Determining the Role of Spontaneous Adenosine as a Neuromodulator using Fast-Scan Cyclic Voltammetry

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Abstract:

Adenosine is an important signaling molecule in the central nervous system that modulates neurotransmitter release and can provide neuroprotective effects. Fast-scan cyclic voltammetry (FSCV) is an electrochemical technique used with carbon fiber microelectrodes and is the primary method of measuring spontaneous adenosine release. Previous research has focused on the mechanisms of formation and release for spontaneous transient adenosine in males only. This thesis will investigate adenosine's role as a neuromodulator and its variation in males and females using FSCV to measure these events *in vivo*.

Adenosine is introduced in Chapter 1, covering formation and release, metabolism, receptor interactions, and function in the central nervous system. The techniques used to measure adenosine are also briefly covered with the main focus being on FSCV, due to its rapid time scale. Data analysis innovations are discussed for the processing of both adenosine and dopamine data. There is also extensive review of the state of current adenosine research on rapid adenosine signaling, sex differences, adenosine/dopamine interactions, as well as predictions of where the field is heading.

Chapter 2 investigates sex differences in spontaneous transient adenosine in the prefrontal cortex, caudate-putamen, and hippocampus as well

as during the four stages of estrous. These sex differences are characterized for the first time *in vivo* in the magnitude and frequency of transients, showing that not only are these differences present, but that they are heterogenous by brain region. When stage of estrous is examined, there is an increase in adenosine transient concentration during proestrus stage when progesterone and estradiol are elevated.

Chapters 3 and 4 investigates adenosine/dopamine signaling and characterized how these systems interact in real time. Chapter 3 describes simultaneous coincident release of adenosine and dopamine in the caudate-putamen. Dopamine release is determined to coincide with adenosine release 86% of the time, suggesting a common origin for about 1/3 of spontaneous adenosine transients. It is also observed that A_{2A} receptors play a large role in regulating this release by measuring these events in global A_1 and A_{2A} receptor knockout mice. Chapter 4 examines dopamine's ability to affect spontaneous adenosine release through the use of cocaine and haloperidol, drugs that restrict the uptake of dopamine and antagonize D_2 receptors, respectively. Increased dopamine activity from these drugs causes a 20% reduction in the frequency of spontaneous dopamine transients, which is contrary to the expected effect.

Overall, this thesis highlights the need for a wider range of studies into sex differences in adenosine signaling and expands knowledge of adenosine/dopamine interactions in the caudate. In understanding these

differences, better and more effective therapeutic treatments may be developed for diseases that currently have no good treatment options.

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Chapter 1
Introduction

Every thing must have a beginning … and that beginning must be linked to something that went before.
-Mary Shelley

1.1 Overview of adenosine

Adenosine is an endogenous purine nucleoside that is the main breakdown product of adenosine triphosphate (ATP) and is ubiquitous throughout the body. [1] In the brain, adenosine acts as a neuromodulator and neuroprotective agent, and is being investigated as a therapeutic avenue for several illnesses, such as Parkinson's disease, schizophrenia, and ischemia. [2] Adenosine has two main modes of signaling, one that works on the span of minutes to hours and one that works on the span of seconds. [3–6] In its neuromodulatory role, the slow mode of adenosine signaling is well studied and understood, while there has been much less effort to understand the rapid mode of adenosine signaling. Fast adenosine transients can be observed spontaneously, but may also be stimulated artificially. [7,8] To truly observe and characterize these adenosine transients and the effect that they have on the neurological microenvironment in real time, a rapid analytical detection method is required. Over the last decade, fast scan cyclic voltammetry (FSCV) with carbon fiber microelectrodes has been the primary method in characterizing these transients on a subsecond timescale with sufficient sensitivity and selectivity to make these observations effective and informative. Most studies have focused on formation and the mechanism of adenosine release, thus the specific role of neuromodulator of other neurochemicals and how sex might affect these roles are still unknown.

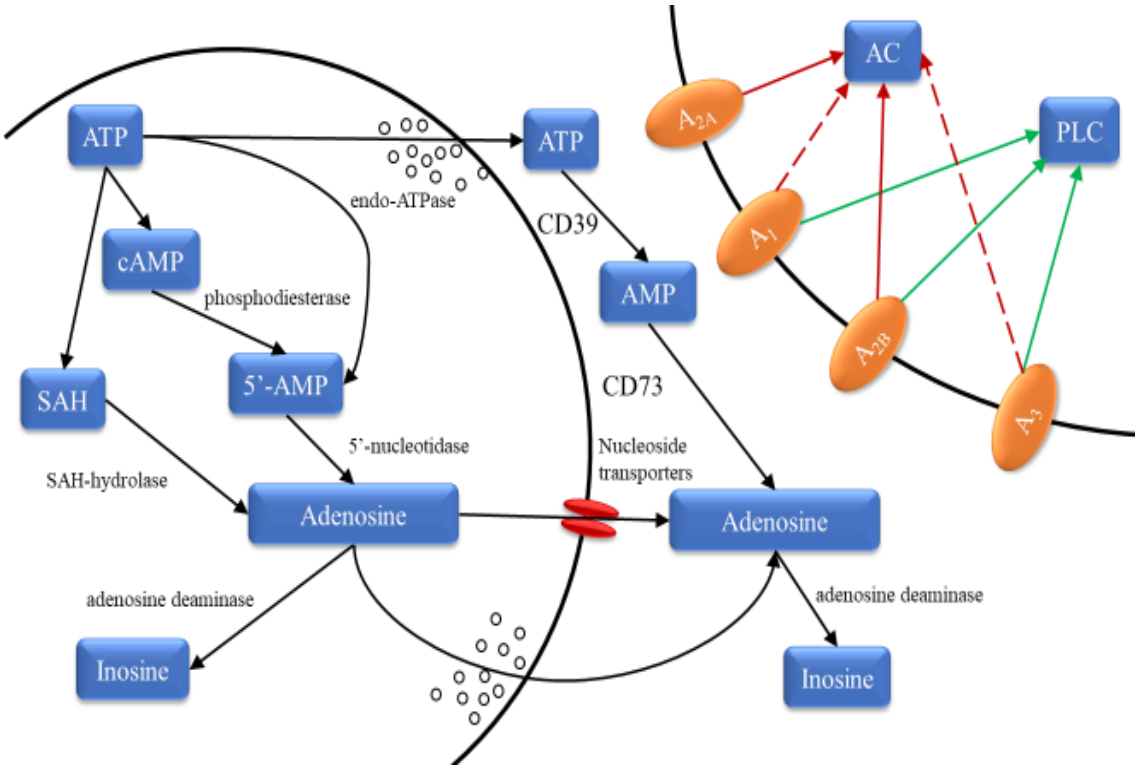

Figure 1.1 Metabolism of intra and extracellular adenosine with adenosine receptor signaling pathways. Adenosine is formed intra and extracellularly. Intracellularly, adenosine is formed from ATP either through the SAH or 5'-AMP pathways. Adenosine can then be transported to the extracellular space via nucleoside transporters, release via exocytosis, and release via other methods such as pannexin channels. Extracellularly, adenosine is formed from the breakdown of ATP. The extracellular adenosine then interacts with adenosine receptors in the membrane of other cells. A_1 and A_3 receptors inhibit the adenylyl cyclase (AC) pathway, while A_{2A} and A_{2B} receptors activate it. A_1, A_{2B} and A_3 receptors also activate the phospholipase C (PLC) pathways. ⟶ – activation ⇢ – inhibition

1.1.1 Adenosine formation and metabolism

There are two main classifications of adenosine formation, intracellular and extracellular. Intracellular adenosine formation is mainly due to the dephosphorylation of adenosine monophosphate (AMP) by cytosolic 5' nucleotidase. [1] The AMP itself is formed from the catabolism of intercellular ATP and adenosine diphosphate (ADP) from a variety of metabolic sources as shown in Figure 1.1. Adenosine is also formed from the hydration of S-

adenosylhomocysteine (SAH) through SAH-hydrolase, however the contribution of this pathway to overall adenosine volume is small. [9] Adenosine can be broken down to inosine by adenosine deaminase, phosphorylated into AMP by adenosine kinase or released into the extracellular space. In the extracellular space, adenosine is formed mainly from the breakdown of extracellular ATP by ecto-ATPase (CD39) and ecto-5'-nucleotidase (CD73). [10] The ATP is released into the extracellular space through exocytosis, pannexin and connexin channels, and through P2X7 activated channels and pores. [11] Adenosine is released synaptically in vesicles by Ca^{+2} dependent excitation[12] and through nucleoside transporters. In the extracellular space, adenosine is broken down to inosine by adenosine deaminase. Extracellular adenosine will spread by diffusion and interact with adenosine receptors imbedded in cell membranes to cause downstream effects specific to the type of receptors activated.

1.1.2 Adenosine receptors

Adenosine regulation in the body is controlled by four main G-protein coupled receptors, A_1, A_{2A}, A_{2B}, and A_3. A_1 and A_{2A} are the most highly expressed of these receptors in the brain, as well as the highest affinity for adenosine, with dissociation constants of 73 and 150 nM, [13] respectively. A_{2B} and A_3 have a much lower expression in the central nervous system and lower dissociation constants, with A_{2B} at 5100 nM [14] and A_3 at 6500 nM. [15] Functionally,

the A_1 and A_3 receptors have an inhibitory role on neurotransmission, including mediating inhibition of synaptic transmission. [16] The receptors block adenylyl cyclase activity through G proteins and inhibit neuronal firing by activating phospholipase C (PLC). [17] The A_{2A} and A_{2B} receptors, in contrast, are excitatory in neurotransmission, activating the adenylyl cyclase pathway and canceling A_1 receptor inhibition (Fig. 1.1). [18] To assist in the study of A_1 and A_{2A} receptors and their effects on adenosine signaling, genetically modified global knockout mice have been developed. [19,20] The pathways or secondary messengers are key to understanding the interconnectivity of neurotransmitter excitation and inhibition. In presynaptic neurons, adenosine receptors and dopamine receptors are often collocated, which allows adenosine release to have a high impact on dopamine release. [21] Membrane estrogen and androgen receptors are also G-protein coupled receptors that could be impacted by adenosine receptor secondary messengers. [22]

Neuromodulation by adenosine receptors has previously been observed for several neurochemicals. A_1 receptors regulate evoked adenosine transients through reduction in calcium influx and secondary messengers. [23] A_1 receptors inhibit serotonin release through calcium channels, and regulation of protein kinase C (PKC), protein kinase A (PKA), syntaxin and synaptobrevin, while A_{2A} receptors enhance serotonin through regulation of calcium channels, PKA, and synaptobrevin [24] and modulate A_1 receptor inhibition. A_{2A} receptors facilitate GABA release in the hippocampus through activation of adenylyl cyclase/cAMP

pathway and subsequently activation of PKA and PKC pathways, while A_1 receptors modulate GABA release by inhibition of the same pathways. [25] These studies demonstrate adenosine's established role as a neuromodulator, not just of synaptically released neurotransmitters due to regulation of calcium channels, but also of other neurochemical release through secondary messengers facilitated by coupling to inhibitory ($G_{i/o}$) and excitatory (G_s) G proteins.

1.1.3 Adenosine transporters

Membrane transport proteins, or transporters, are proteins that exist within and span the cell membrane. They facilitate the movement of molecules and ions through the cell membrane by either active transport or concentrative diffusion. Adenosine transporters, specifically, have two main classifications, equilibrative and concentrative. Equilibrative nucleoside transporters (ENTs) are energy dependent, bidirectional transporters. The release of adenosine by ENTs 1-4, with ENT 4 being adenosine specific, is also Ca^{2+} dependent. Concentrative nucleoside transporters (CNTs) are Na^+ dependent. CNTs 1-3 can transport adenosine, but only CNT 1 and 2 are primarily found in the brain. Adenosine release through pannexin hemi-channels is activated by P2X7 receptors. [26] Adenosine is also moved through the cell membrane by exocytosis. [12] Both adenosine transporters and metabolism control the concentration of adenosine in the extracellular space.

1.1.4 Adenosine in the central nervous system

In the central nervous system, adenosine serves two main functions: neuroprotection and neuromodulation. In its neuroprotective role, adenosine is closely tied to the inhibitory nature of A_1 receptors. [2] The receptors accomplish this protection presynaptically via the inhibition of excitatory responses and postsynaptically through cell hyperpolarization. [27–29] The ability to selectively enable or disable these responses has been a much-researched topic in pharmacology as an avenue to new therapeutic mechanisms. Adenosine protection during ischemic attack is one of its most widely researched functions, with research not only focusing on the role of A_1 receptors, but also how A_{2A} receptors can be utilized as a therapeutic avenue as well. Adenosine pretreatment reduced cell death and damage in brain slices following a hypoxic event, [30] while a high affinity A_1 receptor antagonist will remove the ischemic protection normally afforded by preconditioning. [31] By utilizing A_{2A} antagonist SCH58261, brain damage following an ischemic event was significantly reduced in rodents. [32] These findings support the role of adenosine in neuroprotection, but much of how it accomplishes this is still unknown.

In its neuromodulatory role, adenosine regulates many neurotransmitters, such as glutamate, serotonin, and dopamine. Adenosine and adenosine derivatives inhibit stimulated aspartate and glutamate release in rat hippocampal slices. [33] Adenosine also inhibits glutamate induced calcium channel influx and voltage gated calcium currents through A_1 receptor activation in retinal ganglion

cells. [34] Additionally, it inhibits glutamate reuptake in astrocytes and gliosomes through A_{2A} receptors. [35] Mechanical stimulation of adenosine modulates electrically stimulated dopamine in brain slices through A_1 receptors. [8] Mechanically stimulated adenosine also regulates serotonin levels in enterochromaffin cells through A_{2B} receptor activation of the PKA and cAMP signaling pathways. [36] By manipulating the glutamatergic system, adenosine therapeutics could mitigate the excitotoxicity of glutamatergic pathways. In serotonin systems, use of adenosine therapeutics could provide another avenue to alleviate depression and anxiety, or digestive disorders. By modulating the dopaminergic system, adenosine shows promise as a treatment for dopamine indicated illnesses, such as addiction, Parkinson's disease, and schizophrenia and provide alternatives to current pharmacology, which has only been marginally effective due to the effective half-life of directly manipulating the dopaminergic system. Adenosine is also the key molecule in vasodilation through binding with A_{2A} receptors. [37] In order to utilize adenosine for these applications however, there must be an understanding of the adenosine system and how external factors, such as hormones, affect that system. Additionally, the interactions between the adenosine system and the systems targeted for modulation must also be understood.

1.2 Electrochemical detection of adenosine *in vivo*

Adenosine was first measured in the brain using radiometric labeled adenosine with HPLC coupled to microdialysis and the technique is still utilized today. [38,39] The time scale of this method is minutes however, which makes it difficult to use this technique to determine fast changes in real time. In investigating adenosine's role in modulating glutamate and dopamine, microdialysis measurements were taken at 20 min intervals. [40] Therefore, it was necessary to develop a technique that was capable of operating on a much faster time scale and could directly measure the desired analytes. The main methods developed for electrochemical detection of adenosine to fulfill these requirements are fast scan cyclic voltammetry (FSCV) and enzyme-based biosensors using amperometry. [41,42] FSCV is well suited for rapid biological measurements and is effectively combined with electrophysiology and pharmacology.

1.2.1 Electrochemical detection of adenosine

FSCV is typically performed using a carbon fiber microelectrode (CFME) as the working electrode of a two-electrode voltammetry setup. A triangular waveform is applied to the CFME and the resulting current is measured. Dopamine was the most common analyte observed with this method for many years. For dopamine, with a formal oxidation potential of 0.3 V, this waveform begins at a holding potential of -0.4 V to adsorb as much of the positively

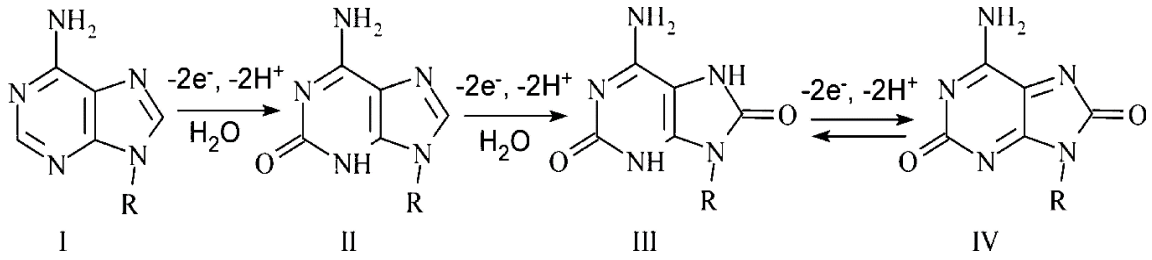

Figure 1.2 Electrochemical reaction of adenosine. Adenosine has three oxidation reactions, only two of which are normally observed with FSCV. Each oxidation results in the loss of two electrons. The first two steps are irreversible, while the last step is reversible though it is seldom observed *in vivo*. (Reprinted from Swamy and Venton, 2007)

charged analyte to the electrode surface as possible. It then scans to a switching potential of 1.3 V vs. a Ag/AgCl reference electrode to completely oxidize the 'adsorbed dopamine before scanning back to the holding potential at 400 V/s. [43] This high switching potential also serves to activate the electrode's carbon surface by adding oxide groups. [44] Adenosine undergoes a series of three oxidation reactions, which are shown in Figure 1.2. The primary oxidation occurs at about 1.3 V. The actual peak for the primary oxidation occurs on the descending scan due to slow molecular kinetics. This reaction is irreversible, so no reduction peak is observed. The secondary oxidation occurs at 1.0 V and will only occur after the first scan, when there is primary oxidation product present to produce it. The secondary peak is always smaller than the primary peak, usually 40% of the magnitude. [41] Due to adenosine having a higher primary oxidation potential (1.3 V) over dopamine, the waveform must scan to 1.45 V as the switching potential, while the other parameters remain the same as shown in Fig. 1.3 A. Adenosine detection using FSCV was first demonstrated in 2000 by the Brajter – Toth group, [45] however our group was the first to identify both the

primary and the secondary oxidation peaks. [41] It is also possible to detect dopamine with the adenosine waveform, since we scan through the voltage of dopamine oxidation. [41,46] These waveforms have an 8.5 ms scan time for the dopamine waveform and a 9.25 ms scan time for that of adenosine followed by an ~90 ms rest period, for a 10 Hz repetition rate (Fig. 1.3 A). This comparatively long rest time allows time for new analyte to adsorb to the electrode before the next subsequent pulse. FSCV is run at scan rates greater than 100 V/s, and the increase in scan rate also creates an increase in background current. The magnitude of the background current is 10-1000x larger than that of the faradaic

