorphic with the human disease, thus, pilocarpine animal models can be used to study the mechanism of epilepsy and predict the response to antiepileptic treatment.[38] The concentration of extracellular adenosine increases upon seizure activity,[39] which is a critical sign to determine the susceptibility of brain to seizure activity.[40] While research has focused on the basal concentration changes of adenosine, the time course of the actual signaling during seizure activity is not defined. In contrast to the increased basal concentration of adenosine during seizure, my preliminary data showed no change in concentration but a decreased frequency of spontaneous adenosine release events during pilocarpine (200 mg/kg, *i.p.*)-induced seizures in the caudate-putamen of anesthetized rats (Fig. 6.1). In order to reveal if spontaneous adenosine release has neuroprotection effects during seizure activity, FSCV and video-EEG could be used simultaneously in pilocarpine-treated animals , where spontaneous adenosine release in real time is monitored by FSCV while seizure onset and severity could be assessed using video-EEG. Moreover, deep brain stimulation reduces the frequency of spontaneous recurrent seizure in pilocarpine-induced animal model,[41] where adenosine released after stimulation may activate A_1 receptors to decrease

brain excitability.[42] Direct injection of adenosine into the affected tissue improves seizure control.[43] Thus, by using FSCV and video-EEG, mechanically-stimulated adenosine release could be performed locally and daily in the brain of pilocarpine-induced animals to investigate whether it exhibits antiepileptic effects.

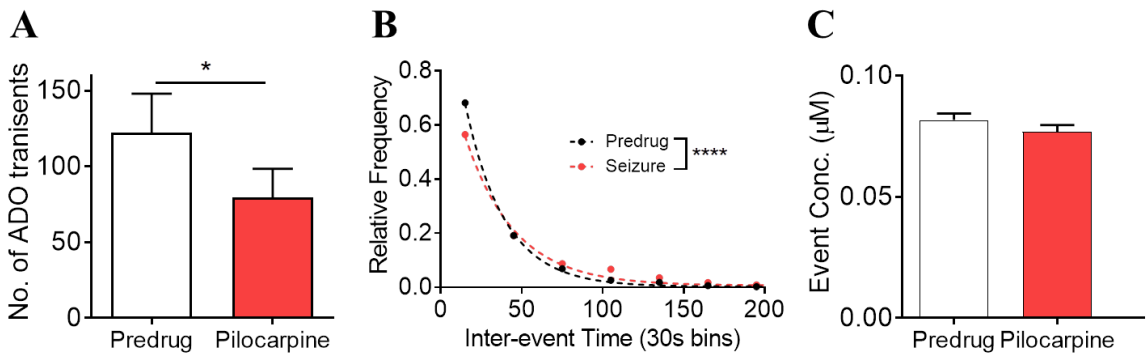

Figure 6.2 Effect of pilocarpine (200 mg/kg, *i.p.*) on spontaneous adenosine release in the caudate-putamen. (A) The number of adenosine events per hour was significantly decreased after pilocarpine administration (paired *t*-test, n = 5 animals, $p = 0.011$). (B) Inter-event time distribution. The underlying distribution after pilocarpine was significantly different (K-S test, $p < 0.0001$). (C) The event concentration of adenosine was significantly different after pilocarpine (unpaired *t*-test, $p = 0.023$)

6.2.2 Detection of adenosine with FSCV and optogenetics

Chapter 3 tested the extent to which adenosine is formed from ATP metabolism by using CD73 and CD39 knockout mice. Deletion of CD73 and CD39 did not affect the concentration of either spontaneous or mechanically-stimulated adenosine release; therefore, there are other mechanisms that modulates the concentration of adenosine. Optogenetics can be combined with FSCV to study the release and formation of spontaneous and mechanically-stimulated adenosine. Optogenetics optically controls genetically targeted biological systems with millisecond temporal resolution and millimeter to micrometer spatial resolution.[44–46] Optogenetics manipulates the activity of individual neurons via turning on or off genetically-modified light sensitive ion channels,

where light sensitive microbial or animal opsins are expressed in target cells and can be activated by certain wavelengths of light.[47] Optogenetics is able to reversibly control the channels on the second time scale and has much higher spatial and temporal resolution than traditional drug administration methods, as drugs need time to transport and are distributed globally in the body to irreversibly manipulate the channels. Therefore, integration of FSCV with optogenetics is able to provide real-time, highly localized information under rapid-changing physiological conditions (Fig. 6.2A).