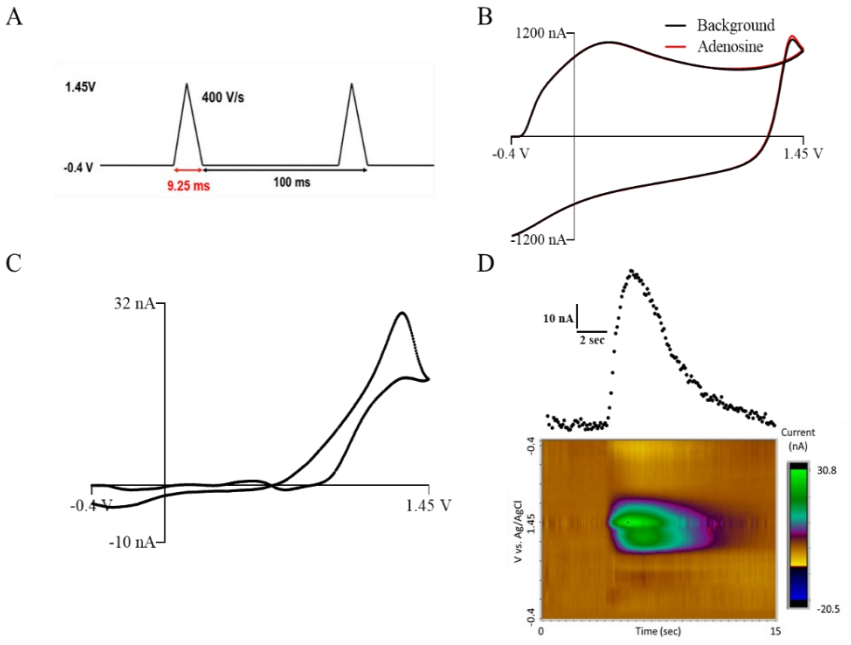

Figure 1.3 FSCV of adenosine. (A) Applied potential waveform for adenosine. The voltage is held at -0.4 V, ramped up to 1.45 V at a scan rate of 400 V/s and back to -0.4 V at a frequency of 10 Hz. (B) The waveform generates a large stable background which can then be subtracted to give (C) the characteristic CV of adenosine. This CV shows the primary oxidation at 1.3 V and secondary oxidation at 1.0 V. (D) 3D color plot of *in vivo* adenosine in a 15 sec window. Time is plotted on the x-axis, applied potential on the y-axis, current is displayed in false color.

current (Fig. 1.3 B) and very stable, which allows the background current to be subtracted from the signal and to more readily observe the faradaic current signal. Due to the background subtraction of FSCV, the technique is not useful for determining basal levels of analyte and only allows the measurement of changes in concentration. To compensate for this, there has been a technique developed, fast-scan controlled absorption voltammetry (FSCAV) that is capable of measuring basal levels of analytes using the same equipment as standard FSCV. [47]

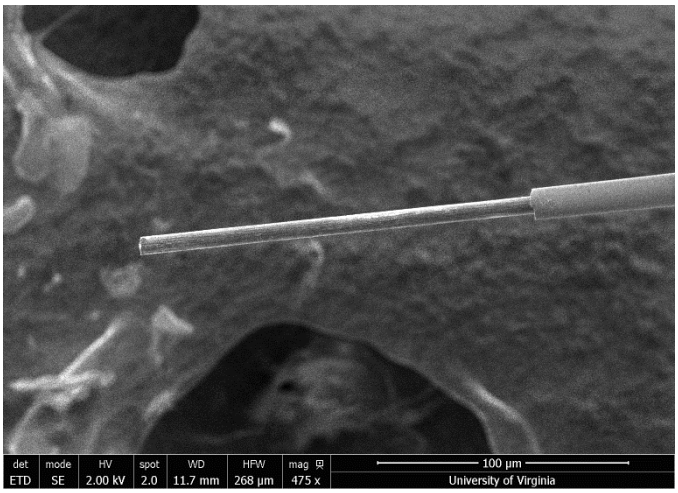

Figure 1.4 Scanning electron microscope image of a carbon fiber electrode. The carbon-fiber was vacuum aspirated into a glass capillary and pulled into two tapered electrodes (one shown). The fiber was cut ~ 120 µm from the end of the glass for a cylinder electrode.

The CFME can be a cylindrical or disk-shaped electrode made from carbon fiber aspirated through a glass capillary (Fig. 1.4). Though many experiments utilize epoxy to seal the electrode [6,48,49] or employ a coating such as Nafion [50,51] or carbon nanomaterials, [52] for spontaneous adenosine it is more advantageous to use bare carbon fiber. There have been many recent developments in materials for CFMEs in order to improve on the classic electrode, such as surface treatments for the electrode, of which there are two basic types. sp^2-Hybridized materials, such as carbon

nanotubes [53–55] and graphene, [56,57] can improve the surface area without increasing the footprint of the electrode and therefore increase sensitivity and adsorption. For longer experiments, sp^3-hybridized materials, such as boron doped diamond and nanodiamonds, [58–60] can be used as coatings to increase the antifouling properties of electrodes. The fiber we currently utilize is 7 μm in diameter and cut to approximately 150 μm in length. This small size is beneficial for biological experiments due to the limited cell damage that electrode implantation incurs and also allows for region specificity when used *in vivo* in the brain.

The faradaic current is recorded over time and with the applied voltage is used to construct a background subtracted cyclic voltammogram (CV). These CVs can be used as an electronic “fingerprint” to identify the analytes under study due to each electroactive molecule’s unique oxidation and reduction reactions. These peaks are shown in Figure 1.3 C. There is also a tertiary oxidation that can occur at 0.2 V, but this peak is not typically observed in FSCV *in vivo*, due to the low concentration. Many standard FSCV waveforms operate at 10 Hz, allowing for temporal resolution of less than 10 ms. FSCV is particularly effective in measuring spontaneous transient adenosine and other rapidly released neurotransmitters (*e.g.,* dopamine, serotonin) and neuromodulators due to its short time scale. Recent advances in electrode materials and fabrication may allow for even faster response times.

Another consideration that must be made when using FSCV, is that when measuring anything *in vivo*, there will be interferents and other chemicals that will be present in the environment besides the analytes of interest. Some of the interferents of note in the measurement of adenosine are pictured in Figure 1.5. Though ATP (Fig. 1.5B) and adenosine undergo the same redox reaction, ATP is not a concern due to its negative charge and the negative holding potential of -0.4 V. This is also the case for other adenine molecules such as AMP and ADP, since the negative charge prevents adsorption to the negatively charged

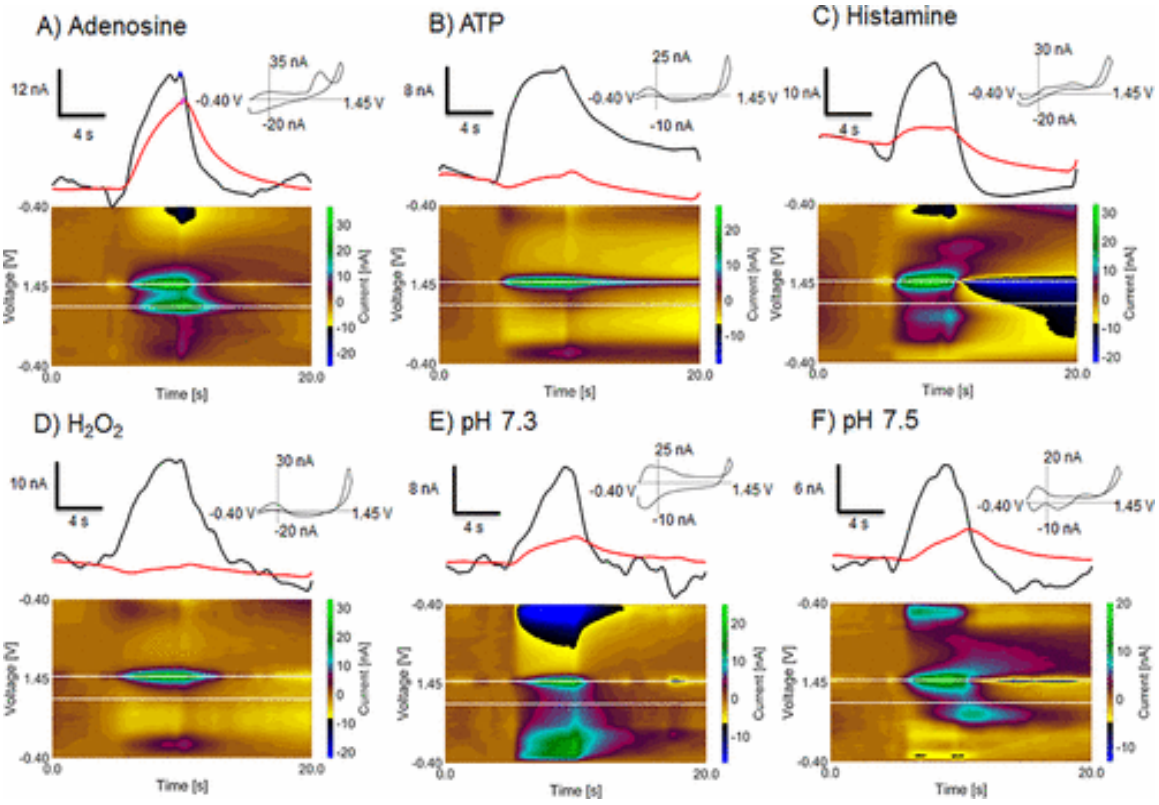

Figure 1.5 In vitro testing of biologically relevant interferents. Current vs time traces, cyclic voltammograms, and false color plots for (A) 1 µM adenosine, (B) 1 µM ATP, (C) 1 µM histamine, (D) 1 µM hydrogen peroxide, (E) pH 7.3 shift, (F) pH 7.5 shift. The black lines represent the current at ADO 1° peak potential, while the red lines are the current at the 2° peak potential. (Reprinted from Borman *et al*, 2017)

electrode. Inosine, which is a product of adenosine metabolism is another possible interferent, however it is not electroactive at the potentials utilized for adenosine detection and therefore not shown. Histamine, pH shifts and hydrogen peroxide can interfere with adenosine measurement as well, but are accounted for utilizing data analysis techniques.

Originally, these spontaneous adenosine transients were identified manually using principal component regression, from a training set of five transients selected by a human being. However, this process was very labor intensive and required around 18 hrs to identify all of the transients in one 4 hr data set. It was therefore necessary to design an algorithm that could be used to identify adenosine transients in these data files that would both recognize adenosine and discriminate it from possible interferents. [61] This method, known going forward as the “Borman Method”, utilized an algorithm that analyzed the data for a peak at adenosine’s primary oxidation potential. Once identified the algorithm would then look at the secondary peak potential and confirmed the existence of a peak and that it lagged the primary. The ratio of the height of the secondary peak to the primary peak was required to be between 0.49 and 0.89 or the peak would not be counted. The duration of the secondary peak must be greater than that of the primary, the transient must be between 0.6 – 15 s long, and have a signal to noise ratio of 3 or greater. By imposing these rules, the Borman Method was not only able to identify spontaneous adenosine transients, but also eliminate interference from histamine, hydrogen peroxide, and pH shifts.

Histamine (Fig. 1.5C), would not be selected because though there is a primary and secondary peak, the secondary peak occurs at 0.76 V instead of 1.06 V as seen in adenosine. As can be seen in Figure 1.5D, hydrogen peroxide does not have a secondary peak, and would be excluded by the selection criteria. Though pH changes (Fig, 1.5E & F) have both primary and secondary peaks, the peak height ratios are below the threshold for the selection criteria. This method was beneficial in reducing labor related data analysis time and in reducing interferents.

Though the Borman Method was effective at automating data analysis, reducing interferents, and increasing throughput, it did have its flaws. Since this method only utilized the current vs time traces at the primary oxidation peak potential and the secondary peak potential, it ignores a large portion of the data. The automated algorithm was also vulnerable to spurious electrical noise and background drift, so another data analysis method was developed. In order to utilize the full available data in the analysis, we in the Venton Lab employed an image recognition method called the structural similarity index method to select adenosine and dopamine transients. It accomplishes this by comparing the visual similarity of the input color plots to a preprogrammed library of example adenosine and dopamine transients, then records transients within a similarity threshold. Spurious electronic noise around the switching potential was addressed using a Savitzy-Golay smoothing filter to reduce false positives in identified peaks. [44] The program also employs a high-pass Butterworth filter, as

demonstrated in DeWaele *et al*., [62] to deal with background drift. This new analysis method further eliminates the possible interferents, keeps background and electronic noise from skewing the data, and allows us to focus solely on the analytes being measured.

1.2.2 Spontaneous transient adenosine

The first studies of adenosine *in vivo* utilized stimulations to evoke adenosine. The stimulation was either accomplished by applying electrical pulses to dopaminergic neurons to stimulate release, or by mechanical means by moving the working electrode in small, controlled increments and agitating the tissue. Through electrical stimulation, it was learned that A_1 receptors act as autoreceptors in the striatum, [23] and that the mechanism of adenosine release varied by brain region. [63] Mechanical stimulation experiments have also been productive showing that the evoked adenosine modulates dopamine release (Fig 1.8) [64] and that it is not correlated to spontaneous release. [65]

Stimulation methods were the standard for many years, until in 2011, when Wightman and Zylka discovered spontaneous transient adenosine in murine spinal cord slices. [66] The transients were irregular in frequency, appearing on average, once every 3 minutes. The average peak concentration was 530 nM with a duration of 1.5 seconds. The fascinating thing about these transients was that they occurred without the need for outside stimuli, allowing the experimenter

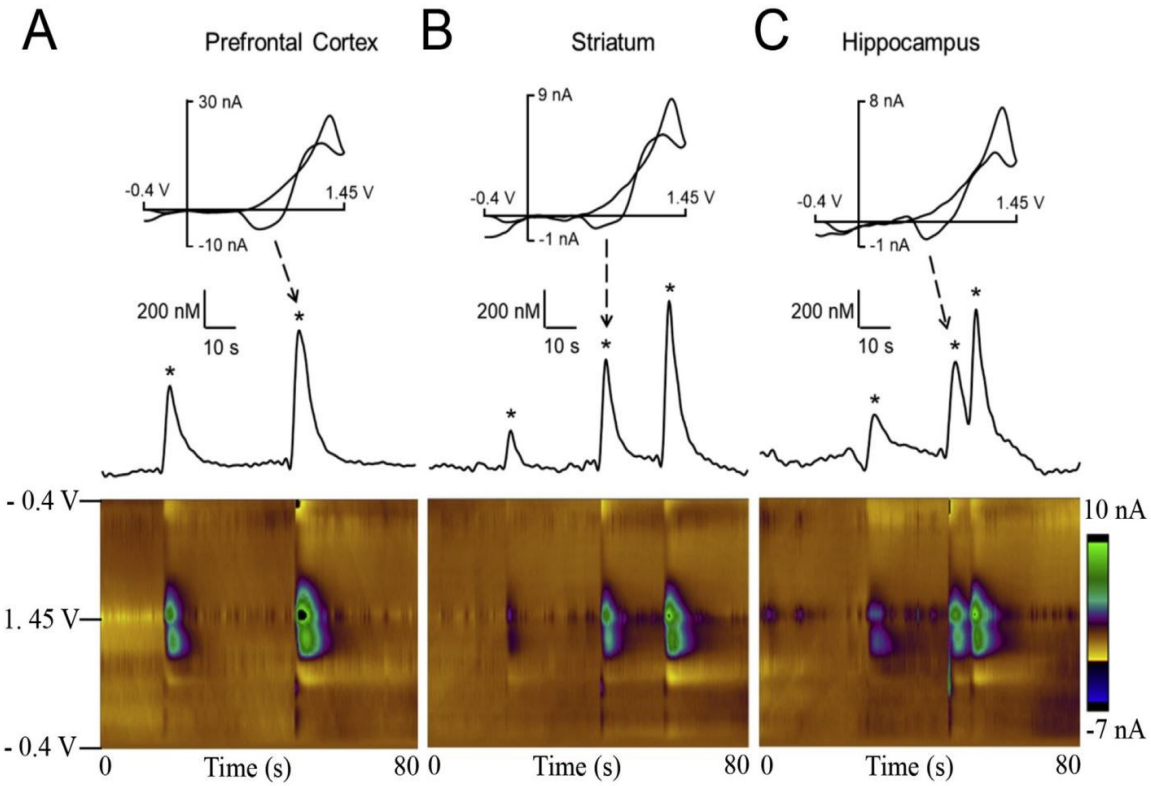

Figure 1.6 Spontaneous, transient adenosine release in various brain regions. Example release in the A) prefrontal cortex (PFC), B) striatum (STR), and C) hippocampus (HPC). Cyclic voltammograms of adenosine have the two characteristic oxidation peaks. Concentration vs. time traces derived from primary oxidation of adenosine (top); adenosine transients marked with stars. Example 3-D color plots show release events in an 80 s time window. Adenosine oxidation is the green/purple area in the middle of the plot. (Adapted from Wang & Venton, 2019)

to insert an electrode and observe this natural process. In our lab, we were concurrently investigating these spontaneous transients *in vivo* in the rat brain, in order to pursue the mechanisms of action that controlled these transients. [6] The Venton group initially observed these transients in the prefrontal cortex (PFC) and the caudate putamen. Figure 1.6 shows spontaneous adenosine transients *in* vivo in these two regions, as well as the hippocampus. [65] The PFC and caudate had similar average concentrations of spontaneous adenosine transients

at 0.19 ± 0.01 μM and 0.17 ± 0.01 μM, respectively. [6] The frequency of transients in the PFC was higher than those in the caudate. Modulation of spontaneous transient adenosine frequency was due to A_1 receptors, since A_1 antagonist DPCPX increased adenosine event frequency in both brain regions. There is an activity dependent element to adenosine release since adenosine events significantly decreased with Ca^{+2} removed. We have shown that although removal of the ATP to adenosine breakdown path via CD73 or CD39 KO reduces the frequency of spontaneous adenosine transients, it does not change the

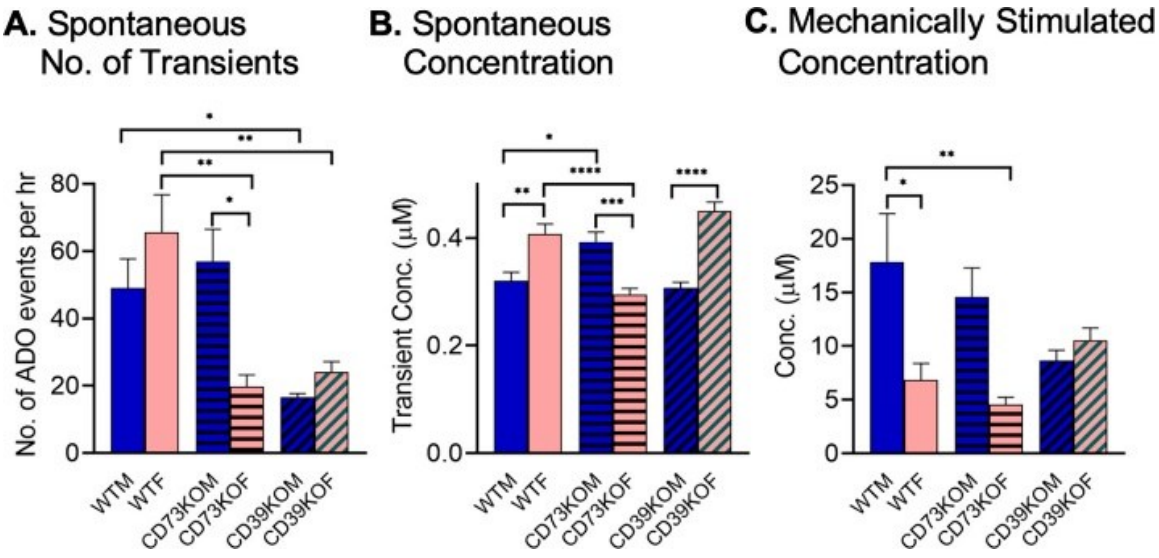

Figure 1.7 Sex differences in spontaneous and mechanically stimulated adenosine. (A) Number of spontaneous adenosine events per hour, broken down by sex in each genotype. There is a significant interaction of genotype and sex on number of adenosine events (two-way ANOVA, $n = 4$ animals/group, $F_{(2, 18)} = 7.9$, $p = 0.0034$). (B) Concentration of individual adenosine transients in each genotype. There is a significant interaction of genotype and sex on concentration of adenosine events (two-way ANOVA, $n = 200$ transients/group, $F_{(2, 1194)} = 32$, $p < 0.0001$). (C) Concentration of mechanically stimulated adenosine. There is a significant interaction of genotype and sex on concentration of mechanically stimulated adenosine (two-way ANOVA, $n = 4$ animals/group, $F_{(2, 18)} = 4.7$, $p = 0.023$). *$p < 0.05$ **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (Reprinted from Wang *et. al* 2020)

concentration. [67] This compensation is due to the upregulation of tissue non-specific alkaline phosphatase (TNAP) in the male mice; however, the compensation was not observed in females (Fig. 1.7). These differences illustrate the importance of planning studies to look at sex differences, as no previous CD73 studies had included female mice. The disparity between male and female reactions to the loss of the typical ATP breakdown pathway also implies that there is also another mechanism by which these transients may occur.