There are several ion channels related to the release of adenosine, such as pannexin channels, sodium channels, and calcium channels.[10,48–50] Thus, it is useful to modify these channels with optogenetic methods to investigate their roles in adenosine release. The changes of adenosine concentration and frequency can be monitored by FSCV after manipulating the ion permeability of channels by light. The pannexin channel is an ATP releasing channel[51], where turning on this channel is expected to result in a increase in extracellular concentration of ATP and thus would increase adenosine formed by extracellular ATP breakdown. If the rapid adenosine signaling is from breakdown of extracellular ATP, the concentration of rapid adenosine release will increase after turning on the pannexin channel (Fig. 6.2B). Another ion channel that is critical for adenosine release is sodium channels. Activation of Na^+-K^+ ATPase by Na^+ influx leads to the breakdown of intracellular ATP and results in more adenosine production, which can be released to the extracellular space through ENTs.[52] Administration of a sodium channel blocker, TTX, decreases electrically- and mechanically-stimulated adenosine release but does not affect spontaneous adenosine release in brain slices.[10,48,53] Future research could use optogenetics to open sodium channels in anesthetized or freely moving animals to

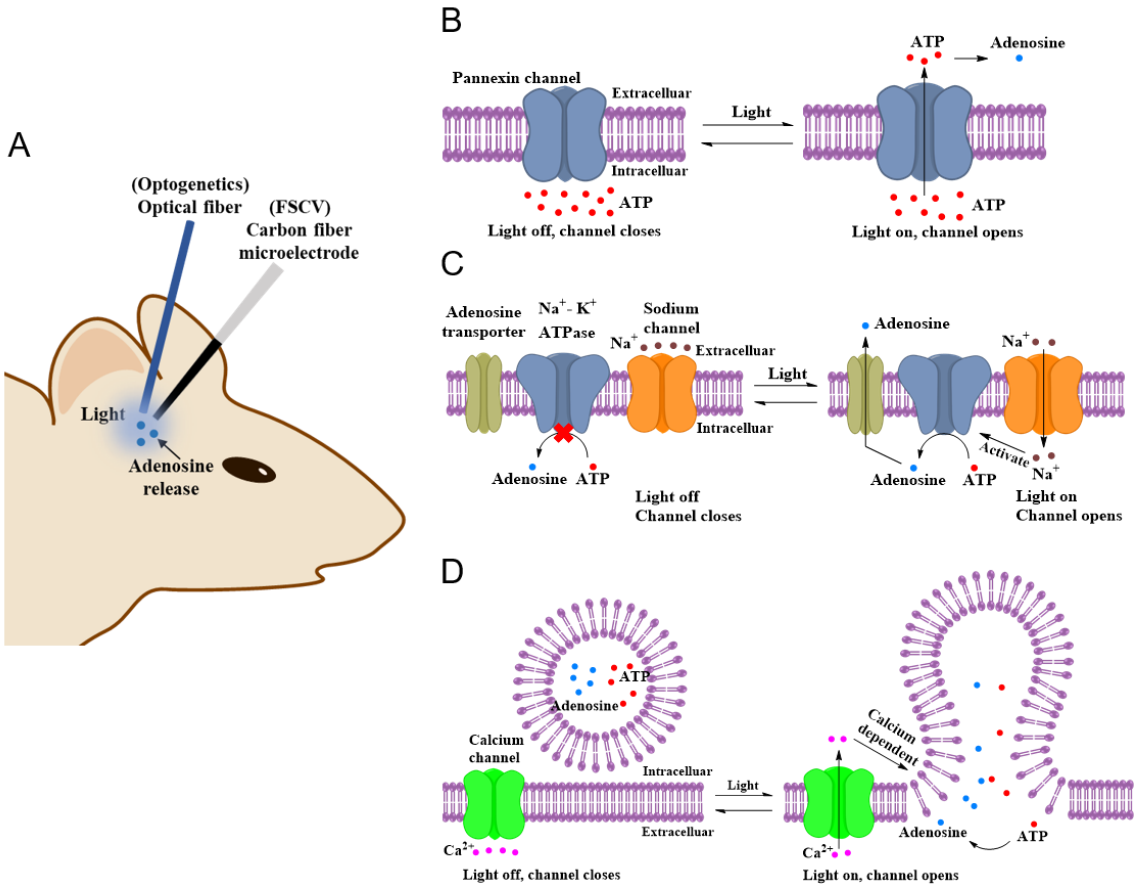

Figure 6.3 Proposed mechanisms of adenosine release manipulation by light-gated ion channels. (A) General scheme to combine optogenetics with FSCV. Optogenetics is used to modulate adenosine release and FSCV is applied for adenosine detection. (B) Pannexin channel is an ATP release channel. With optogenetically-controlled channels, the channel would open when the light is on, and that intracellular ATP would flow through the channel to extracellular space and hydrolyze to adenosine. The concentration of rapid adenosine release would increase if it is from breakdown of ATP. (C) Light-gated sodium channel is closed when light is off. When light is on, the channel opens to allow more Na^+ into the cell to further activate Na^+-K^+ ATPase, leading to hydrolysis of more intracellular ATP and production of more adenosine through breakdown of ATP, which can be released to extracellular places by ENTs. (D) Adenosine and ATP can also be released by exocytosis, which is calcium-dependent. Calcium channels are closed when the light is off. Opening the channel by light allows more Ca^{2+} into the cell, which facilitates exocytosis and causes more adenosine and ATP release.