1.2.3 Sex differences

Adenosine based therapeutics have been proposed for the treatment of several neurological conditions, [68] such as ischemia, [69] schizophrenia, [70] and Parkinson's disease, [71] all of which have demonstrable sex differences in onset and severity. And yet, there is a woeful lack of published material on sex differences in adenosine. This is well illustrated by a comprehensive review of adenosine, published in 2005, that has been highly cited. Though the review is quite extensive and cites over 500 papers on adenosine research, it bears no mention of sex differences at all and the word "female" is only used twice in reference to a single paper dealing with the differences in ethanol sensitivity and consumption between A_{2A} KO mice. [2,72] This gap in our understanding of adenosine signaling can only be filled by adding female subjects to studies of the adenosine system, but fortunately, some work has been done since the 2005

review to this end. A_1 receptor regulation of heart rate and body temperature differ between male and female mice. Female mice had higher heart rate, locomotion, and body temperature than males regardless of time of day. [73] A_{2A}/A_1 receptor antagonists reduce the motivation to self-administer cocaine in rats and A_1 and A_{2A} receptors can regulate ADHD learning deficit severity, which varies by sex. [74,75] Males have been found to have reduced adenosine deaminase activity compared to females following a stroke. [76] Though some receptor-based studies have had sex differences included or been their focus, until recently none had dealt with adenosine signaling directly. This is of particular concern, as much of the interest in adenosine research is pointed at adenosine's use as a therapeutic for many diseases and disorders, which are known to display differences in both incidence and severity between sexes. [70,71,76]

1.2.4 Adenosine-dopamine interactions

Differences between the sexes is not the only remaining unknown aspect of adenosine signaling. Adenosine's role as a neuromodulator, as previously discussed, is of much current interest and study. The role that adenosine plays in the dopaminergic system is of interest due to the desire to use adenosine as a dopaminergic therapeutic avenue. For many diseases of the dopamine system, treatments that directly manipulate dopamine are only temporarily effective or do not actually alleviate the root issue. [77–79] For this reason, many have turned to the

adenosine system as an alternative since manipulating the adenosine system does not seem to suffer the same effects as manipulating the dopamine system directly.[80] To that end, the relationship between the adenosine and dopamine systems needs to be thoroughly investigated, in order to better understand the function and interaction of these two systems.

Dopamine is the most studied neurotransmitter in the world and is investigated in addiction, [81,82] depression, [83,84] Parkinson's disease, [85,86] and schizophrenia. [48,87] Dopamine regulates cognitive function, maternal and reproductive behaviors, motivation, motor control, and reward. [88] Dopamine has three modes of release, tonic, phasic without burst-fire, and phasic with burst-fire. [48] Phasic dopamine release due to burst firing occurs in response to addiction cues or food rewards.[89,90] Spontaneous, phasic dopamine release has been observed,[91] although the frequency is generally low and heterogeneous in the brain. [92] However, these studies only observed dopamine and have not explored interaction with other neurotransmitters or neuromodulators.

Adenosine and dopamine receptors are co-located and adenosine can modulate dopamine release in the brain, which makes it important to understand how both systems will respond when studying either one.[93,94] Adenosine/dopamine receptor pairs work antagonistically, allowing adenosine to control the level of dopamine through activation of adenosine receptors. [40] These receptors, specifically A_1-D_1 and A_{2A}-D_2 receptor pairings in the brain, [95,96] cause

allosteric changes in the receptor complex that alter affinity and G-protein coupling in the connected receptor. [97] In the striatum, A_1 receptors are expressed on GABAergic neurons with both D_1 and D_2 receptors, and A_{2A} receptors expressed on neurons with D_2 receptors.[93] While these receptor interactions are known, it is not known how they affect adenosine and dopamine release in real time. Previously, our lab has measured adenosine and dopamine simultaneously in order to understand how adenosine regulates dopamine *in vitro* [41] and *in vivo* following electrical stimulation. [98] Adenosine was artificially introduced prior to electrical stimulation of dopamine with the results compared to stimulation without adenosine (Fig. 1.8a), which reduced stimulated dopamine response. The effect of this adenosine addition was also time dependent, decreasing steadily over the course of a minute (Fig. 1.8b),

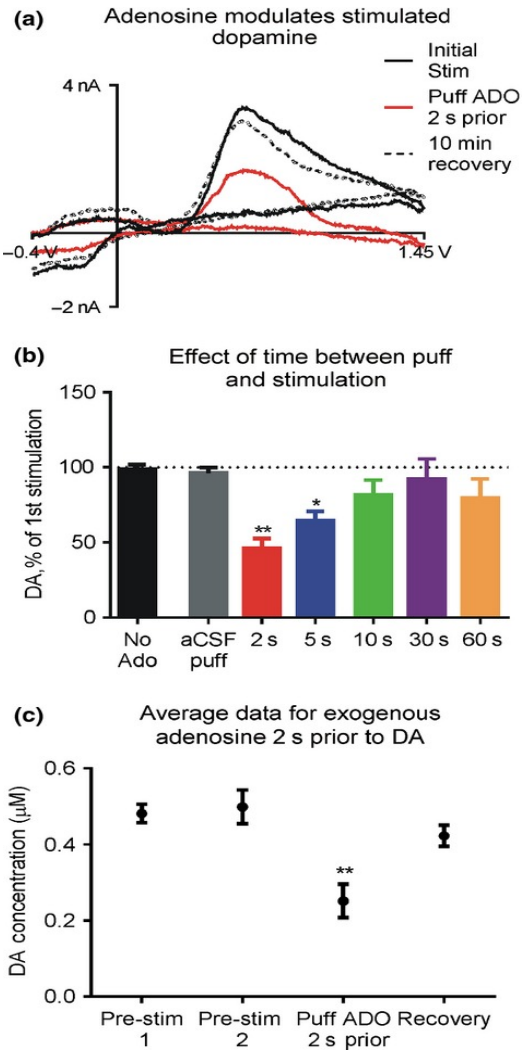

Figure 1.8 Effect of time interval between exogenous application and dopamine stimulation. (a) Example of the optimized procedure, the black trace indicates the initial dopamine stimulation, red trace is the dopamine 2 s after adenosine application, and the dotted trace shows dopamine on the subsequent stimulation (10 min later). (b) Effect of time between adenosine application and stimulation on dopamine inhibition. (c) The average pre-adenosine stimulations, the dopamine stimulation 2 s after adenosine application, and recovery is shown. $*p < 0.05$ $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. (Reprinted from Ross *et. al* 2015)

and modulated through A_1 receptors. [64] This finding prompted further investigation into the adenosine/dopamine interaction *in vivo* in real time. Manipulation of the dopaminergic system *in vivo* using pharmaceuticals did not affect stimulated adenosine transients. [7,23] However, no studies have measured spontaneous adenosine and dopamine transients simultaneously to discover how adenosine affects dopamine release on a rapid time scale. The logical next step is to take these measurements *in vivo* and observe the natural process of how adenosine and dopamine release affect each other in real time.

1.3 Introduction to the thesis

Adenosine signaling is complex and tightly interwoven with the signaling of other neurochemicals due to its ubiquity. Because of this, adenosine is an attractive avenue for the treatment of neurological conditions and disorders that still lack effective or long-term treatments options. In order to develop these treatments however, real time adenosine signaling *in vivo* must be better understood. Rapid detection methods like FSCV have been instrumental in observations in neurotransmitter signaling on a subsecond time scale. Though *in situ* work has laid the foundations of our understanding and still has effective uses, for us to truly further the field, it is necessary to make measurements and observations *in vivo* in real time.

In this thesis, I start by investigating the role of sex differences in the characterization of spontaneous adenosine transients in Sprague-Dawley rats and demonstrate that because of the complexity of the adenosine system, these differences must be taken account in adenosine studies going forward (Chapter 2). I then move into the investigation of adenosine's role as a neuromodulator of dopamine. I observed the effect of spontaneous adenosine transients on the spontaneous release of dopamine in the caudate putamen of mice. I found that spontaneous dopamine release is correlated to adenosine release (Chapter 3). Chapter 4 further illuminates the findings of the previous chapter by observing the results of adding dopamine pharmacology, specifically cocaine and haloperidol, to the system. This resulted in the surprising finding that cocaine/haloperidol induced dopamine increases reduced the frequency of spontaneous adenosine transients. In Chapter 5, I will reflect on the future of the field and propose further experiments for filling in the gaps of knowledge in the field that still remain.

In conclusion, this thesis pushes forward the edges of our understanding of rapid adenosine release. By expanding our knowledge of the interactions of adenosine signaling in the brain, it paves the way for more effective and individualized adenosine-based treatments of diseases, such as Parkinson's disease and schizophrenia.

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Chapter 2

Complex Sex and Estrous Cycle Differences in Spontaneous Transient Adenosine

"What you learn from a life in science is the vastness of our ignorance."
– David Eagleman

Chapter 2: Complex Sex and Estrous Cycle Differences in Spontaneous Transient Adenosine

Abstract

Adenosine is a ubiquitous neuromodulator that plays a role in sleep, vasodilation, and immune response and manipulating the adenosine system could be therapeutic for Parkinson's disease or ischemic stroke. Spontaneous transient adenosine release provides rapid neuromodulation; however, little is known about the effect of sex as a biological variable on adenosine signaling and this is vital information for designing therapeutics. Here, we investigate sex differences in spontaneous, transient adenosine release using fast-scan cyclic voltammetry to measure adenosine *in vivo* in the hippocampus CA1, basolateral amygdala, and prefrontal cortex. The frequency and concentration of transient adenosine release were compared by sex and brain region, and in females, stage of estrous. Females had larger concentration transients in the hippocampus (0.161 ±0.003 μM) and the amygdala (0.182 ±0.006 μM) than males (hippocampus: 0.134 ±0.003, amygdala: 0.115 ±0.002 μM), but males had a higher frequency of events. In the prefrontal cortex, the trends were reversed. Males had higher concentrations (0.189 ±0.003 μM) than females (0.170 ±0.002 μM), but females had higher frequencies. Examining stages of the estrous cycle, in the hippocampus, adenosine transients are higher concentration during proestrus and diestrus. In the cortex, adenosine transients were higher in concentration during proestrus, but were lower during all other stages. Thus, sex and estrous cycle differences in spontaneous adenosine are complex, and not completely consistent from region to region. Understanding these complex differences in spontaneous adenosine between the sexes

and during different stages of estrous is important for designing effective treatments manipulating adenosine as a neuromodulator.

2.1 Introduction

Adenosine is an endogenous purine nucleoside that plays roles in sleep, vasodilation, and immune response. [1,2] In the brain, adenosine builds up in the extracellular space after the breakdown of ATP, but is also directly released through synaptic vesicles and membrane transporters. [3–5] Adenosine builds up on the timescale of minutes during stressful events in the brain; [6] for example, acting in a neuroprotective role during ischemia. [7] Adenosine also signals on a rapid timescale, lasting a few seconds. [8–11] The faster mode of adenosine transiently modulates phasic dopamine [12] and blood flow, which is correlated to oxygen release. [13,14] Adenosine based therapeutics have been proposed as treatments for several neurological diseases, [15] and many of these diseases where adenosine could act as neuroprotective agents have sex differences. For example, ischemia occurs less frequently in women until the age of 80, [16] schizophrenics have sex differences in onset, severity of symptoms, and response to therapeutics, [17] and Parkinson's disease is twice as prevalent in men than women, as estrogen has a protective effect on striatal dopamine loss in females. [18] Rapid adenosine might be important in providing neuromodulation in these diseases, and for schizophrenia and Parkinson disease, could provide a non-dopaminergic drug target, which reduces the development of tolerance to the treatment. [19] However, almost all adenosine research in the brain has been performed in males, so there is little to no information about how adenosine signaling varies with regard to sex as a biological variable or during the female estrous cycle.

A few studies have examined sex differences regulated by adenosine, typically focusing on differences in adenosine receptors. In mice, there are sex differences in the

regulation of heart rate, body temperature, and locomotor activity caused by differences in adenosine A_1 receptor expression. [20] Additionally, A_1 and A_{2A} receptors regulate the level of severity of learning deficits that accompany attention-deficit hyperactivity disorder, and those deficits vary between the sexes. [21] Adenosine has also been implicated in the differences in cocaine addiction between males and females, with an A_{2A} antagonist having greater effects on motivation in females. [22] There are sex differences in the metabolic enzyme adenosine deaminase after stroke, with males having reduced adenosine deaminase activity compared to females, which may provide males with more neuroprotection because less adenosine is broken down. [23] These studies suggest there are sex differences in the adenosine system but no studies have monitored adenosine levels as a function of biological sex.

Sex differences are found in both regional brain structure and neurochemistry that result in behavioral changes. [24–26] In the prefrontal cortex, sex differences are found in microglial colonization, [26] corticolimbic dopamine and serotonin systems, [27] stress-induced dysfunction, [28] and cognitive function. [29] In the hippocampus, sex differences have been observed in the effects of stress on dendritic spine density [30] and the expression of serotonin receptors. [31] In the basolateral amygdala, there are sex differences in extracellular concentrations of dopamine and serotonin. [32] Given that many sex differences have been observed for neurotransmitters and neuromodulators, we examined sex differences in adenosine in these three regions.

In this work, we explored sex differences in spontaneous, transient adenosine release *in vivo* for the first time. The concentration and frequency of transient adenosine release events was compared in the CA1 region of the hippocampus, the medial

prefrontal cortex, and the basolateral amygdala. There were significant differences in both transient concentration and frequency between the sexes in all three regions, with females having larger, less frequent transients in the hippocampus and amygdala, and males having larger, less frequent transients in the prefrontal cortex. We then explored the effect of estrous cycle in the hippocampus and prefrontal cortex, finding concentration and frequency of spontaneous adenosine transients vary with changes in stage of estrous. In the hippocampus, adenosine concentrations are highest during proestrus, when estrogen levels peak. These results reveal complex sex and estrous cycle differences in the frequency and concentration of spontaneous adenosine release which should be considered in developing treatments targeting the adenosine system.

2.2 Materials & Methods

2.2.1 Materials

All reagents were purchased from Fisher Scientific (Fair Lawn, NJ, USA) unless otherwise noted. Phosphate buffered saline was adjusted to pH 7.4 and was made with sodium chloride (NaCl, S271-3, 131.25 mM), sodium phosphate from Sigma Aldrich (St. Louis, MO, USA) (NaH_2PO_4, monohydrate, S9683-250G, 10.0 mM), potassium chloride (KCl, P217-500, 3.0 mM), sodium sulfate (Na_2SO_4, anhydrous, S421-1, 2.0 mM), calcium chloride, ($CaCl_2$, dihydrate, Sigma Aldrich, 223506-500G, 1.2 mM), magnesium chloride ($MgCl_2$, hexahydrate, M33-500, 1.2 mM).

2.2.2 Electrodes and fast-scan cyclic voltammetry

Carbon-fiber microelectrodes were prepared as previously described. [33] In brief, a carbon fiber (T-650, Cytec Engineering Materials, West Patterson, NJ, USA) of 7 μm in

diameter was aspirated into a glass capillary (1.2 x 0.68 mm) and pulled using a vertical pipette puller (model PE-21; Narishige, Tokyo, Japan) into two electrodes. The protruding carbon fiber was cut to 100–125 µm with a scalpel. Electrical connection was made by backfilling the capillary with 1 M KCl. The silver-silver chloride reference electrodes were made in house by electrodepositing chloride onto a silver wire (7440-22-4, Acros Organics, New Jersey, USA). Fast-scan cyclic voltammetry (FSCV) was used to detect and quantitate adenosine on a subsecond time scale. [34,35] The FSCV waveform was applied and data were collected through computer controlled HDCV software (University of North Carolina, Chapel Hill, NC, USA). A Dagan ChemClamp potentiostat (Dagan Corporation, Minneapolis, MN, USA) was used to apply the potential in conjunction with a Pine Research WaveNeuro headstage (AB01HS2-P, Pine Research

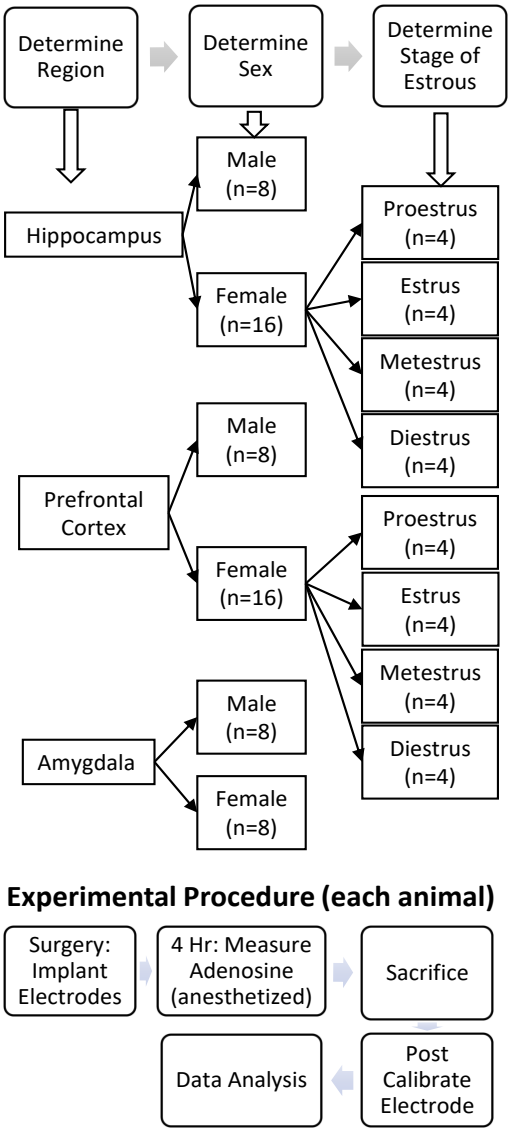

Figure 2.1. Flow chart of experimental procedure and groups for animal experiments. The experimenter decided the region that would be studied and the sex of the animal was recorded. If the animal was female, stage of estrous was determined by vaginal swab after the animal had been anesthetized. Surgery was performed under urethane anesthesia and the electrodes implanted. Spontaneous adenosine was measured for 4 hr. under anesthesia in the selected brain region. The animal was sacrificed by decapitation and a post experiment calibration of the electrode performed. The data were analyzed by software to find the adenosine transients and then statistical analysis performed.

Instrumentation, Durham, NC, USA). The applied waveform was from –0.40 V to 1.45 V and back at 400 V/s, for every 100 milliseconds against Ag/AgCl reference. Applying the waveform produces a large background current, thus data were background subtracted (10 cyclic voltammograms averaged) to remove non-Faradaic currents. Electrodes were post-calibrated with 1.0 μM adenosine in phosphate buffered saline immediately following animal experiments and the currents were used to estimate the concentrations of adenosine *in vivo*.

2.2.3 Animal experiments

All animal experiments were conducted in accordance with protocol number 3517-10-17 approved by the Institutional Animal Care and Use Committee of the University of Virginia. A flowchart of the general animal experiment workflow can be seen in Figure 2.1. Animal welfare was monitored daily by animal care staff. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) between 250–425 grams and female Sprague-Dawley rats (RRID: RGD_734476 Charles River Laboratories, Wilmington, MA, USA) between 225-375 grams were housed in 12/12-hour light/dark cycles, fed *ad libitum*, and provided environmental enrichment. A power analysis was conducted to calculate acceptable sample size. In this determination, it was assumed that there was a confidence level of 95% and that minimum observable difference would be 20%. When calculated using a comparison of means with the cortex data, it determined that for a mean concentration of 0.244 μM with a standard deviation of 0.310, a minimum of 1055 transients would have to be observed, which equated to about 4 rats. The data presented are from 8 males per brain region and 8 females in the amygdala and 16 females from both the cortex and the hippocampus. Surgeries were

performed in the morning, during the beginning of the light cycle. Estrous cycle determination was made by vaginal swab with sterile calcium alginate tipped applicators moistened with saline. Swabs were taken in the morning during the beginning of the light cycle, smeared on glass slides, and examined under light microscope in accordance with the Organization for Economic Cooperation and Development Histopath guidance, Part 5. [36] No randomization was performed to allocate subjects in the study. Animals were assigned to different experimental groups by the experimenter without any blinding procedure. Study was not pre-registered.

Experiments were performed while animals were anesthetized to minimize impact to the animals. Urethane is a commonly used anesthesia for non-survival voltammetry surgery and fits well with our experimental timeline. Prior to the anesthetic injection with urethane (1.5 g/kg, i. p.), rats were initially anesthetized with isoflurane (1 mL/100 g rat weight). Surgical areas were exposed by shaving around the surgical sites. The rat was then placed in a stereotaxic frame and 250 µL of bupivacaine (Sensorcaine, MPF, APP Pharmaceuticals, LLC; Schaumburg, IL, USA) was injected subcutaneously at the top of the skull prior to incision to ensure no pain was inflicted. Holes were drilled in the skull for the placement of both working and reference electrodes. [37] The working carbon-fiber microelectrode was placed in the CA1 region of the hippocampus (in mm from bregma): AP: -2.5, ML: +2.0, DV: –3.0, the prefrontal cortex (in mm from bregma): AP: +2.7, ML: +0.8, DV: –3.0, or the basolateral amygdala (in mm from bregma): AP: -2.8, ML: +4.5, DV: –8.2. The Ag/AgCl reference electrode was placed on the contralateral side. The rat's body temperature was regulated with a temperature-controlled heating pad and thermistor probe (FHC; Bowdoin, ME, USA). Rats were

sacrificed via decapitation under anesthesia. One male and four female rats died during the course of the experiment.

2.2.4 Data collection and analysis

Electrodes were implanted and equilibrated for at least 30 min with the applied waveform prior to data collection. Data were excluded if fewer than 10 transients were observed within the initial 30 min. If robust transients were not found, a new electrode was inserted, up to five new electrodes for each animal. Eighty-two animals were excluded based on these criteria. After adenosine transients were identified, the electrode placement was optimized and data were collected for 4 h. An automated algorithm was previously developed to identify transient adenosines without bias using MATLAB, which resulted in a more rapid analysis than was possible manually. [38] Here, to further separate the signal from the noise, data was also analyzed with a second custom-written MATLAB program that verified the presence of the secondary oxidation peak at an acceptable prominence. The original program analyzed the data for the presence of a secondary oxidation peak, but did not evaluate the quality and position of those peaks, and the secondary program cut down on false positives (i.e., identifying changes as adenosine that were not).

2.2.5 Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Welch's t-test was used to compare the mean adenosine transient concentrations and inter-event times of males and females. The distribution of concentrations and inter-event times of adenosine transients were analyzed using a Kolmogorov–Smirnov (K-S) test. The mean adenosine transient concentrations and

inter-event times of the various stages of estrous were analyzed by one-way ANOVA. The distributions of the adenosine transient concentrations and inter-event times were analyzed using a Kruskal-Wallace test. Outlier tests were conducted using the ROUT method and eight rats scattered throughout the different groups were excluded due to these tests. With the addition of the eight animals removed due to outlier testing to the five animals that died during testing, the 82 removed due to exclusion criteria, and the 64 included in the study, 159 total animals were utilized in this study. Statistical significance was designated at $p < 0.05$ and all data are presented as mean ± standard error of the mean (SEM).

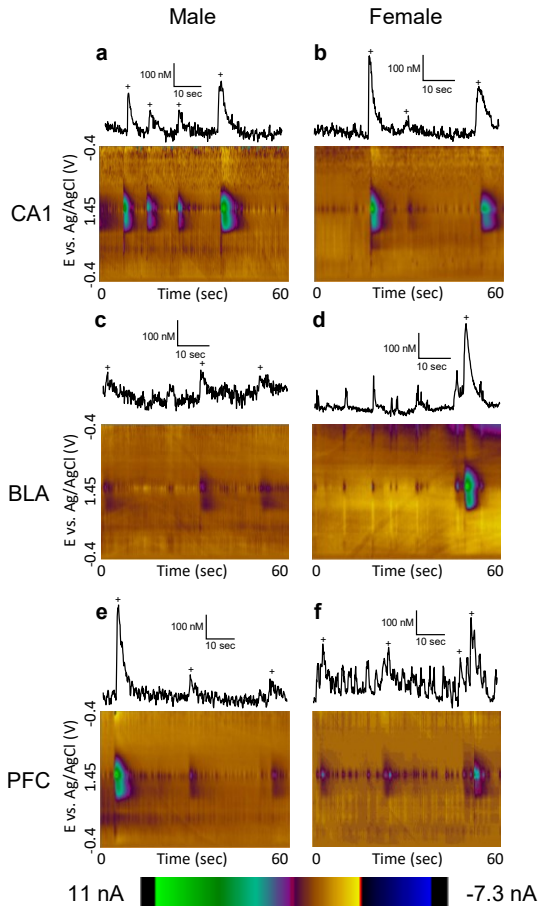

Figure 2.2. Example data from male and female rats from each brain region. Concentration traces (top) and 3D color plots (bottom) compare adenosine release in the CA1 region of the hippocampus in (a) male) and (b) female rats, the basolateral amygdala in (c) male and (d) female rats, and the prefrontal cortex in (e) male and (f) female rats. Adenosine transients are marked with plus (+) signs in the concentration traces, which are all scaled the same to highlight the varying concentrations.

2.3 Results

2.3.1 Spontaneous Adenosine Detection *in vivo*

Adenosine was detected using fast-scan cyclic voltammetry at a carbon-fiber microelectrode, which allows real-time measurement of adenosine changes with subsecond temporal resolution. [10,34,39,40] This technique is best at identifying fast

changes, due to background subtraction of data, and basal levels are not determined. Fig. 2.2 shows representative color plot data for each sex, by respective brain region as well as an extracted current vs. time trace for each color plot at the peak potential for adenosine oxidation (1.3 V on the back scan). Adenosine is identified from background subtracted cyclic voltammograms and false color plots by the two unique peaks produced by two sequential, two electron oxidation peaks characteristic of this compound. [34] Automated programs are used to identify adenosine transients from the data (see Methods). [38] Peaks identified as adenosine are marked with + signs. Because there can be noise fluctuations at the primary oxidation voltage, an important part of the identification of a transient as adenosine is the presence of a peak at the secondary oxidation potential (1.0 V on the forward scan) that starts after the primary peak.

2.3.2 Comparison of Spontaneous Adenosine Transients in Males and Females

Three brain regions were chosen to examine the effect of sex on spontaneous adenosine: the hippocampus, prefrontal cortex, and basolateral amygdala. Our lab has previously studied transient adenosine release in the hippocampus and the cortex, [41,42] but not in the amygdala. The amygdala was chosen because previous studies saw sex differences in neurochemistry and because it is also a part of the limbic system, controlling the formation of fear and emotional memory. [25,43–46] As shown in Fig. 2.2, the frequency of transients is highest in the hippocampus and lowest in the amygdala, with higher concentration transients in the cortex and amygdala, and lower in the hippocampus. Transients in the hippocampus tended to be larger and less frequent in females, compared to males (Fig. 2.2a, b). This relationship was also seen in the amygdala (Fig. 2.2c, d), although the transient frequency was greatly reduced compared

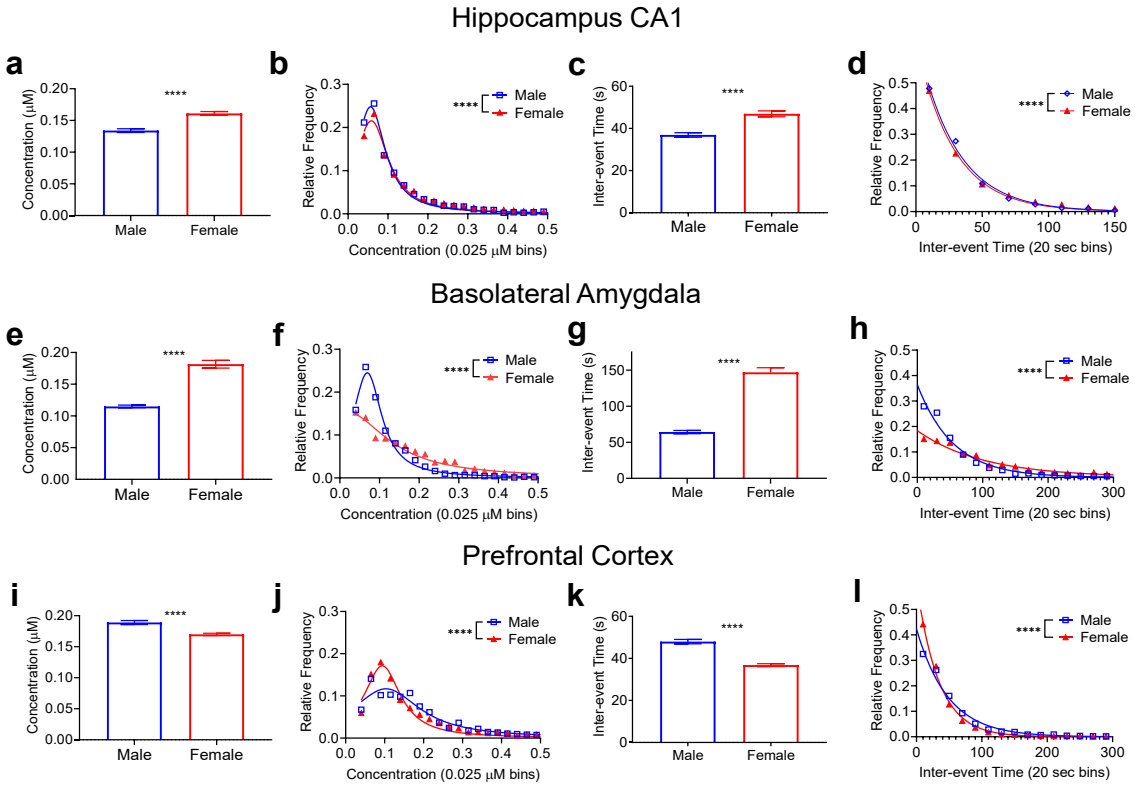

Figure 2.3. Comparison of spontaneous adenosine between males and females in the hippocampus, basolateral amygdala, and prefrontal cortex. Mean transient concentrations (a) in females were significantly larger in the hippocampus (Welch's t-test, n=3080 M/4639 F transients, $p<0.0001$, n is same for panels a-d) and (b) the distribution of transient concentrations was wider in the females vs. males (K-S test, $p<0.0001$). (c) Mean inter-event time was significantly longer in females in the hippocampus (Welch's t-test, $p<0.0001$) (d) and the distribution of inter-event times was significantly wider in females (K-S test, $p<0.0001$). In the basolateral amygdala, e) mean concentration (Welch's t-test, n=1934 M/1057 F transients, $p<0.0001$, n is same for panels e-h) is significantly larger in females and the (f) frequency distribution is wider in females (K-S test, $p<0.0001$). The BLA (g) inter-event time is also significant longer in females (Welch's t-test, $p<0.0001$) and (h) the distribution of inter-event times is wider in females (K-S test, $p<0.0001$). In the prefrontal cortex, the opposite is true. (i) Mean transient concentrations were significantly larger in males (Welch's t-test, n=2393 M/6233 F transients, $p<0.0001$, n is same for panels i to l) and (j) the distribution was wider for males (K-S test, $p<0.0001$), while (k) inter-event time (Welch's t-test, $p<0.0001$) was significantly longer in males with (l) the distribution was wider for males (K-S test, $p<0.0001$). All error bars are SEM. **** $p<0.0001$

to the other regions in females (Fig. 2.2d). In the cortex, sex differences were reversed, with males showing larger, less frequent transients and females having smaller, more frequent transients (Fig. 2.2e, f).

Figure 2.3 compares the average concentration and frequency of spontaneous adenosine transients in each brain region. Fig. 2.3a shows the average concentration of

each event in the hippocampus, which was 0.134 ±0.003 μM for males and 0.161 ±0.003 μM for females, a significant difference (*n*=3080 and 4639, respectively Welch's *t*-test, p<0.0001). A histogram of the distribution of the concentration values is shown in Figure 2.3b. Male rats had significantly more small concentration transients compared to females (K-S test, p<0.0001), which had a wider distribution of concentrations. The frequency of the transient events was also evaluated using inter-event time, the time in between two consecutive transients. The average inter-event time was significantly greater in females, 47 ±1 s, compared to males, 36 ±1 s (Fig. 2.3c, Welch's t-test, n=3080 m/4639 f, p<0.0001). The distribution of inter-event times was also significantly different (K-S test, p<0.0001) (Fig. 2.3d). Thus, in the hippocampus, female rats had larger concentration adenosine transients, but these transients occurred less frequently than in males.

In the basolateral amygdala, the average transient concentration was 0.115 ±0.002 μM for males, significantly less than 0.182 ±0.006 μM for females (*n*=1934 and 1057 transients, Welch's *t*-test, p<0.0001) (Fig. 2.3e). The distribution of the concentration values (Fig. 2.3f) shows that males had significantly more smaller concentration transients (K-S test, p<0.0001). The inter-event times in females were longer than those in males with a mean of 147 ±6 s for females and 64 ±2 s for males (Fig. 2.3g, Welch's t-test, p<0.0001). The distributions of inter-event times were also significantly different (K-S test, p<0.0001) with males having more frequent transients (Fig. 2.3h). These relationships are similar to those observed in the hippocampus, with the female rats showing larger, but less frequent transients, than the males.

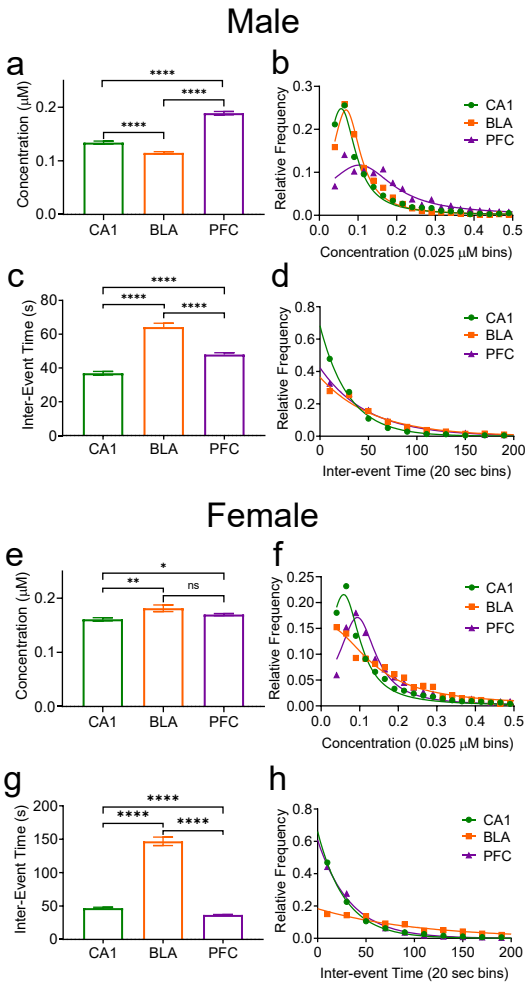

Figure 2.4. Comparison of spontaneous adenosine transients in the hippocampus, basolateral amygdala, and prefrontal cortex in male (top, a-d) and female (bottom, e-h) rats. (a) In males, there was an overall effect of region on the mean transient concentrations and all pairs of columns were significantly different from each other (One-way ANOVA, p<0.0001 Tukey's multiple comparisons test, significance marked, n=3080 CA1/2393 PFC/1934 BLA transients, n is the same for all statistics, panels a-d). (b) There was a main effect of region on distribution of concentrations, and comparisons between individual regions were significant except between the CA1 and the BLA (Kruskal-Wallace test, p<0.0001, CA1-PFC & PFC-BLA p<0.0001). (c) There was a significant effect of region on inter-event time in males, with significant differences between all columns (One-way ANOVA, p<0.0001, Tukey's multiple comparisons test, p<0.0001). (d) There was a significant main effect of frequency distribution in males, with all regions being significantly different (Kruskal-Wallace test, p<0.0001). (e) In females, there is a significant main effect of region on transient concentration and the concentration differed between the CA1-BLA and CA1-PFC, but not the PFC-BLA (One-way ANOVA, p=0.0009, with Tukey's multiple comparisons test, n=4639 CA1/6258 PFC/1057 BLA transients, CA1-PFC p=0.0321, CA1-BLA p=0.0076, n is same for all female statistics, panels e-h). (f) In females, there is a main effect of region on concentration distribution and the CA1 is significantly different from both the PFC and the BLA, but the PFC and the BLA were not different (Kruskal-Wallace test, p<0.0001, CA1-PFC & CA1-BLA p<0.0001). (g) There is a significant main effect of region on inter-event time in females and comparison between all regions are significantly different (One-way ANOVA, p<0.0001, Tukey's multiple comparisons test, p<0.0001). (h) Overall, there was a main effect of region on inter-event time distribution. However, the frequency distributions (Kruskal-Wallace test, p<0.0001, CA1-BLA/PFC-BLA p<0.0001, CA1-PFC) were not significantly different between the CA1 and the PFC, but were significantly different between all other regions and overall. All error bars are SEM. **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05

The prefrontal cortex shows the opposite relationship between adenosine concentration and frequency for males and females. In males, the average transient concentration of 0.189 ±0.003 μM was significantly larger than the concentration of 0.170 ±0.002 μM in females (*n*=2393 and 6258 transients, respectively, Welch's *t*-test, p<0.0001) (Fig. 2.3i). The concentration distributions confirm this relationship, with a wider distribution in males than in females (K-S test, p<0.0001) (Fig. 2.3j). For

frequency, mean inter-event time was significantly longer in males (48 ±1 s) than in females (37 ±1 s) (Welch's *t*-test, p<0.0001) (Fig. 2.3k). For the inter-event time distributions in Figure 2.3l, the distribution is wider in males (K-S test, p<0.0001). Thus, in the amygdala, transient concentration in the males was higher but the events were less frequent, and the trends were opposite of those observed in the other two regions.

2.3.3 Comparison of Spontaneous Adenosine Transients among Brain regions

While Fig. 2.3 compared males and females for adenosine release per region, the associations in males and females were also compared among the three regions (Fig. 2.4). In males, the mean transient concentration was significantly different among all brain regions studied (*n*=3080/CA1, 1934/BLA, 2393/PFC transients, One-way ANOVA with Tukey's multiple comparisons test, p<0.0001). The average concentration is highest in the prefrontal cortex (0.189±0.003 μM) followed by the hippocampus (0.134 ±0.003 μM) and finally the basolateral amygdala (0.115±0.002 μM) (Fig. 2.4a). The distributions for the transient concentrations were significantly different overall. The hippocampus and cortex were significantly different as well as the cortex and amygdala (Kruskal-Wallace test, p<0.0001), but not the hippocampus and amygdala (p>0.9999) (Fig. 2.4b). For inter-event time, the longest mean inter-event time was in the amygdala at 64±2 s, followed by the cortex at 48±1 s, and the hippocampus at 37±1 s, all of which were significantly different from each other (One-way ANOVA with Tukey's multiple comparisons test, p<0.0001) (Fig. 2.4c). The inter-event time distributions were all significantly different with the amygdala having the widest distribution (Kruskal-Wallace test, p<0.0001) (Fig. 2.4d). In males, the highest concentrations were in the cortex, but the lowest frequency was in the amygdala.