further study the mechanism of adenosine formation during different behaviors (Fig. 6.2C). In addition, previous studies showed that adenosine release is calcium dependent. For example, chelating Ca^{2+} with EDTA decreases mechanically-stimulated adenosine

release.[10] Ca^{2+} influx causes the depolarization of cell membrane and exocytosis of ATP and adenosine.[49] Thus, opening light-gated calcium channel allows more calcium influx into the cell, which facilitates exocytosis and causes more adenosine and ATP release. Future research could investigate whether rapid adenosine signaling is from exocytosis by opening calcium channels and simultaneously inhibiting the breakdown of ATP (Fig. 6.2D).

6.2.3 Interaction of adenosine and dopamine

Adenosine is also known to modulate dopamine responses in the central nervous system. Exogenously applied adenosine and mechanically-stimulated adenosine transiently modulate electrically-stimulated dopamine release through A_1 receptors.[54] Dopamine is oxidized to dopamine-o-quinone at 0.6 V and reduced back to dopamine at approximately - 0.25V *vs* an Ag/AgCl reference electrode using FSCV (Fig. 6.3) A triangular waveform, -0.4 V to 1.45 V and back every 100 ms at 10Hz, can be used to quantitatively and simultaneously measure dopamine and adenosine release. Spontaneous adenosine and dopamine releases are found in the striatum in anesthetized animals using FSCV (Fig. 6.4). Thus, spontaneous adenosine and dopamine can be simultaneously detected *in vivo*.

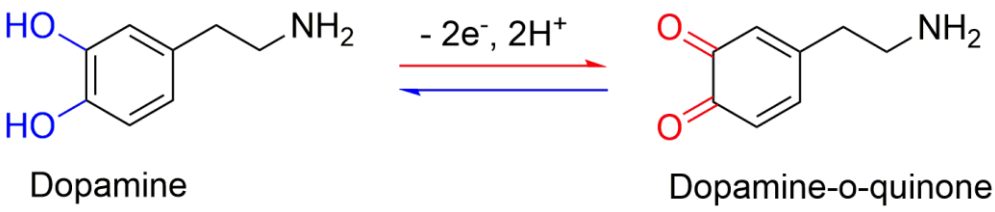

Figure 6.4 Oxidation and reduction of dopamine. Dopamine is oxidized to dopamine-o-quinone by losing two electrons and two protons. This process is reversible as dopamine-o-quinone can be reduced back to dopamine.

An automated algorithm has been built to identify rapid changes of adenosine signaling *in vivo*.[55] Adenosine is identified based on its two oxidation peaks, the current *vs.* time peak ratio as well as the time delay between two peaks. Rapid changes of dopamine and pH *in vivo* can be separated and quantified using principal component regression (PCR).[56] PCR is multivariate calibration methods that combines principal component analysis and least-squares regression, which identifies chemicals from a mixture and removes noise from data.[57,58] Dopamine can be processed using PCR to