In females, the amygdala has the highest concentration (0.182±0.006 μM), followed by the cortex (0.170±0.002 μM) and the hippocampus (0.161±0.003 μM) (Fig. 2.4e). Overall, there was a main effect of region on concentration (*n*=4639/CA1, 1057/BLA, 6258/PFC transients, One-way ANOVA with Tukey's multiple comparisons test, p=0.0009). Individually, the hippocampus was significantly different from both other regions, but the amygdala and the cortex were not significantly different. For transient concentration distribution (Fig. 2.4f), there was also a main effect of region (Kruskal-Wallace test, p<0.0001). By region, the distributions followed the same pattern as the concentration with the hippocampus being significantly different from both other regions, but the cortex and amygdala were not. For inter-event time, the means (Fig. 2.4g) were significantly different among all regions, with the amygdala having the longest inter-event time at 147±6 s, followed by the hippocampus at 47±1 s, and the cortex at 37±1 s (One-way ANOVA with Tukey's multiple comparisons test, p<0.0001). There was an overall effect of region of the distributions of the inter-event times (Fig. 2.4h), but the distributions of the cortex and the hippocampus were not significantly different from each other, while the amygdala was significantly wider than both (Kruskal-Wallace test, p<0.0001). In females, the highest concentration transients were in the amygdala, which also had the lowest frequency.

Both males and females had a much lower frequency in the amygdala than the other two regions but they differed greatly in the transient concentration in the amygdala, with males having a much smaller concentration compared to females. The relationship between the average concentration in the cortex and the hippocampus are similar between males and females, however the trends for inter-event times for those regions

are reversed. Thus, the patterns in concentration and inter-event time were not the same between sexes for the different regions.

2.3.4 Estrous Cycle Comparison of Spontaneous Adenosine Transients

Female hormones vary throughout the estrous cycle, and these fluctuations in hormones could cause differences in neurochemistry. [47,48] Thus, we examined how

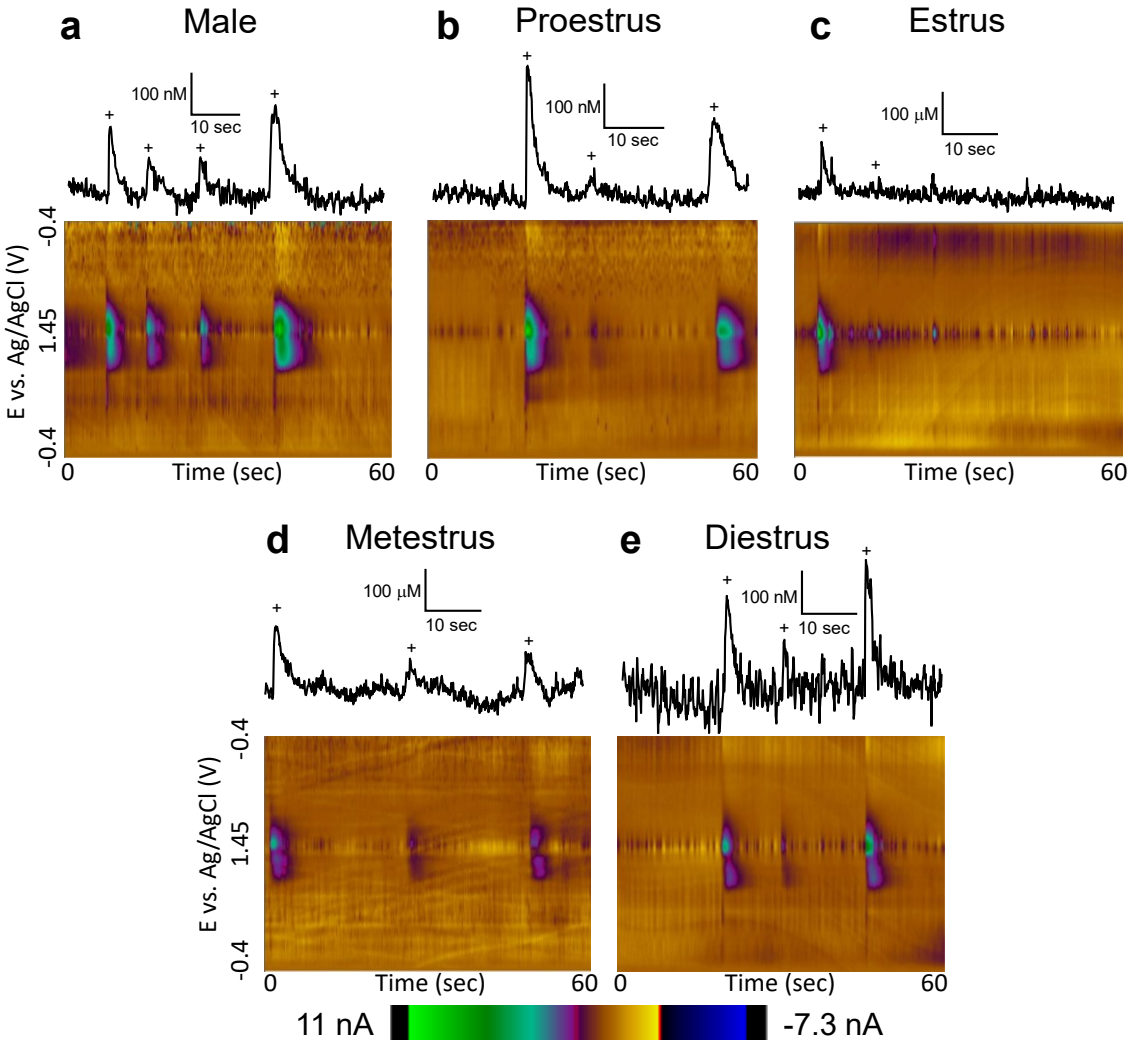

Figure 2.5. Example male data and female data by estrous cycle in the hippocampus. Concentration traces (top) and 3D color plots (bottom) for adenosine transients in the CA1 region of the hippocampus in (a) male and female rats during (b) proestrus, (c) estrus, (d) metestrus, and (e) diestrus highlighting the differences in transient concentration and frequency between estrous stages. Adenosine transients are marked with plus (+) signs on the concentration traces, which are all scaled the same to highlight the variety of concentrations.

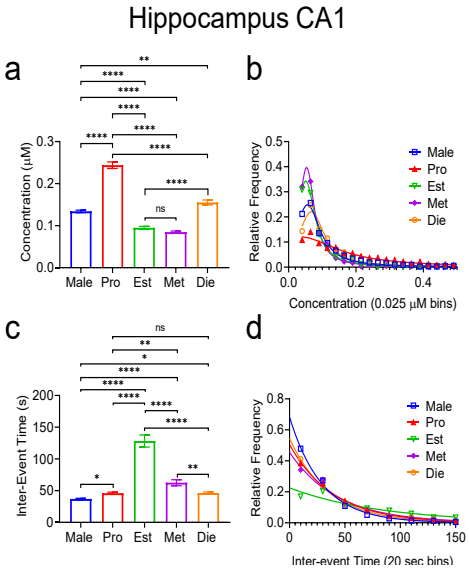

Figure 2.6. Spontaneous adenosine transient comparison in the hippocampus between male rats and female rats in various stages of estrous. (a) Overall, there was a main effect of stages of estrous/sex on mean transient concentration (One-way ANOVA with Tukey's multiple comparisons test, n=3080 Male/1237 Pro/558 Est/858 Met/1232 Die transients, $p<0.0001$, n is the same for all statistics in this figure). Differences between groups are marked (Tukey's multiple comparison). (b) There is a main effect of stage of estrous/sex on the concentration distribution (Kruskal-Wallace, $p<0.0001$). Pairwise comparisons of different stages were also significantly different, except between estrus and metestrus, where there was no significance (Kruskal-Wallace test, Male-Pro/Male-Est/Male-Met/Male-Die/Pro-Est/Pro-Met/Pro-Die/Est-Die/Met-Die $p<0.0001$). (c) There was a significant main effect of estrous cycle/sex on mean inter-event time (One-way ANOVA, $p<0.0001$), with the mean inter-event time of the stages being significantly different, except between proestrus and diestrus (Tukey's multiple comparisons test, $p<0.0001$, Pro-Met $p=0.0010$, Met-Die $p=0.0014$, Male-Pro $p=0.0425$, Male-Die $p=0.0313$). (d) There was a significant main effect of estrous stage/sex on inter-event time distribution (Kruskal-Wallace, $p<0.0001$) and pairwise comparisons were significantly different except between proestrus and metestrus, proestrus and diestrus, and metestrus and diestrus (Male-Pro/Male-Est/Male-Met/Male-Die/Pro-Est/Est-Male, Est-Die $p<0.0001$). All error bars are SEM. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$

adenosine transients vary with the estrous cycle. Two regions were compared: the hippocampus, which had a high frequency of transients, and the cortex, because it had a different pattern of frequency and concentration sex differences from the hippocampus and amygdala. Fig. 2.5 shows representative color plots in the hippocampus from a male and from females in the proestrus, estrus, metestrus, and diestrus stages. An extracted concentration vs. time trace is shown with adenosine transients marked with a + sign. The frequency of transients was generally higher in males than any of the individual stages of estrous in females (Fig 2.5a). The concentration of the proestrus stage was the highest and proestrus and diestrus had greater concentrations than males (Fig. 2.5b, e). The estrus stage showed both the lowest frequency and the lowest concentration (Fig. 2.5c).

Figure 2.6a summarizes the hippocampus data. Overall, there was a significant main

effect of estrous cycle on mean adenosine transient concentration (*n*=3080/Male, 1237/Pro, 558/Est, 858/Met, 1232/Die transients, One-way ANOVA with Tukey's multiple comparisons test, $p<0.0001$). The highest mean concentration was during the proestrus stage at 0.243±0.008 μM, followed by diestrus at 0.155±0.006 μM, then males at 0.134±0.003 μM. Transient adenosine concentration in the hippocampus is lowest during estrus at 0.095±0.003 μM and metestrus at 0.085±0.002 μM, which were not significantly different from each other. Estrus and metestrus were, however, significantly smaller than all other stages and males. There were a variety of differences between the stages, which are categorized and summarized in Figure 2.6. The distributions of transient concentrations overall were significantly different, but individually, estrus and metestrus distributions were not significantly different, while all other stages were significantly different from each other (Fig. 2.6b, Kruskal-Wallace test, n=3080 Male/1237 Pro/558 Est/858 Met/1232 Di, $p<0.0001$). In Fig. 2.6c, there was a significant main effect of estrous cycle on average inter-event time (One-way ANOVA with Tukey's multiple comparisons test, n=3080 Male/1237 Pro/558 Est/858 Met/1232 Die, $p<0.0001$). Males had the shortest inter-event time at 37±1 s, followed by proestrus and diestrus at 46±2 s, metestrus at 63±5 s, and estrus at 128±9 s. Proestrus and diestrus were not significantly different from each other, but all other comparisons were (One-way ANOVA with Tukey's multiple comparisons test, $p<0.0001$, Pro-Met $p=0.0010$, Met-Die $p=0.0014$, Male-Pro $p=0.0425$, Male-Die $p=0.0313$). For the distribution of the inter-event times, there was a significant main effect of estrous stage/sex on inter-event time distribution and the distributions of proestrus, diestrus, and metestrus were not significantly different, while estrus and males were significantly different from each other and all other stages (Kruskal-Wallace test, $p<0.0001$) (Fig. 2.6d).

Due to the differences in patterns between the cortex and the other two regions, we also examined how stage of estrous affects adenosine in the cortex. Overall, there was a significant effect of estrous stage/sex for both mean concentration (Fig. 2.7a, One-way ANOVA with Tukey's multiple comparisons test, n=2367 Male/1699 Pro/1668 Est/1765 Met/1776 Die, p<0.0001) and for concentration distribution (Fig. 2.7b, Kruskal-Wallace test, n=2367 Male/1699 Pro/1668 Est/1765 Met/1776 Die, p<0.0001). As shown in Figure 2.7a, the mean transient concentration for males (0.245±0.006 μM) and for proestrus (0.243±0.004 μM) are not significantly different. The mean concentrations for estrus (0.136±0.003 μM), metestrus (0.144±0.003 μM), and diestrus (0.141±0.003 μM) are also not significantly different from each other,

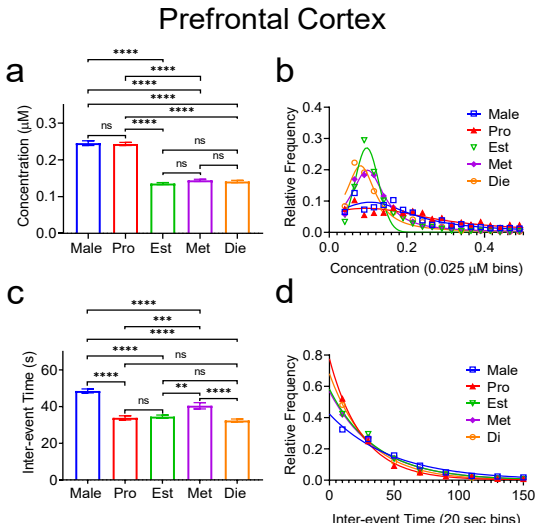

Figure 2.7. Spontaneous adenosine transient comparison in the prefrontal cortex between male rats and female rats in various stages of estrous. (a) There is a main effect of stage of estrous and sex on mean transient concentrations (One-way ANOVA, p<0.0001, n=2367 Male/1699 Pro/1668 Est/1765 Met/1776 Die transients, n is same for all statistics in this figure). Pairwise comparisons are marked (Tukey's multiple comparisons test). (b) Overall, there was a main effect of stage of estrous/sex on transient concentration distribution (Kruskal-Wallace, p<0.0001). Pairwise comparisons of distributions were also significantly different, except between estrus and metestrus, and estrus and diestrus where there was no significant difference (Kruskal-Wallace test, Male-Est/Male-Met/Male-Die/Pro-Est/Pro-Met/Pro-Die p<0.0001, Male-Pro p=0.0001, Male-Die p=0.0453). (c) There was an overall main effect of stage of estrous/sex on inter-event time (One-way ANOVA, p<0.0001). Individual comparisons are marked (Tukey's multiple comparison test). (d) There was a significant main effect of estrous stage/sex on the distribution of inter-event times (Kruskal-Wallace, p<0.0001). Pairwise comparisons of distributions were significantly different except between proestrus and diestrus, and estrus and metestrus (Kruskal-Wallace test, Male-Pro/Male-Est/Male-Met/Male-Die/Pro-Est/Pro-Met p<0.0001, Met-Die p=0.0001, Est-Die p=0.0101). All error is in SEM. **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05

but each stage is significantly different from the other groups (One-way ANOVA with Tukey's multiple comparisons test, n=2367 Male/1699 Pro/1668 Est/1765 Met/1776 Die,

p<0.0001). For the transient distributions, estrus and diestrus are not significantly different, and estrus is not significantly different from metestrus. All other distributions were significantly different from each other (Kruskal-Wallace test, p<0.0001, Fig. 2.7b). There was also a significant main effect of estrous cycle on both the mean inter-event time and the frequency distribution (Fig. 2.7c, One-way ANOVA with Tukey's multiple comparisons test, n=2367 Male/1699 Pro/1668 Est/1765 Met/1776 Die, p<0.0001, Fig. 2.7d, Kruskal-Wallace test, n=2367 Male/1699 Pro/1668 Est/1765 Met/1776 Die p<0.0001). Average inter-event times (Fig. 2.7c) in proestrus (34±1 s), estrus at (34±1 s), and diestrus (32±1 s) were not significantly different from each other, but were significantly shorter than both males at 49±1 s and metestrus at 40±2 s (One-way ANOVA p<0.0001 with Tukey's multiple comparisons test, Male-Pro/Male-Est/Male-Met/Male-Die/Met-Die p<0.0001, Pro-Met p=0.0015, Est-Met p=0.0062). For the inter-event time distributions, there was an overall significant effect of stage of estrous/sex (Kruskal-Wallace test, p<0.0001, Fig. 2.7d). By sex/region, there was no significance between proestrus and diestrus or between estrus and metestrus. All female stages were significantly different than males (Kruskal-Wallace test, Male-Pro/Male-Est/Male-Met/Male-Die/Pro-Est/Pro-Met p<0.0001, Met-Di p=0.0001, Est-Di p=0.0101).

The results of estrous cycle data were compared to see if any patterns emerged between regions. In both regions, the mean concentration of transients in the proestrus stage was significantly higher than that of the other stages. Otherwise, the patterns in the regions were different and not predictable, with variations in both concentration and inter-event time across the remainder of the stages.

2.4 Discussion

This study shows that the concentration and frequency of spontaneous adenosine release differs between male and female rats but that the patterns of sex differences is not the same in all brain regions. For example, females have larger, but fewer transients, in the hippocampus and amygdala, but in the cortex, females have smaller, but more frequent adenosine transients than males. Changes during the female estrous cycle were examined, and in the hippocampus, the concentration and frequency were high during diestrus and proestrus. In the cortex, the pattern was different, with a high frequency release for all stages vs. males, but lower concentrations for estrus, diestrus, and metestrus. Thus, we found that spontaneous adenosine release varies across the estrous cycle in females. These results show that there are complex sex differences for adenosine which may lead to differences in neuromodulation, and that sex as a biological variable should be considered in studies of developing treatments targeting the adenosine system.

2.4.1 Spontaneous Adenosine Transient Varies by Sex

Sex differences have been observed in many neurochemicals; for example, dopamine release is larger and uptake is faster in females. [49] Adenosine regulates phasic dopamine, [12] and so we hypothesized that adenosine might also have sex differences. Table 2.1 summarizes the major findings for sex differences in adenosine. We studied different brain regions

Table 2.1. Relative comparison of transient adenosine concentration and frequency between males and females in the hippocampus, basolateral amygdala, and prefrontal cortex

	Concentration			Frequency		
	CA1	BLA	PFC	CA1	BLA	PFC
Male	Low	Low	High	High	High	Low
Female	High	High	Low	Low	Low	High

because our previous studies showed that adenosine release varied greatly among regions. [9,42,50] In the hippocampus and amygdala, males had smaller concentration, more frequent transients while, in females, the transients were larger but at a lower frequency. In the cortex, the pattern was the opposite with larger,

less frequent transients for males and smaller, more frequent transients for females. Thus, sex differences were not the same in every region.

One trend evident from Table 2.1 is a trend towards higher concentrations of adenosine associated with lower frequencies of release. While the mechanism of release is not yet fully elucidated, adenosine is likely formed from extracellular metabolism of released ATP, [51,52] and it is possible that more ATP builds up and is available for release when the frequency is lower. Changes in frequency of ATP release could affect the quantal size of the vesicles allowing them more time to accumulate ATP from within the cell. Exocytotically released ATP is packaged together with other neurotransmitters, such as acetylcholine or glutamate, or on its own, [53] and release from different types of vesicles could also account for some of the observed discrepancies in concentration. However, the observed trend between high concentration and low frequency was not found when the stages of estrous were compared, and was not apparent in previous research. [9,54] Thus, higher frequency leading to low concentrations is a trend that could be investigated further to determine if there is a clear mechanism to link frequency and concentration.

Multiple brain regions were investigated to determine if sex differences were consistent throughout the brain, and Fig. 2.4 shows that the patterns between regions were not the same in males and females. The concentrations varied significantly from

region to region in males, while in females, the mean concentrations in the cortex and amygdala were similar. One of the biggest differences is that the amygdala has the lowest concentration in males but the highest concentration in females. The frequency was lowest (i.e., inter-event time was highest) in the amygdala for both sexes. Comparing the hippocampus and cortex, the frequency for males was higher in the hippocampus than the cortex, while the trend was flipped in females, which had a higher frequency in the cortex. Thus, the trends in frequency and concentration are complex and not consistent between the sexes.

The differences in patterns for females from males might be caused by different levels of estradiol. Progesterone and estrogen modulate neurological functions including oxidative stress, glial function, and mitochondrial function. [55] Barker and Galea showed that the estradiol concentrations in the hippocampus and the amygdala are much larger than in the cortex. [25] Estradiol also increases cyclic adenosine monophosphate (cAMP) concentrations through the adenylate cyclase pathway in some estrogen dependent cells, although the brain was not studied. [56] Increased cAMP is generally excitatory and might increase the concentration of the adenosine transients in the amygdala and hippocampus, which have higher levels.

While hormone levels could affect receptor sensitivity, differences in spontaneous adenosine might also simply be due to differences in receptor density between males and females. Both A_1 and A_{2A} receptors regulate spontaneous adenosine release, [9,13,14] with A_1 receptors antagonists increasing the frequency of adenosine release [57] and A_{2A} receptor antagonists decreasing the frequency. [58] For example, if there were more A_1 receptors in one sex or a brain region, more inhibition

would be expected to lower frequency of adenosine release. The opposite would be true if there were more A_{2A} receptors. Receptor activation is complicated because the two main types of adenosine receptors work against each other. There is currently no published information on A_1 and A_{2A} receptor densities in females, so it is not possible to match the neurochemistry data with receptor density and future studies could examine sex differences in receptors.

2.4.2 Spontaneous Adenosine Transients vary during the estrous cycle

Female hormone levels change over the stages of estrous, with concentrations of estrogen and progesterone peaking during proestrus, then dropping during estrus and the beginning of metestrus, and rising again during late metestrus and diestrus. [46] We examined the patterns of spontaneous adenosine release in the two regions with higher frequencies (hippocampus and cortex) that had opposite patterns in terms of concentration and frequency in males and females. The overall trends for these findings are summarized in Table 2.2. In the hippocampus, the highest concentration is during the proestrus stage, followed by diestrus, with estrus and metestrus the lowest. The concentration in the hippocampus correlates with the fluctuation of estrous cycle hormones, [46] as adenosine transients are higher concentration when estrogen and progesterone levels are high. Thus, estradiol might have a stimulatory effect, activating the same pathway as excitatory A_{2A} receptors, [13,56] that affects the concentration of the transients. The frequency of adenosine events is also affected as the frequency is higher when estradiol and progesterone are

Table 2.2. Relative comparison of transient adenosine concentration and frequency between males and stages of estrus in the hippocampus and prefrontal cortex

	Concentration		Frequency	
	CA1	PFC	CA1	PFC
Proestrus	High	High	High	High
Estrus	Low	Low	Low	High
Metestrus	Low	Low	Med	High
Diestrus	Med	Low	High	High

higher and lower when the hormones drop during estrus. Therefore, in the hippocampus, frequency and concentration of adenosine transients are both elevated when estrogen and progesterone levels are high.

In the cortex, the highest concentration adenosine events also occur during proestrus, a similar trend as in the hippocampus. The frequency of events in the cortex is higher in females for all stages of estrous than in males. Thus, in the cortex, the effect of estrogen on the concentration and frequency is not as pronounced as in the hippocampus. Previous studies showed low levels of estrogen in the cortex [25] and thus estrogen might change less and cause fewer changes during the estrous cycle. Future experiments should examine the role of estrogen in the brain to regulate spontaneous adenosine concentration and the frequency of release events. If estrogen does regulate spontaneous adenosine release, this has implications for its role as a neuromodulator and shows that it might vary during the estrous cycle for regions that have more estrogen.

2.4.3 Implications of Sex Differences in Adenosine Release

Adenosine is a neuromodulator that can modulate other neurotransmitters and blood flow. For example, rapid adenosine release can modulate phasic dopamine release via the A_1 receptor; [12] when adenosine is present, phasic dopamine decreases by about 50%. Serotonin release is inhibited by A_1 receptors or stimulated by A_{2A} receptors [59] and similar observations have been made for adenosine regulation of glutamate, [60] acetylcholine, [61] and norepinephrine. [62] Thus, a change in either the frequency or concentration of adenosine in males vs females might lead to variety of downstream effects on neurotransmitter signaling. Changes in concentration are

important because larger adenosine signals would be able to activate lower affinity receptors, diffuse further, and have a longer lasting effect. Targeting adenosine as a treatment for disease has been proposed for schizophrenia and Parkinson's disease, [63,64] as it would have downstream effects on the dopamine system, and these studies show it is important to consider sex and estrus cycle in any treatment strategy, as they affect adenosine levels. Previous work has shown that changes in frequency are the most common regulation of spontaneous adenosine release. [9,65] The higher the frequency of spontaneous adenosine release, the tighter the regulatory control over the neurotransmitters in that area. The inverse correlation between frequency and concentration observed shows that while release may be more frequent, each event is typically smaller and that might mitigate some of the effects of higher frequency. Larger adenosine events also affect blood flow, causing vasodilation and increases in brain oxygen. [13] Thus, larger but low frequency transients might affect blood flow more. Adenosine plays a role in neuromodulation and neuroprotection during ischemia, [14,41] and sex differences in adenosine might cause less neuroprotection in a given area if the frequency is low. Understanding these complex differences in spontaneous adenosine between the sexes and during different stages of estrous is important for understanding variations in the endogenous neuromodulation by adenosine and designing effective treatments targeting adenosine as a neuromodulator.

2.5 Conclusions

The concentration and frequency of spontaneous adenosine transients varies with sex and by region. In the hippocampus and amygdala, females had higher

concentration but lower frequency adenosine release but the pattern was the opposite in the cortex, where females had lower concentrations and higher frequencies. In addition, spontaneous adenosine release also varies with different stages of the estrous cycle. While the patterns were not the same for the hippocampus and the cortex, higher concentrations were observed during proestrus, where estrogen and progesterone concentrations are high. Thus, it is important that sex and estrous cycle differences are closely examined in future studies in order to better understand adenosine neuromodulation. In the future, it would be beneficial to examine how sex affects both the fast and slow methods of adenosine release or how basal levels of extracellular adenosine may vary between the sexes. It would also be valuable to examine gonadectomized males and females to do a more in-depth study of how hormones, such as estradiol, testosterone, and progesterone affect spontaneous adenosine release.

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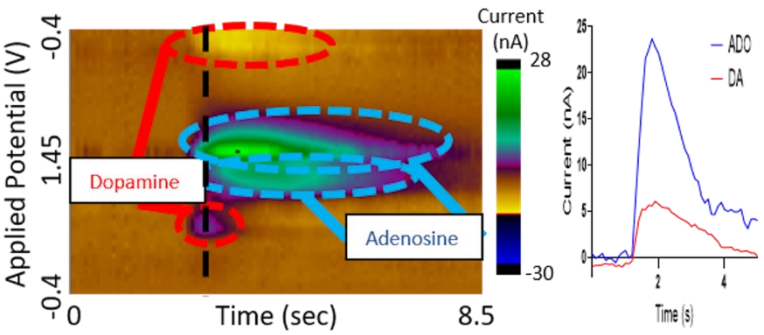
Chapter 3

Characterization of Coincident Spontaneous Adenosine and Dopamine Transients: Evidence for Co-release

One never notices what has been done; one can only see what remains to be done.
— Marie Curie

Chapter 3: Characterization of Coincident Spontaneous Adenosine and Dopamine Transients: Evidence for Co-release

Abstract



Rapid changes in adenosine and dopamine have been measured *in vivo,* but no studies have examined if these transient changes occur simultaneously. In this study, we characterized spontaneous adenosine and dopamine events in anesthetized mice, examining coincident release in the caudate putamen for the first time. We find that most (86 %) of the dopamine events were coincident with adenosine, but fewer (27%) of adenosine release events were coincident with a dopamine event. There was a positive correlation (R^2=0.53) of adenosine and dopamine concentrations during coincident release, with larger dopamine events having a higher incidence of coincidence. Dopaminergic vesicles may be packaged with ATP, which is co-released with dopamine, and then broken down to adenosine quickly in the extracellular space. However, the high frequency of adenosine events in comparison with dopamine events suggests adenosine is also released from non-dopaminergic vesicles as well. We investigated how A_1 and A_{2A} adenosine receptors regulate adenosine and dopamine transients using A_1 and A_{2A} KO mice. In A_1KO mice, the frequency of adenosine and dopamine transients increased, but concentration was unchanged. For A_{2A}KO mice, adenosine frequency and concentration did not change, but dopamine increased in concentration (but not frequency). Adenosine receptors

modulate coincident transients and could be drug targets to modulate both dopamine and adenosine release. Spontaneous dopamine transients nearly all have coincident adenosine release and thus the neuromodulatory properties of adenosine should be examined for designing treatments for dopamine diseases, such as Parkinson's or addiction.

3.1 Introduction

Adenosine is an endogenous nucleoside that plays roles in sleep, vasodilation, and immune response.[1,2] In the brain, adenosine is a neuromodulator and a rapid mode of adenosine transiently modulates phasic dopamine release[3] as well as oxygen changes, which are correlated with blood flow.[4,5] Spontaneous transient adenosine last only a few seconds, with concentrations in the hundreds of nM. The frequency of these transients is random, but modulated by A_1 receptors.[6] In fact, in A_1KO mice, the frequency of adenosine transients increases, while in A_{2A}KO mice have higher concentrations of adenosine during spontaneous release.[7] The mechanism of spontaneous adenosine release is not fully elucidated, but the predominant mechanism of release appears to be exocytosis of ATP,[8–10] which is then broken down to adenosine in the extracellular space in about 200 ms.[11] Adenosine may also be directly released from synaptic vesicles or through membrane transporters.[8,12,13] ATP is packaged into vesicles with neurotransmitters such as dopamine,[14–16] but measurements of adenosine and dopamine co-release have not been made.

Dopamine is a catecholamine neurotransmitter that has been investigated for its role in Parkinson's disease[17,18] schizophrenia[19,20], addiction,[21,22] and depression.[23,24] Phasic dopamine release, due to burst firing, occurs in many behavioral experiments, particularly in response to addiction cues or food rewards.[25,26] Spontaneous, phasic dopamine release has been observed,[27] although the frequency is generally low. The frequency of spontaneous dopamine release increases after pharmacological agents such as cocaine[28] or nomifensine and haloperidol,[29] even in anesthetized animals, which means phasic dopamine is not always behaviorally evoked. Transient dopamine release is heterogenous within the brain; while some brain regions have active dopamine

transients, others do not despite dopamine being available.[30] This heterogeneous response could be due to neuromodulation by other neurochemicals. However, these studies only observed dopamine and have not explored the possibility of coincident release with other neurotransmitters or neuromodulators.

The adenosine and dopamine systems in the brain are inextricably linked, as their receptors are co-located and adenosine can modulate dopamine release in the brain.[31,32] Adenosine and dopamine receptors in the striatum are co-located with A_1 receptors being expressed on GABAergic neurons with both D_1 and D_2 receptors, and A_{2A} receptors being expressed on neurons with D_2 receptors.[31] Adenosine and dopamine receptors also exist in A_1-D_1 and A_{2A}-D_2 heteromers, where they work antagonistically.[33–35] While these receptor interactions are known, it is not known how they affect adenosine and dopamine release. Previously, our lab has measured adenosine and dopamine simultaneously in order to understand how adenosine regulates dopamine *in vitro*[36] and *in vivo* following electrical stimulation.[37] Manipulation of the dopaminergic system *in vivo* using pharmaceuticals did not affect stimulated adenosine transients.[38,39] However, no studies have measured spontaneous adenosine and dopamine transients, to examine if they are co-released and how adenosine affects dopamine release on a rapid time scale.

The goal of this study was to characterize adenosine/dopamine coincident release *in vivo* for the first time. Adenosine and dopamine transients were measured in C57BL/6J (WT) mice and in A_1 and A_{2A} knockout mice, in order to observe how changes in the adenosine receptors impact the transients. We found that dopamine release was coincident with adenosine 86% of time. The dopamine concentration was also significantly higher during coincident release and was correlated with adenosine

transient concentration. The frequency of adenosine and dopamine release events increased in A_1KO mice, while A_{2A}KO mice had higher dopamine concentrations, but a lower rate of coincident release for adenosine. These results demonstrate coincident release of adenosine and dopamine and modulation of dopamine by the adenosine system in real time, which could be important for designing treatments for dopamine diseases, such as Parkinson's or addiction.

3.2 Results

3.2.1 Co-Detection of Spontaneous Adenosine and Dopamine *In Vivo*

Adenosine and dopamine were co-detected using fast-scan voltammetry (FSCV) at a carbon-fiber microelectrode, which allows real-time measurement of discrete concentration changes of both analytes with subsecond temporal resolution. This technique is ideal for observing rapid changes in analyte concentration, but is unable to measure basal level concentrations due to background subtraction.[45] Simultaneous detection of dopamine and adenosine with FSCV was demonstrated previously by our lab[37,46] and by Kendall Lee's lab[47]; however, this is the first study of spontaneous adenosine and dopamine codetection *in vivo*. Measurements were made in the caudate putamen because it is rich in dopaminergic neurons, has been the target of dopamine research[3,43,48], and also exhibits spontaneous adenosine release and high expression of adenosine receptors[3,4,6,37].

The carbon-fiber microelectrode was scanned from -0.4 V to 1.45 V and back at 400 V/s and 10 Hz. Adenosine undergoes two oxidation reactions to produce the characteristic two peaks at 1.3 and 1.0 V. Dopamine undergoes a redox reaction where dopamine is oxidized to dopamine-o-quinone, with a peak around 0.6 V, and DOQ is

reduced back to dopamine with a peak around -0.2 V. Figure 3.1 shows a representative false color plot of a typical adenosine/dopamine coincident release. The magnitude and duration of the adenosine (ADO) and dopamine (DA) transients are observed in the current vs. time (i vs t) traces, taken at the oxidation peak maximums for each analyte (Fig. 3.1A). The false color plot allows all the data to be visualized in time, and peaks for both DA and ADO are evident at different voltages (Fig. 3.1B). The ADO primary oxidation peak appears around 1.3 V on the back scan 3 s into the plot, lasting about 4 s. The DA oxidation peak appears at 0.5 V, concurrent with the ADO peak and lasts for about 2 s. When the cyclic voltammogram is examined (Fig. 3.1C), you can see the characteristic peaks of both ADO and DA. Using *in vitro* calibration factors, currents are converted to calibration and in this example, the ADO transient peak concentration is 1.7 μM, while the DA concentration is 0.14 μM. In order to distinguish ADO and DA peaks, our lab

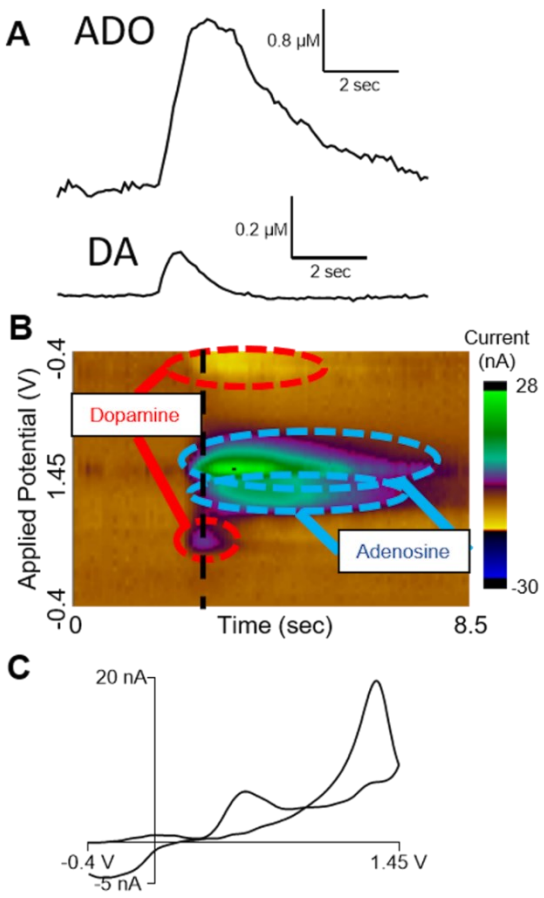


Figure 3.1. Example data of spontaneous adenosine/dopamine coincident release transient *in vivo*. (A) Current vs. time curves of a representative coincident release transient showing both the ADO and DA concentrations. (B) A representative example of a false color plot of an ADO/DA coincident release event in the caudate putamen. The y-axis is applied voltage, the x-axis is time, and the current in represented in false color. The ADO transient shows the characteristic primary and secondary oxidation peaks at the center of the plot. The DA oxidation peak is below that with the reduction peak above. (C) A current vs. voltage graph of the event that illustrates both the representative shape of ADO and that of DA.

previously developed an automated image processing program that uses image analysis techniques to identify the ADO and DA transients from the color plot.[44]

3.2.2 Characterization of Coincident Spontaneous Adenosine and Dopamine Transients in the Caudate Putamen

Figure 3.2 shows a typical three-minute duration false color plot used in this study with current vs time traces (above) for the primary oxidation peak potential of adenosine (top) and the oxidation peak of dopamine (middle). Transients identified by the automated algorithm are marked with + for ADO and ‡ for DA. Spontaneous ADO transients were more frequent than DA transients in WT mice. Fig. 3.2 shows a number of ADO transients, one of which also has a coincident DA transient (the left most ADO and DA transient). In this study, we defined coincident transients as ones where a DA transient occurred within ± 5 s of an ADO transient, but normally they occurred at the same time as shown in Fig. 3.2.

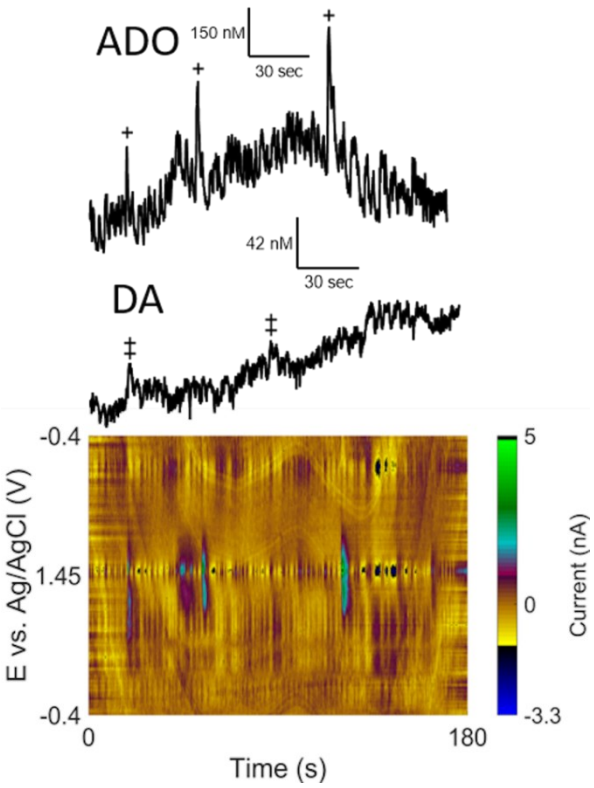


Figure 3.2. Example data from the caudate putamen of WT, showing separate and coincident transients. Concentration traces for both adenosine (top) and dopamine (middle), and a 3D color plot (bottom) compare ADO and DA release. ADO transients are marked with plus (+) signs and DA transients are marked with double dagger (‡) symbols in the concentration traces.