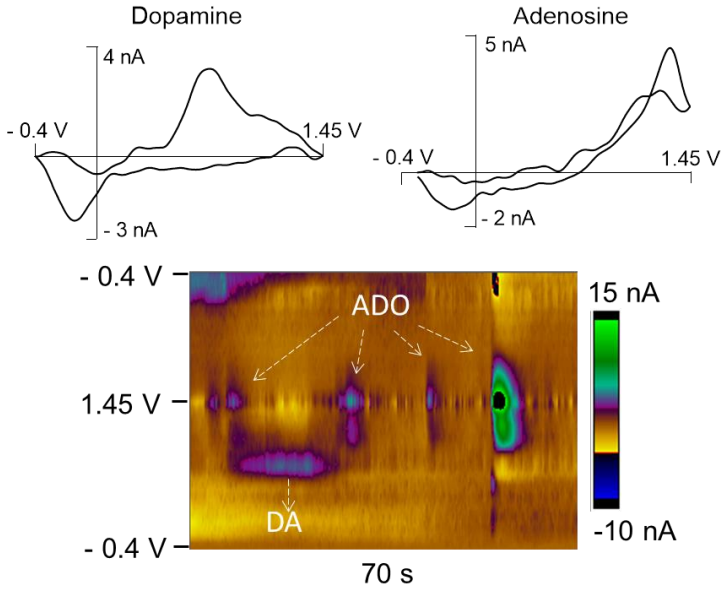

Figure 6.5 Spontaneous adenosine and dopamine release in the caudate-putamen *in vivo*. Adenosine and dopamine events are shown in a 70 seconds window of the 3-D color plot, the adenosine and dopamine events are marked. Cyclic voltammograms above the color plot show characteristics of dopamine and adenosine. The oxidation peak of dopamine occurs at about 0.65 V and reduction peak occurs at – 0.2 V. The primary oxidation of adenosine is at 1.3 V on the cathodic scan and secondary oxidation peak is at 1.2 V on the anodic scan.

separate from pH and noise, and then the automated algorithm could be used to pick the oxidation and reduction peaks based on the its characteristics. The combination of the automated algorithm and PCR for dopamine detection will reduce the time spent on data analysis and be able to automatically process both adenosine and dopamine data. Future

research could focus on the extent to which rapid adenosine signaling modulates transient dopamine release and how it influences dopamine-affected reward and relapse behavior, alcohol abuse, and drug addiction using A_1 and A_{2A} agonist and antagonist in freely moving animals or transgenic models.

Dopamine exerts its effects in the brain through postsynaptic D_1 and D_2 receptors that is involved in various psychostimulant-mediated effects, such as reward-related incentive learning[59], alcohol abuse[60], and drug addiction[61]. Adenosine A_1 and A_{2A} receptors are located presynaptically on glutamatergic nerve terminals to modulate glutamate release. Postsynaptically, A_1 receptors are co-expressed with dopamine D_1 receptors on neurons of direct pathway, where striatal cells project directly to globus pallidus (internal) to excite neuron activity. A_{2A} and D_2 receptors are co-expressed on neurons of indirect pathway, where striatal cells project to globus pallidus (external) through subthalamic nucleus to inhibit neuron activity.[62,63] Previous studies showed functional interactions between A_1 and D_1 and between A_{2A} and D_2. For example, stimulation of A_{2A} receptors leads to decreased affinity of D_2 agonist binding sites in the rat striatum.[64] Activation of A_1 receptors attenuated the high-affinity binding of dopamine to D_1 receptors.[65] Thus, adenosine A_1 and A_{2A} receptors can antagonistically modulate dopaminergic neurotransmission and therefore psychostimulant-mediated effects. The modulation role of adenosine receptors on dopamine has been studied on a slow time scale. However, how rapid adenosine signaling interacts with dopamine involved psychostimulant-mediated effects is not fully known.

Parkinson's disease (PD) is a neurodegenerative disorder that causes tremor, stiffness, and slow decreased movement.[66] Widespread dopamine deficiency is

associated with PD and inactivation of adenosine receptors using caffeine shows neuroprotection in an animal model of PD.[67] Future research could examine the interaction between spontaneous adenosine and dopamine signaling simultaneously in PD animals at different stages of PD and the effect of inactivating adenosine receptors on dopamine release. Deep brain stimulation is used to treat movement disorders, such as the tremor produced by PD patients, which is associated with a marked increase in the release of ATP and extracellular accumulation of adenosine.[68] Deactivation of A_1 receptors with the antagonist DPCPX (4 mg/kg, *i.p.*) increases the tremor, while intrathalamic infusion of A_1 receptor agonist CCPA (1.0 μM), directly reduces tremor.[68] Thus, adenosine may have potential therapeutic and neuroprotective effects on PD. However, the extent to which rapid adenosine plays a neuroprotective role is not known and future studies could examine the time course of adenosine modulation of dopamine. Future research could also explore the role of electrically and mechanically-stimulated adenosine release in PD.

6.3 Final Conclusions

In conclusion, the discovery of rapid adenosine signaling by FSCV is a paradigm shift for understanding the time course of adenosine. Real time monitoring of spontaneous adenosine release reveals its role as local metabolic cerebral vasodilator by transiently adjusting blood flow to the oxygen and metabolic requirements of the tissue on a second time scale. This local vasodilation is neuroprotective and more active in response to ischemia, even after the blockage of major arteries. To better manipulate the neuroprotective effects of spontaneous adenosine release, investigating regional variation of release and the mechanism of formation is essential to establish better strategies.

Adenosine agonist/antagonist and enzyme activator/inhibitor can be used to target specific regions to modulate the frequency and concentration of rapid adenosine signaling. Further research is required to resolve different pathways involved in adenosine generation and identify more functions of adenosine under various diseases, including sleep disorders, Parkinson's disease, and epilepsy, which will give valuable insights and lead to the identification of new therapeutic strategies for the treatment of adenosine-related diseases.

6.4 Reference

1. Löffler M, Morote-Garcia JC, Eltzschig SA, Coe IR, Eltzschig HK. Physiological roles of vascular nucleoside transporters. *Arterioscler Thromb Vasc Biol*. 2007;27(5):1004-1013. doi:10.1161/ATVBAHA.106.126714.

2. Anderson CM, Xiong W, Geiger JD, et al. Distribution of Equilibrative, Nitrobenzylthioinosine-Sensitive Nucleoside Transporters (ENT1) in Brain. *J Neurochem*. 2002;73(2):867-873. doi:10.1046/j.1471-4159.1999.0730867.x.

3. Anderson CM, Baldwin SA, Young JD, Cass CE, Parkinson FE. Distribution of mRNA encoding a nitrobenzylthioinosine-insensitive nucleoside transporter (ENT2) in rat brain. *Mol Brain Res*. 1999;70(2):293-297. doi:10.1016/S0169-328X(99)00164-3.

4. Hyde RJ, Cass CE, Young JD, Stephen A. Baldwin JD. The ENT family of eukaryote nucleoside and nucleobase transporters: recent advances in the investigation of structure/function relationships and the identification of novel isoforms. *Mol Membr Biol*. 2001;18(1):53-63. doi:10.1080/09687680118799.

5. Decking UK, Schlieper G, Kroll K, et al. Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. *Circ Res*. 1997;81(2):154-164. doi:10.1161/01.res.81.2.154.

6. Gray JH, Owen RP, Giacomini KM. The concentrative nucleoside transporter family, SLC28. *Pflugers Arch Eur J Physiol*. 2004;447(5):728-734. doi:10.1007/s00424-003-1107-y.

7. Lu H, Chen C, Klaassen C. Tissue distribution of concentrative and equilibrative nucleoside transporters in male and female rats and mice. *Drug Metab Dispos*. 2004;32(12):1455-1461. doi:10.1124/dmd.104.001123.

8. Basheer R, McKenna JT, McCarley RW. Pharmacology of Sleep: Adenosine. *Encycl Neurosci*. January 2009:601-610. doi:10.1016/B978-008045046-9.01764-2.

9. Nguyen MD, Ross AE, Ryals M, Lee ST, Venton BJ. Clearance of rapid adenosine release is regulated by nucleoside transporters and metabolism. *Pharmacol Res Perspect*. 2015;3(6). doi:10.1002/prp2.189.

10. Ross AE, Nguyen MD, Privman E, Venton BJ. Mechanical stimulation evokes rapid increases in extracellular adenosine concentration in the prefrontal cortex. *J Neurochem*. 2014;130(1):50-60. doi:10.1111/jnc.12711.

11. Genzen JR, Yang D, Ravid K, Bordey A. Cerebrospinal Fluid Research Activation of adenosine A2B receptors enhances ciliary beat frequency in mouse lateral ventricle ependymal cells. *Cerebrospinal Fluid Res*. 2009;6(6). doi:10.1186/1743-

8454-6-15.

12. Söllner T, Whiteheart SW, Brunner M, et al. SNAP receptors implicated in vesicle targeting and fusion. *Nature*. 1993;362(6418):318-324. doi:10.1038/362318a0.