In order to understand adenosine and dopamine transients, we first looked at the frequency that these events occurred. We hypothesized that if ADO and DA are co-released from the same

vesicle, that their frequencies would be similar. In FSCV, there are two measures of frequency, the number of transients per hour and the inter-event time. Figure 3.3A illustrates the number of transients per hour for all ADO, all DA, and ADO-DA coincident transients. ADO transients (including coincident transients with DA) are frequent in WT mice, with an average of 81 ± 11 per hour, while there were 14 ± 4 per hour DA transients (including coincident transients with ADO). The number of coincident transients averaged 10 ± 3 per hour, which shows that most of the DA transients occur simultaneously with ADO. Overall, there was a significant main effect of neurotransmitter on the number of transients per hour (Brown-Forsythe ANOVA, p<0.0001, *n*=11) with ADO significantly more frequent than both DA and coincident transients, which were not significantly different from each other. This trend of more frequent transients for ADO is also observed by examining the inter-event time (IET). Inter-event time is a measure of the amount of time between the end of one

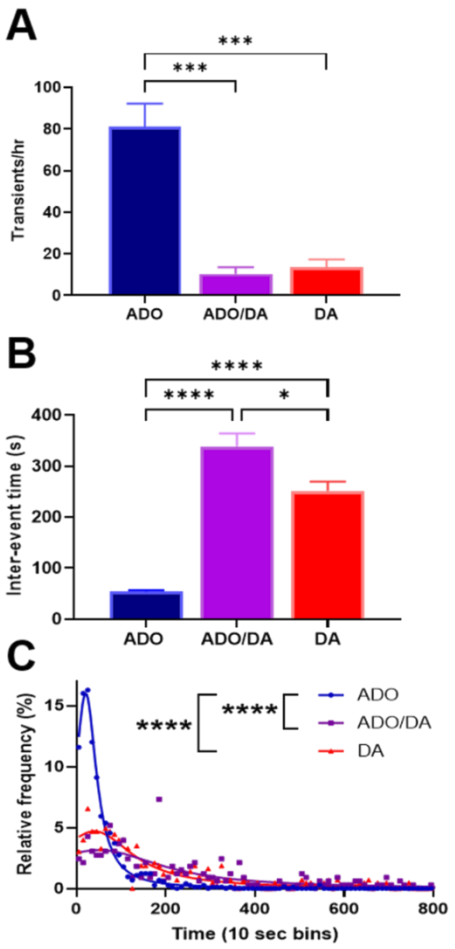


Figure 3.3. Characterization of adenosine/dopamine coincident release frequency in the caudate putamen of C57BL (WT) mice. (A) Number of transients per hour for all ADO, ADO coincident with DA (ADO/DA) and all DA. There was an overall main effect of neurotransmitter on frequency (Brown-Forsythe ANOVA, p<0.0001, Dunnett's T3 post-test, *n*=11). (B) Mean inter-event times. There was a significant main effect of group (Brown-Forsythe ANOVA, Games-Howell's multiple comparisons, p<0.0001, *n*=725 ADO, *n*=427 DA, *n*=328 ADO/DA). (C) Inter-event time distributions. There is an overall main effect of transient type on inter-event time distribution (Kruskal-Wallis test, p<0.0001, *n*=725 ADO, *n*=427 DA, *n*=328 ADO/DA). The distributions of the IET show a significantly thinner distribution of inter-event times for ADO, while that of DA and coincident release is wider. The coincident release and DA inter-event time distributions are not significantly different. Graphs are marked *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001

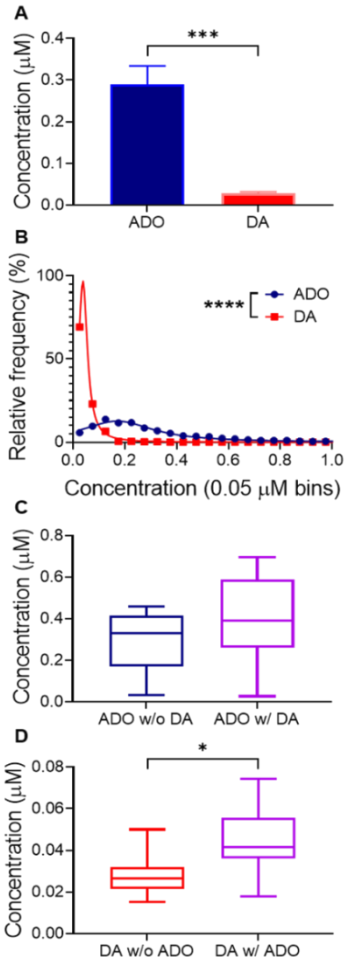


Figure 3.4. Comparison of spontaneous adenosine, dopamine, and coincident release transient concentrations in the caudate putamen of C57BL (WT) mice. (A) Concentration of transients. Spontaneous ADO transients had a significantly higher mean concentration than DA transients overall (paired t-test, p=0.0001, *n*=11). (B) Concentration distributions. The distribution of transient concentrations was also significantly different (K-S test, p<0.0001, *n*=2675 ADO, *n*=445 DA). (C) Concentration of ADO with and without DA co-release. The concentration of spontaneous ADO transients alone is not significantly different from the concentration of ADO transients that were coincident released with DA (Welch's t-test, p=0.1627, *n*=11). (D) Concentration of DA with and without ADO co-release. The concentration of spontaneous DA transients alone was significantly different from the concentration of DA transients coincident released with ADO (Welch's t-test, p=0.0125, *n*=11). Graphs are marked *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001

transient and the beginning of the next; therefore, longer IET indicates a lower frequency. There was a significant main effect of group on IET (Brown-Forsythe ANOVA, p<0.0001, *n*=725 ADO, *n*=427 DA, *n*=328 ADO/DA), with ADO (54 ± 2 s), DA (252 ± 18 s), and coincident release transients (339 ± 25 s) significantly different (Games- Howell's multiple comparison's test, ADO vs ADO/DA and ADO vs DA p<0.0001, ADO/DA vs DA p=0.0147, Fig. 3.3B). For the distributions of the IETs, there was an overall effect of group, ADO had a very narrow distribution at shorter times with DA and coincident release transients having wider distributions at longer times (Kruskal-Wallis test, p<0.0001, *n*=725 ADO, *n*=427 DA, *n*=328 ADO/DA) (Fig. 3.3C). ADO transients overall occur about once per minute and coincident release transients occur about once every 6 min. Coincident release is only slightly less frequent than DA overall with a large percentage of DA transients (86%) happening simultaneously with ADO.

Next, we examined the concentration of each event. In WT, average ADO transient concentration (290 ±4 nM) was significantly higher than the average DA concentration (28 ±0 nM) (paired t-test, p=0.0001, *n*=11) (Fig. 3.4A). The concentration distributions were also significantly different (K-S test, p<0.0001, *n*=2675 ADO, *n*=445 DA) as the dopamine distribution was more compact around lower concentrations than the broad distribution for ADO at higher concentrations (Fig. 3.4B). The average concentration of each ADO transient during coincident release was 406 ± 67 nM, while ADO transients with no DA averaged 290 ± 44 nM, but the values were not significantly different (Fig. 3.4C) (Welch's t test, p=0.1625, *n*=11). For DA, mean concentration for coincident release was 44 ± 5 nM and the mean for DA without adenosine was 28 ± 3 nM, which was significantly different (Fig. 3.4D) (Welch's t-test, p=0.0125, *n*=11).

To determine if there was a correlation between the concentration of ADO and DA

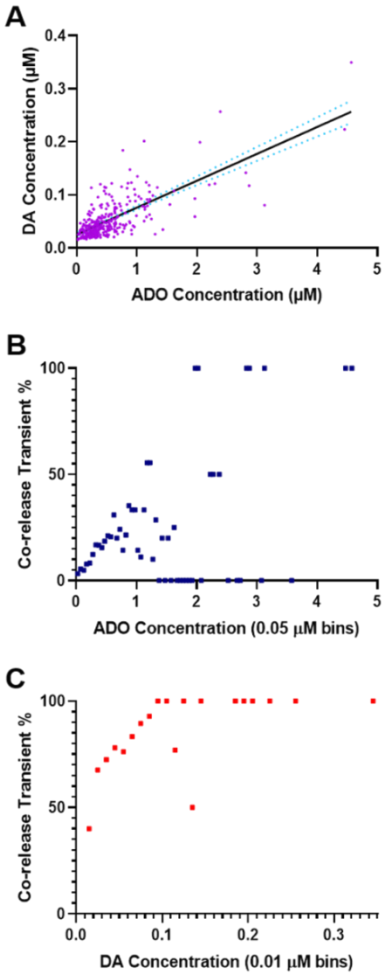


Figure 3.5. Characterization of coincident release for adenosine and dopamine transients in the caudate putamen of mice. (A) Correlation of adenosine and dopamine concentrations during coincident events. There is a significant positive correlation between the ADO concentration and the DA concentration of coincident release transients (Pearson's correlation, r=0.7292, *n*=328) (B) Percentage of ADO events with dopamine co-released by ADO concentration. As the concentration of spontaneous ADO transients increased, the instance of DA coincident release also increased, but was not significantly correlated (Spearman's correlation, r=0.0880, *n*=55). (C) Percentage of DA events with ADO co-released by DA concentration. As the concentration of spontaneous DA transients increased, the instance of coincident release with ADO also increased and was significantly correlated to transient concentration (Spearman's correlation, r=0.7632, *n*=20).

transients during a coincident release event, a correlation plot was made. The DA concentration for each event is on the y-axis and ADO concentration is on the x-axis. In Figure 3.5A, there is a positive correlation between ADO and DA concentrations, with an R^2 value of 0.5317 (Pearson's correlation, $p<0.0001$, $n=328$).

In order to discern if there was a correlation between the incidence of coincident release and transient concentration, the concentration of all transients was binned and plotted vs the percentage of transients that were coincident. In Figure 3.5B, ADO concentration is plotted in 50 nM bins and the y-axis is the percent of transients in that bin that had DA coincident release. As concentration of the ADO transient increases, the percentage of transients with DA coincident release also increases. Above 3 μM, ADO had coincident DA release more than 60% of the time, despite the average rate of coincident release being 27 ± 4%. Thus, larger ADO transients are more likely to have simultaneous DA release than the smaller events. However, the correlation of the ADO concentration with the coincident release percentage is not significant (Spearman's correlation, $p=0.5228$, $n=55$ transients). Fig. 3.5C plots a similar plot, but with DA concentration is on the x-axis 10 nM bins. Above 90 nM for DA, 93% of DA events have simultaneous ADO, which is larger than the mean coincident release rate of 86 ±4%. There was a significant correlation between the rate of coincident release and DA concentration (Spearman's correlation, $p<0.0001$, $n=20$ transients). Thus, large DA transients were more likely to coincide with ADO events and in general, most DA transients observed in these anesthetized mice also had ADO transients.

3.2.3 Adenosine Receptors Modulate Coincident Release Events

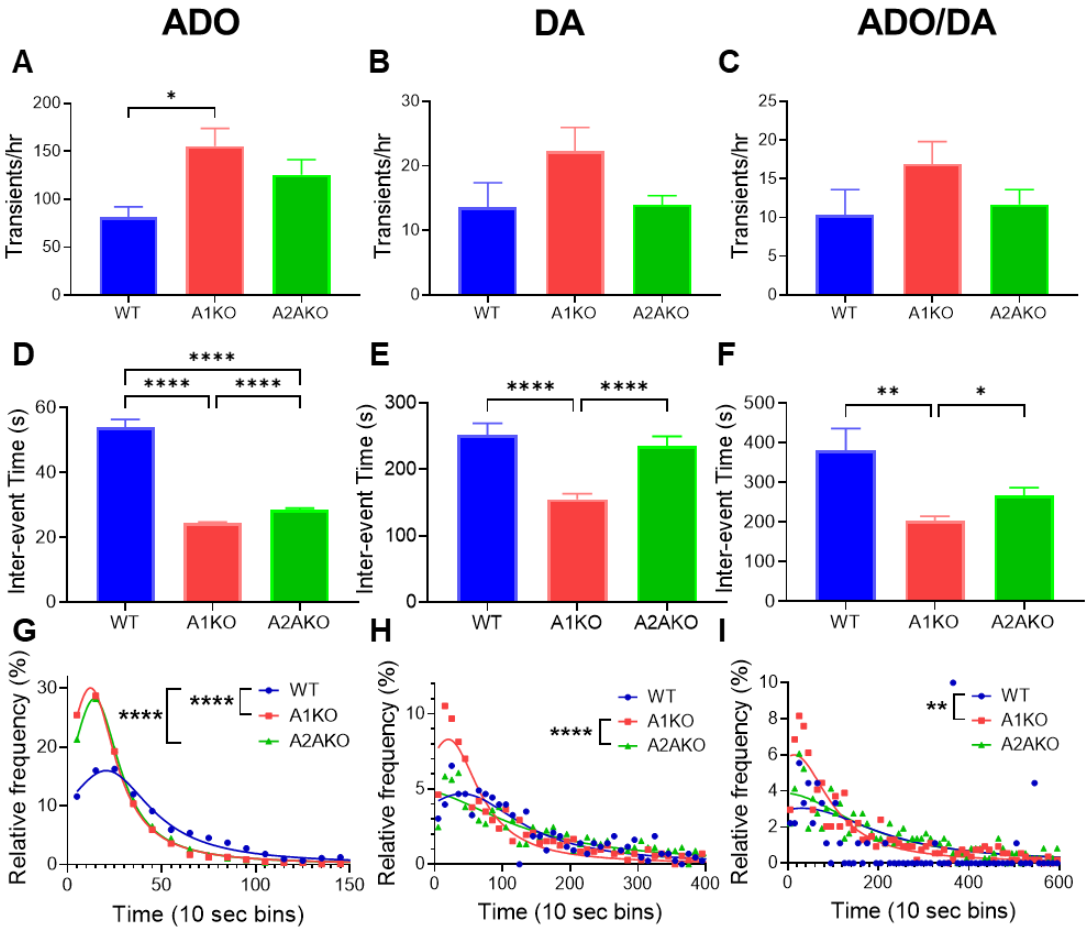


Figure 3.6. Characterization of adenosine/dopamine coincident release frequency in the caudate putamen of A_1KO /A_{2A}KO mice. (A) ADO transients per hour. There was a main effect of number of ADO transients per hour (Brown-Forsythe ANOVA, p=0.0066, *n*=11 WT, *n=8* A_1KO & A_{2A}KO) and A_1KO was significantly higher than WT (Dunnett's T3 post-test, p=0.0136, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). (B) DA transients per hour. For DA, there were no significant differences found (Brown-Forsythe ANOVA, p=0.1379, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). (C) ADO/DA coincident release transients per hour. In coincident release, no significant differences were found (Brown-Forsythe ANOVA, p=0.2441, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). (D) ADO inter-event time. There was an overall effect of mean inter-event time for ADO transients (Brown-Forsythe ANOVA, Games-Howell's test p<0.0001, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). WT was significantly longer than both A_1KO and A_{2A}KO, which were also significantly different from each other. (E) DA inter-event time. For DA, there was a significant main effect of mean IET (Brown-Forsythe ANOVA, Games-Howell's test, p<0.0001, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO), with WT being significantly longer than A_1KO, but not A_{2A}KO, and A_1KO being significantly shorter than A_{2A}KO. (F) ADO/DA coincident release inter-event time. The mean IET of the coincident release transients had a significant main effect (Brown-Forsythe ANOVA, Games-Howell's test, p=0.0006, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO), with A_1KO being significantly different from WT and A_{2A}KO. (G) ADO inter-event time distribution. The IET distributions for ADO had a significant overall effect (Kruskal-Wallis, Dunn's post-test, p=0.0189, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO) with WT being significantly wider than A_1KO. (H) DA inter-event time distribution. The distributions for DA had no significant difference for IET distribution (Kruskal-Wallis, p=0.6102, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). (I) ADO/DA coincident release inter-event time distribution. The coincident release transient IET also showed no significant differences (Kruskal-Wallis, p=0.6102, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). Graphs are marked *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001

In order to better understand the regulation of ADO and DA coincident release, we also collected data on adenosine and dopamine release in A_1KO and A_{2A}KO mice.

We predicted that global knockout of the A_1 receptors would result in lower concentrations of phasic DA transients, because previous studies showed transient adenosine decreased electrically-stimulated dopamine release[3]. We also hypothesized that global KO of A_{2A} receptors would have no effect on DA. Overall, there was a main effect of number of ADO transients per hour by genotype (Brown-Forsythe ANOVA, p=0.0066, *n*=11 WT, *n=8* A_1KO & A_{2A}KO) and the number of transients in A_1KO (155 ± 18 transients/hr) was significantly higher than WT (81 ± 11 transients/hr) (Brown-Forsythe ANOVA, p=0.0136, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO), but the A_{2A}KO (126 ± 16 transients/hr) was not different than WT (Fig. 3.6A). For DA (Fig. 3.6B), the number of transients per hour was 14 ± 4 for WT, 22 ± 4 for A_1KO, and 14 ± 1 for A_{2A}KO. There was not a significant main effect of genotype on number of DA transients (Brown-Forsythe ANOVA, p=0.1379, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). For coincident events, the findings were similar to that of DA with 10 ± 3 transients/hr for WT, 17 ± 3 for A_1KO, and 12 ± 2 for A_{2A}KO, with no significant main effect (Brown-Forsythe ANOVA, p=0.2441, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO).