13. Fernández-Chacón R, Südhof TC. GENETICS OF SYNAPTIC VESICLE FUNCTION: Toward the Complete Functional Anatomy of an Organelle. *Annu Rev Physiol*. 1999;61(1):753-776. doi:10.1146/annurev.physiol.61.1.753.

14. Kaeser PS, Regehr WG. Molecular mechanisms for synchronous, asynchronous, and spontaneous neurotransmitter release. *Annu Rev Physiol*. 2014;76:333-363. doi:10.1146/annurev-physiol-021113-170338.

15. Ganesana M, Venton BJ. Early changes in transient adenosine during cerebral ischemia and reperfusion injury. *PLoS One*. 2018;13(5):e0196932. doi:10.1371/journal.pone.0196932.

16. Lee ST. Electrochemical methods for determining the mechanism of spontaneous adenosine release (Unpublished doctoral dissertation). University of Virginia, Charlottesville, VA. 2018.

17. O'Sullivan GA, Mohammed N, Foran PG, Lawrence GW, Oliver Dolly J. Rescue of exocytosis in botulinum toxin A-poisoned chromaffin cells by expression of cleavage-resistant SNAP-25. Identification of the minimal essential C-terminal residues. *J Biol Chem*. 1999;274(52):36897-36904. doi:10.1074/JBC.274.52.36897.

18. Geisler JC, Corbin KL, Li Q, Feranchak AP, Nunemaker CS, Li C. Vesicular Nucleotide Transporter-Mediated ATP Release Regulates Insulin Secretion. *Endocrinology*. 2013;154(2):675-684. doi:10.1210/en.2012-1818.

19. Penuela S, Gehi R, Laird DW. The biochemistry and function of pannexin channels. *Biochim Biophys Acta - Biomembr*. 2013;1828(1):15-22. doi:10.1016/J.BBAMEM.2012.01.017.

20. Penuela S, Bhalla R, Gong X-Q, et al. Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins. *J Cell Sci*. 2007;120(Pt 21):3772-3783. doi:10.1242/jcs.009514.

21. Bao L, Locovei S, Dahl G. Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett*. 2004;572(1-3):65-68. doi:10.1016/j.febslet.2004.07.009.

22. Giaume C, Leybaert L, Naus CC, Sáez JC. Connexin and pannexin hemichannels in brain glial cells: properties, pharmacology, and roles. *Front Pharmacol*.

2013;4:88. doi:10.3389/fphar.2013.00088.

23. Rouach N, Avignone E, Même W, et al. Gap junctions and connexin expression in the normal and pathological central nervous system. *Biol Cell*. 2002;94(7-8):457-475. doi:10.1016/S0248-4900(02)00016-3.

24. Taruno A. ATP Release Channels. *Int J Mol Sci*. 2018;19(3). doi:10.3390/ijms19030808.

25. Kukulski F, Lévesque SA, Lavoie EG, et al. Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. *Purinergic Signal*. 2005;1(2):193-204. doi:10.1007/s11302-005-6217-x.

26. Zimmermann H. Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol*. 2000;362(4-5):299-309. doi:10.1007/s002100000309.

27. Robson SC, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal*. 2006;2(2):409-430. doi:10.1007/s11302-006-9003-5.

28. Langer D, Hammer K, Koszalka P, Schrader J, Robson S, Zimmermann H. Distribution of ectonucleotidases in the rodent brain revisited. *Cell Tissue Res*. 2008;334(2):199-217. doi:10.1007/s00441-008-0681-x.

29. Goding JW, Grobben B, Slegers H. Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. *Biochim Biophys Acta - Mol Basis Dis*. 2003;1638(1):1-19. doi:10.1016/S0925-4439(03)00058-9.

30. A. Demenis, Francisco A. Leone M, Leone FA. Kinetic Characteristics of ATP Hydrolysis by a Detergent-Solubilized Alkaline Phosphatase From Rat Osseous Plate. *IUBMB Life (International Union Biochem Mol Biol Life)*. 2000;49(2):113-119. doi:10.1080/15216540050022421.

31. Street SE, Kramer NJ, Walsh PL, et al. Tissue-nonspecific alkaline phosphatase acts redundantly with PAP and NT5E to generate adenosine in the dorsal spinal cord. *J Neurosci*. 2013;33(27):11314-11322. doi:10.1523/JNEUROSCI.0133-13.2013.

32. Zhang D, Xiong W, Chu S, Sun C, Albensi BC, Parkinson FE. Inhibition of Hippocampal Synaptic Activity by ATP, Hypoxia or Oxygen-Glucose Deprivation Does Not Require CD73. Barnes S, ed. *PLoS One*. 2012;7(6):e39772. doi:10.1371/journal.pone.0039772.

33. Zimmermann H. Prostatic acid phosphatase, a neglected ectonucleotidase. *Purinergic Signal*. 2009;5(3):273-275. doi:10.1007/s11302-009-9157-z.

34. Quintero IB, Araujo CL, Pulkka AE, et al. Prostatic acid phosphatase is not a prostate specific target. *Cancer Res*. 2007;67(14):6549-6554. doi:10.1158/0008-5472.CAN-07-1651.

35. Araujo CL, Quintero IB, Kipar A, et al. Prostatic acid phosphatase is the main acid phosphatase with 5=-ectonucleotidase activity in the male mouse saliva and regulates salivation. *Am J Physiol Cell Physiol*. 2014;306:1017-1027. doi:10.1152/ajpcell.00062.2014.

36. Huang Z-L, Urade Y, Hayaishi O. The role of adenosine in the regulation of sleep. *Curr Top Med Chem*. 2011;11(8):1047-1057.

37. Scorza FA, Arida RM, Naffah-Mazzacoratti M da G, Scerni DA, Calderazzo L, Cavalheiro EA. The pilocarpine model of epilepsy: what have we learned? *An Acad Bras Cienc*. 2009;81(3):345-365. doi:10.1590/S0001-37652009000300003.

38. Curia G, Longo D, Biagini G, Jones RSG, Avoli M. The pilocarpine model of temporal lobe epilepsy. *J Neurosci Methods*. 2008;172(2):143-157. doi:10.1016/j.jneumeth.2008.04.019.

39. During MJ, Spencer DD. Adenosine: A potential mediator of seizure arrest and postictal refractoriness. *Ann Neurol*. 1992;32(5):618-624. doi:10.1002/ana.410320504.

40. Boison D. Adenosine kinase, epilepsy and stroke: mechanisms and therapies. doi:10.1016/j.tips.2006.10.008.

41. Covolan L, de Almeida A-CG, Amorim B, et al. Effects of Anterior Thalamic Nucleus Deep Brain Stimulation in Chronic Epileptic Rats. Gross RE, ed. *PLoS One*. 2014;9(6):e97618. doi:10.1371/journal.pone.0097618.

42. Miranda MF, Hamani C, de Almeida A-CG, et al. Role of adenosine in the antiepileptic effects of deep brain stimulation. *Front Cell Neurosci*. 2014;8:312. doi:10.3389/fncel.2014.00312.

43. Anschel DJ, Ortega EL, Kraus AC, Fisher RS. Focally injected adenosine prevents seizures in the rat. *Exp Neurol*. 2004;190(2):544-547. doi:10.1016/J.EXPNEUROL.2004.07.017.

44. Rein ML, Deussing JM. The optogenetic (r)evolution. *Mol Genet Genomics*. 2012;287(2):95-109. doi:10.1007/s00438-011-0663-7.

45. Sun Q-Q, Wang X, Yang W. Laserspritzer: A Simple Method for Optogenetic

Investigation with Subcellular Resolutions. Martinez LM, ed. *PLoS One*. 2014;9(7):e101600. doi:10.1371/journal.pone.0101600.

46. Levskaya A, Weiner OD, Lim WA, Voigt CA. Spatiotemporal control of cell signalling using a light-switchable protein interaction. *Nature*. 2009;461(7266):997-1001. doi:10.1038/nature08446.

47. Pama EAC, Colzato LS, Hommel B. Optogenetics as a neuromodulation tool in cognitive neuroscience. *Front Psychol*. 2013;4:610. doi:10.3389/fpsyg.2013.00610.

48. Lee ST, Venton BJ. Regional Variations of Spontaneous, Transient Adenosine Release in Brain Slices. *ACS Chem Neurosci*. 2018;9(3):505-513. doi:10.1021/acschemneuro.7b00280.

49. Tatur S, Kreda S, Lazarowski E, Grygorczyk R. Calcium-dependent release of adenosine and uridine nucleotides from A549 cells. *Purinergic Signal*. 2008;4(2):139-146. doi:10.1007/s11302-007-9059-x.

50. Miteva AS, Gaydukov AE, Shestopalov VI, Balezina OP. The role of pannexin 1 in the purinergic regulation of synaptic transmission in mouse motor synapses. *Biochem (Moscow), Suppl Ser A Membr Cell Biol*. 2017;11(4):311-320. doi:10.1134/S1990747817040067.

51. Sandilos JK, Chiu Y-H, Chekeni FB, et al. Pannexin 1, an ATP Release Channel, Is Activated by Caspase Cleavage of Its Pore-associated C-terminal Autoinhibitory Region. *J Biol Chem*. 2012;287(14):11303-11311. doi:10.1074/jbc.M111.323378.

52. Sims RE, Dale N. Activity-Dependent Adenosine Release May Be Linked to Activation of Na+-K+ ATPase: An In Vitro Rat Study. Hetman M, ed. *PLoS One*. 2014;9(1):e87481. doi:10.1371/journal.pone.0087481.

53. Pajski ML, Venton BJ. The mechanism of electrically stimulated adenosine release varies by brain region. *Purinergic Signal*. 2013;9(2):167-174. doi:10.1007/s11302-012-9343-2.

54. Ross AE, Venton BJ. Adenosine transiently modulates stimulated dopamine release in the caudate-putamen via A1 receptors. *J Neurochem*. 2015;132(1):51-60. doi:10.1111/jnc.12946.

55. Borman RP, Wang Y, Nguyen MD, Ganesana M, Lee ST, Venton BJ. Automated Algorithm for Detection of Transient Adenosine Release. *ACS Chem Neurosci*. 2017;8(2):386-393. doi:10.1021/acschemneuro.6b00262.

56. Heien MLA V, Khan AS, Ariansen JL, et al. Real-time measurement of dopamine fluctuations after cocaine in the brain of behaving rats. *Proc Natl Acad Sci U S A*.

2005;102(29):10023-10028. doi:10.1073/pnas.0504657102.

57. Keithley RB, Heien ML, Wightman RM. Multivariate concentration determination using principal component regression with residual analysis. *Trends Analyt Chem*. 2009;28(9):1127-1136. doi:10.1016/j.trac.2009.07.002.

58. Keithley RB, Wightman RM. Assessing principal component regression prediction of neurochemicals detected with fast-scan cyclic voltammetry. *ACS Chem Neurosci*. 2011;2(9):514-525. doi:10.1021/cn200035u.

59. Beninger RJ, Miller R. Dopamine D1-like receptors and reward-related incentive learning. *Neurosci Biobehav Rev*. 1998;22(2):335-345.

60. Ruby CL, Adams CA, Knight EJ, Nam HW, Choi D-S. An essential role for adenosine signaling in alcohol abuse. *Curr Drug Abuse Rev*. 2010;3(3):163-174.

61. Filip M, Zaniewska M, Frankowska M, Wydra K, Fuxe K. The importance of the adenosine A(2A) receptor-dopamine D(2) receptor interaction in drug addiction. *Curr Med Chem*. 2012;19(3):317-355.

62. Svenningsson P, Le Moine C, Fisone G, Fredholm BB. Distribution, biochemistry and function of striatal adenosine A2A receptors. *Prog Neurobiol*. 1999;59(4):355-396. doi:10.1016/S0301-0082(99)00011-8.

63. Nambu A. A new dynamic model of the cortico-basal ganglia loop. In: *Progress in Brain Research*. Vol 143. ; 2004:461-466. doi:10.1016/S0079-6123(03)43043-4.

64. Ferre S, von Euler G, Johansson B, Fredholm BB, Fuxe K. Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proc Natl Acad Sci U S A*. 1991;88(16):7238-7241.

65. Ferré S, Torvinen M, Antoniou K, et al. Adenosine A1 receptor-mediated modulation of dopamine D1 receptors in stably cotransfected fibroblast cells. *J Biol Chem*. 1998;273(8):4718-4724.

66. Blesa J, Przedborski S. Parkinson's disease: animal models and dopaminergic cell vulnerability. *Front Neuroanat*. 2014;8:155. doi:10.3389/fnana.2014.00155.

67. Chen JF, Xu K, Petzer JP, et al. Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. *J Neurosci*. 2001;21(10):RC143.

68. Bekar L, Libionka W, Tian G-F, et al. Adenosine is crucial for deep brain stimulation–mediated attenuation of tremor. *Nat Med*. 2008;14(1):75-80. doi:10.1038/nm1693.