When inter-event time is examined, the differences become more pronounced. There was an overall effect of genotype on mean IET for ADO transients (Fig. 3.6D) (Brown-Forsythe ANOVA, p<0.0001, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO) and with multiple comparisons, the mean IET in WT (Games-Howell's post-test, 54 ± 2 s) was significantly longer than both A_1KO (24 ± 0.3 s) and A_{2A}KO (29 ± 0.5 s), which were also significantly different from each other (Fig, 3.6D). For DA, there was also a significant main effect of genotype on mean IET (Brown-Forsythe ANOVA, p<0.0001, *n*=11 WT, *n*=8 A_1KO and A_{2A}KO). A_1KO (155 ± 8 s) and A_{2A}KO (236 ± 14 s) IETs were significantly different, and

A_1KO, but not A_{2A}KO was significantly different from WT (252 ± 18 s) (Games-Howell's post-test, Fig. 3.6E). For ADO/DA coincident transients, there was a main effect of genotype on mean IET (Brown-Forsythe ANOVA, p=0.0006, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO), and WT (339 ± 25 s) was significantly different from A_1KO (203 ± 12 s), but not A_{2A}KO (267 ± 20 s), and there was a significant difference between A_1KO and A_{2A}KO (Games-Howell's test, Fig. 3.6F). Overall, transient ADO had a main effect of frequency, which was higher in A_1KO & A_{2A}KO mice with transients occurring at around twice the

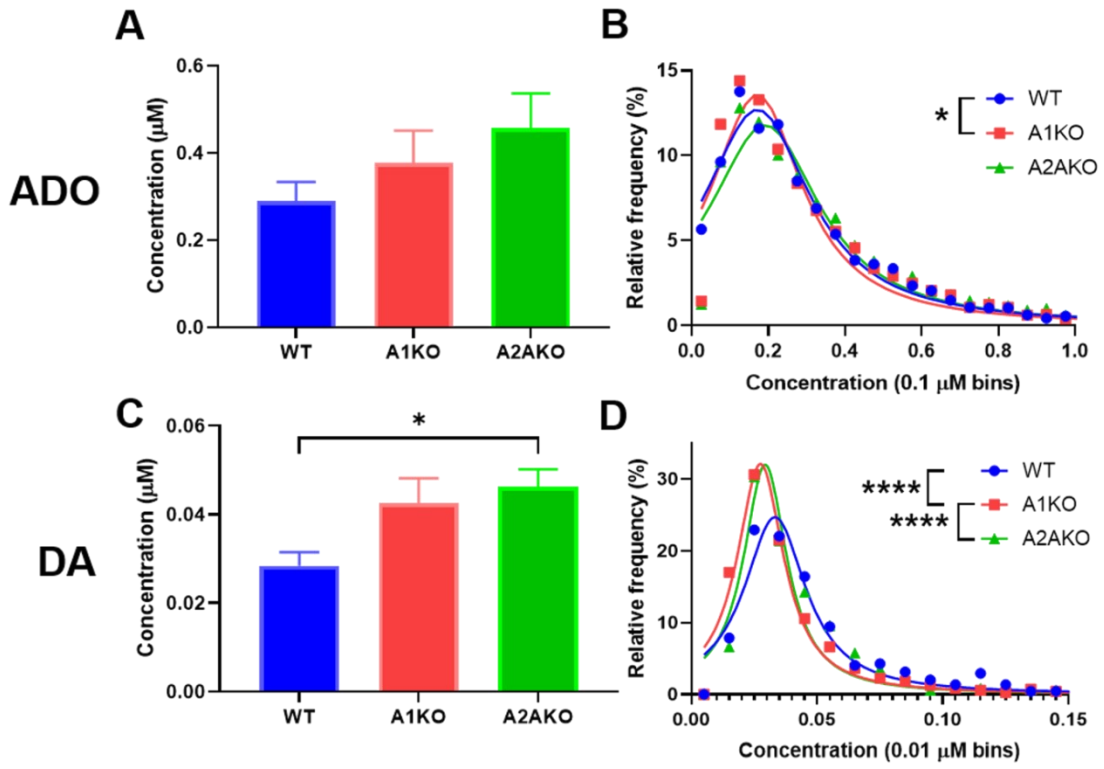


Figure 3.7. Comparison of spontaneous adenosine, dopamine, and coincident release transient concentrations in the caudate putamen of A_1KO /A_{2A}KO mice. (A) ADO concentration. There were not significant differences in ADO transient concentration across all three genotypes (Brown-Forsythe ANOVA, p=0.2193, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). (B) ADO concentration distribution. The distribution of the ADO transient concentrations did show a significant main effect, and A_{2A}KO was also significantly different from both WT and A_1KO by (Kruskal-Wallis, Dunn's post-test, p<0.0001, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO) (right). (C) DA concentration. There was a main effect of mean DA transient concentration (Brown-Forsythe ANOVA, Dunn's post-test, p=0.0160, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO) and A_{2A}KO mean concentration was significantly higher than WT, but neither was significantly different from A_1KO. (D) DA concentration distribution. The distribution also showed a main effect (Kruskal-Wallis, Dunn's post-test, p<0.0001, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO) with A_1KO being significantly different from both WT and A_{2A}KO having more lower concentration transients than both other genotypes. Graphs are marked *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001

rate than in WT (Brown-Forsythe ANOVA, p=0.0005, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). For DA, there was also a main effect of frequency and A_1KO had significantly more transients than A_{2A}KO, but neither KO was significantly different from WT (Brown-Forsythe ANOVA, p=0.0127, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). For the IET distributions, there was an overall significant effect of genotype for ADO (Kruskal-Wallis, p=0.0189, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO) with WT being significantly wider than A_1KO (p=0.0148, Fig. 3.6G). The distributions for DA were not significantly different (Fig. 3.6H, Kruskal-Wallis, p=0.6102, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). The coincident ADO/DA transient IET distributions also showed no significant differences (Figure 3.6I) (Kruskal-Wallis, p=0.6102, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO).

We next compared the transient concentrations in WT, A_1KO, and A_{2A}KO for both ADO and DA. Figure 3.7A shows no significant differences in ADO concentration across all three genotypes with WT at 290 ± 40 nM, A_1KO at 378 ± 70 nM, and A_{2A}KO at 457 ± 80 nM (Brown-Forsythe ANOVA, p=0.2193, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO). However, the distribution of the ADO transient concentrations did show a significant main effect (Kruskal-Wallis, p=0.0349, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO), and A_{2A}KO was significantly different from WT (Dunn's multiple comparisons test, p=0.0348) (Fig. 3.7B). For DA transients, there was a main effect of genotype on mean DA concentration (Brown-Forsythe ANOVA, p=0.0160, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO) and A_{2A}KO (46 ± 4 nM) concentration was significantly higher than WT (28 ± 3 nM, p=0.0100), but A_1KO (43 ± 6 nM) was not significantly different than WT or A_{2A}KO (Fig. 3.7C). The distribution of concentrations also showed a significant main effect of genotype (Kruskal-Wallis, p<0.0001, *n*=11 WT, *n*=8 A_1KO & A_{2A}KO) and A_1KO was significantly different from both

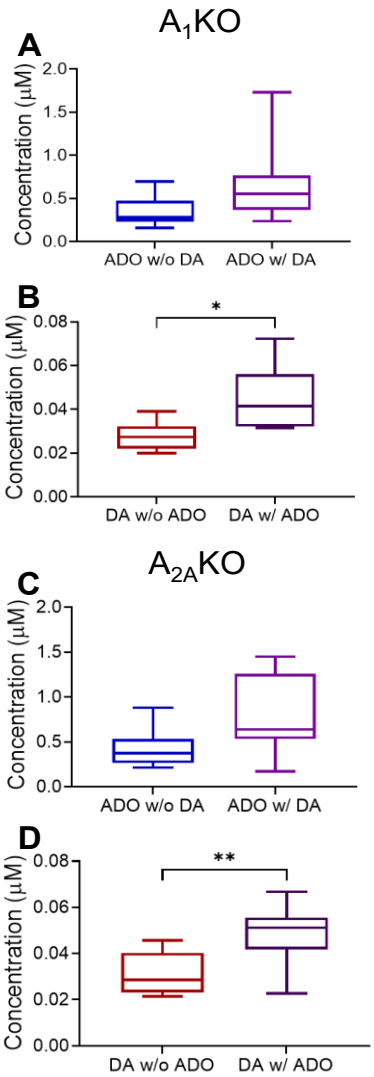


Figure 3.8. Comparison of spontaneous adenosine and dopamine transient concentrations with and without coincident release in A_1KO /A_{2A}KO mice. (A) In A_1KO, ADO transients without DA present were not significantly different than those with coincident release (Welch's t-test, p=0.0967, *n*=8). (B) The DA concentration of coincident release transients for A_1KO was significantly higher than that of DA alone (Welch's t-test, p=0.0121, *n*=8). (C) For A_{2A}KO, coincident release concentration is not significantly different from ADO concentration alone (Welch's t-test, p=0.0702, *n*=8). (D) In A_{2A}KO coincident release concentration was significantly higher than DA alone (Welch's t-test, p=0.0085, *n*=8). Graphs are marked *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001

WT and A_{2A}KO, as it was distributed around lower concentration transients (Dunn's multiple comparisons test, p<0.0001) (Fig, 3.7D).

In Figure 3.8, the concentrations of ADO and DA are compared when they occurred with and without coincident events. In A_1KO, ADO transients without DA present (342 ± 63 nM) were not significantly different than those with DA present (290 ± 44 nM) (Fig. 3.8A) (Welch's t-test, p=0.0967, *n*=8). For A_{2A}KO, the results are similar with no significant differences for ADO alone (426 ± 76 nM) compared to ADO with DA (778 ± 156 nM) (Fig. 3.8C) (Welch's t-test, p=0.0702, *n*=8). The concentration of DA was also compared and in A_1KO the concentration of DA alone (28 ± 2 nM) was lower than when DA occurred with ADO (45 ± 5 nM) (Fig. 3.8B) (Welch's t-test, p=0.0121, *n*=8). This trend was similar in A_{2A}KO, where DA alone (31 ± 3 nM) was also significantly lower than when it occurred with ADO (48 ±

5 nM) (Fig. 3.8D) (Welch's t-test, p=0.0085, *n*=8).

In Figure 3.9, the ADO and DA concentrations are plotted to examine correlations. The WT (blue, r^2=0.5317), A_1KO (red, r^2=0.6223), A_{2A}KO (green, r^2=0.6468) all show a positive correlation between ADO concentration and DA concentration in coincident transients (Pearson's correlation, p<0.0001, *n*=328 WT, *n*=540 A_1KO, *n*=362 A_{2A}KO). Examining percentage of events of coincident events at different concentrations, in A_1KO, 27 ± 3% of ADO events have coincident DA events but above 3 µM ADO, the percentage rises to 64 ± 10%. In A_1KO mice, 91 ± 5% of DA transients are coincident with ADO, and for DA concentrations above 90 nM, that percentage is slightly higher at 94 ± 5%. In A_{2A}KO, 18 ± 3% of ADO transients are coincident with DA, but for ADO transients above 3 µM, only 25 ± 8% have DA coincident, a big difference from A_1KO and WT. For DA, 94 ± 3% of the DA transients in A_{2A}KO have a coincident ADO transient, and above 90 nM DA 100 % had a coincident ADO transient.

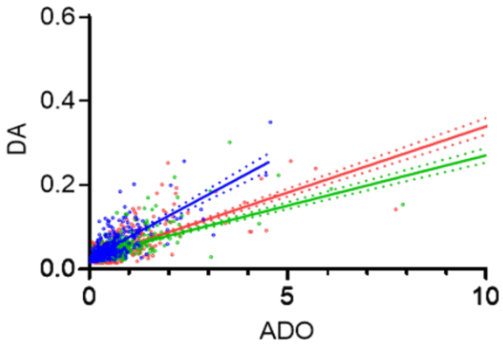


Figure 3.9. Comparison of coincident release transient concentration correlation in C57BL, A_1KO, and A_{2A}KO mice. The WT (blue, r^2=0.5317), the A_1KO (red, r^2=0.6223), A_{2A}KO (green, r^2=0.6468) all show a positive correlation between ADO concentration and DA concentration in coincident release transients (Pearson's correlation, p<0.0001, *n*=328 WT, *n*=540 A_1KO, *n*=362 A_{2A}KO).

3.3 Discussion

In this study, we characterized coincident, spontaneous adenosine and dopamine release events in the caudate putamen for the first time. About one-quarter of ADO transients were accompanied by DA release and the concentration of the DA

correlated strongly to that of the ADO. However, about 86% of DA release events were coincident with ADO release. A_1 receptor deletion increased the frequency of both ADO and DA transients while the A_{2A} receptor knockout increased DA concentration and the occurrence of coincident release with DA. Thus, spontaneous ADO and DA transients occur together, A_1 receptors regulate transient frequency, and A_{2A} receptors regulate DA transient concentration. These results demonstrate coincident release of adenosine and dopamine and modulation of dopamine by the adenosine system in real time and suggest ATP is packaged with dopamine in order to modulate the transient coincidentally.

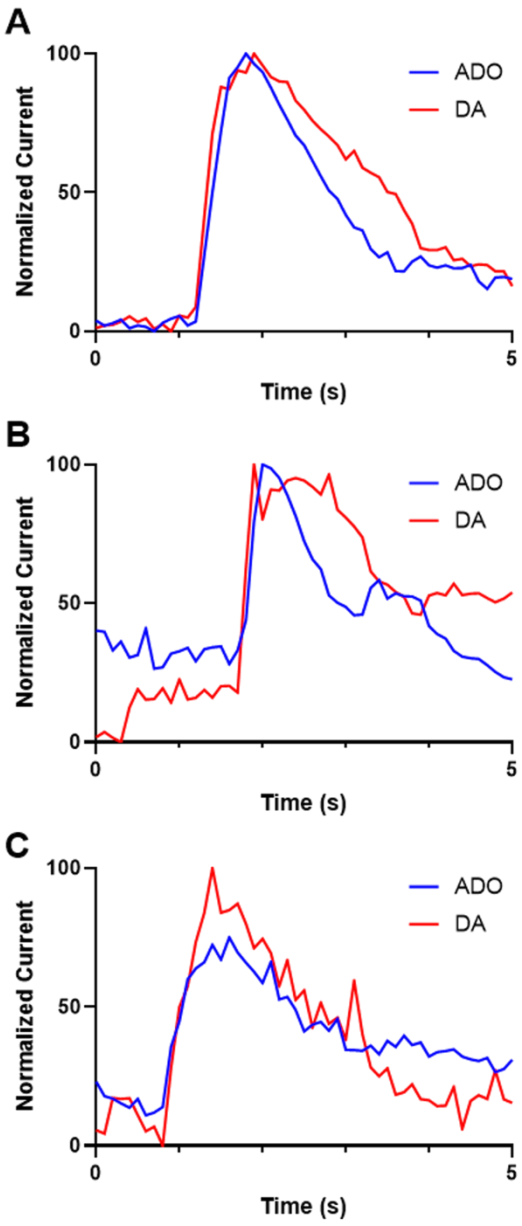


Figure 3.10. Comparison of timing of analyte detection during coincident release in WT mice. Three different coincident transients (A, B, C). Currents are normalized and ADO and DA are overlaid in time. The ADO and DA transients occurred concurrently with our 100 ms time resolution.

3.3.1 Adenosine and Dopamine Transients Occur at the Same Time

Adenosine in the extracellular space is formed mainly from the rapid breakdown of ATP[11,49], which can be released from vesicles by

exocytosis[14,50]. Adenosine is also released by exocytosis, although there is less evidence this is a major mechanism of adenosine release[12]. There is evidence that ATP and DA are co-released from vesicles in dopaminergic neurons,[14–16] and this co-release could cause the coincident transients we observed. Because ATP breakdown to adenosine is so rapid in the brain, we would expect dopamine and adenosine concentrations to rise simultaneously if dopamine was co-released with ATP. We looked at the time course of the coincident events and found that the rise in adenosine and dopamine concentrations happens concurrently, instead of one neurochemical obviously increasing before the other (Fig. 3.10). For both adenosine and dopamine, transients with larger concentrations are more likely to be coincident transients. The larger release events likely happen closer to the electrode, and thus it is less likely that analyte is lost to diffusion or reuptake before being detected, so these events are easier to see. While most of the dopamine events had a coincident adenosine event, due to dopamine co-released with adenosine or ATP, many of the adenosine events (75%) did not have a coincident dopamine event. It is possible that these are coincident dopamine-adenosine events, but the dopamine is taken up quickly and not observed at the electrode. Given the high percentage of events without dopamine, this is not likely. The more probable explanation is that adenosine or ATP is also co-released from other vesicles that do not have dopamine. Indeed, ATP is also co-packaged into other vesicles, such as acetylcholine vesicles.[51]

Another important observation that supports that adenosine or ATP and dopamine may be co-released is that spontaneous ADO does not reduce the concentration of spontaneous DA transients. Previously, when adenosine was puffed on before stimulated dopamine release, the concentration of stimulated dopamine release

was reduced by half.[3] A_1 receptors do not dampen dopamine release that is coincident with adenosine, because large dopamine events are observed when large adenosine events occur and concentrations of dopamine during co-release trend higher than when dopamine is not coincident with adenosine. Thus, the time course and concentration dependence are consistent with co-release of dopamine and adenosine, but additional experiments would be needed to prove the events came from the same vesicular release event.

3.3.2 A_1 Receptors Regulate Coincident Release Frequency

A_1 receptors are the most common adenosine receptor in the brain and inhibit adenylyl cyclase activity[52]. They are located presynaptically[53] and inhibit vesicular release of both ATP and glutamate.[54] Previous studies have shown spontaneous ADO formation is due to rapid breakdown of ATP in the extracellular space,[9] therefore regulating ATP release will regulate ADO. A_1 receptors have also been previously shown to regulate ADO transient frequency, but not concentration, as the A_1 receptor antagonist, DPCPX increases frequency.[55,56]

In this study, we employed global deletion knockout mice in order to study the effects of the A_1 receptors on spontaneous ADO/DA coincident release. The A_1 receptor KO mice are an effective tool in studying the role of adenosine following traumatic brain injury[57] and in regulating sleep[58]. We had previously observed an increase in adenosine transient frequency in A_1KO mice, and since A_1 receptors downregulate excitatory D_1 receptors and inhibit DA release[59], we also hypothesized there would be more DA transients. In fact, this is exactly what we observed, as both ADO and DA frequency were significantly increased in A_1KO mice compared to WT. In the global A_1 knockout,

there is more exocytosis, in general, leading to increased frequency of both ADO and DA transients, and this would be expected if dopamine and adenosine or ATP were co-released. A_1KO does not alter the content of the vesicles and so the concentration remains unchanged. We have shown previously that A_1 receptors acting presynaptically regulate spontaneous ADO frequency[7], but here we now show that these receptors also regulate spontaneous DA and coincident release frequency as well.

3.3.3 A_{2A} Receptors Regulate Dopamine Concentration

A_{2A} receptors are very abundant in the brain, particularly the caudate-putamen, although they are not as abundant as A_1 receptors.[60] A_{2A} receptors are excitatory and increase adenylyl cyclase activity, which in turn activates the cAMP pathway. In the caudate, A_{2A} receptors are most commonly located postsynaptically, but they are also located presynaptically, where they regulate glutamate release through their interactions with A_1 and other receptors in heteromers.[61] A_{2A}KO mice have been successfully used to study behavior, and A_{2A}KO worsens the severity of motor function in mice models of Huntington's disease.[62] A_{2A}KO also causes increased severity in morphine withdrawal symptoms.[63] Here, A_{2A}KO mice had a higher number of ADO transients and shorter inter-event time than WT, but frequency was unchanged for DA and coincident release. This suggests that in A_{2A}KO there is an increase in adenosine or ATP being released, but not an increase in co-release from the same vesicle, as the ADO and DA frequency did not both increase. A_{2A}KO mice did not show a significant difference in ADO concentration, but DA transients were significantly larger on average and had a wider distribution of values. This is likely due to the antagonistic nature of A_{2A} and D_2 receptors in striatal neurons, where in the absence of A_{2A} receptors, D_2 receptors can excite more

DA release.[64] The concentration of ADO transients during coincident release was significantly lower in A_{2A}KO than either WT or A_1KO, which is contrary to our previous findings that A_{2A}KO had higher mean concentration than WT or A_1KO.[7] In the future, in order to better understand these coincident release events, the dopaminergic system could be investigated to see how coincident transients are affected. A D_2 receptor antagonist, such as haloperidol, would further illuminate the role of A_{2A} and D_2 receptors in regulating coincident release and clarify the role they play in neurotransmitter modulation.

3.3.4 Implications of adenosine and dopamine release events

Adenosine-based therapeutics have been proposed as treatments for several neurological diseases,[54] and many of the diseases where adenosine could act as a neuroprotective affect the dopaminergic system. For example, the A_{2A} antagonist istradefylline reduces daily OFF time in Parkinson's patients.[65] In schizophrenia, targeted regional ADO therapies have ameliorated the severity of symptoms and increased the response to therapeutics.[66] Additionally, both A_1 and A_{2A} receptors play a role in modulating psychostimulant addiction.[67] Rapid ADO is important in providing neuromodulation in these diseases, and could provide a non-dopaminergic drug target, which could reduce the development of tolerance to the treatment.[68] Our studies show that adenosine and dopamine transients are interconnected and that modulating adenosine may also modulate rapid dopamine release. In addition, treatments that target dopamine may also affect adenosine or ATP release if they are co-released, which could affect its neuromodulation. Thus, this study provides a better understanding

of interactions of adenosine and dopamine systems, which are critical for development of new therapies.

3.4 Conclusions

In this study, we investigated the coincident release of spontaneous dopamine and adenosine transients to understand how they are regulated. We found that most (86%) of the DA events happened within 5 s of an ADO event, although only about ~30% of ADO events had a coincident DA event. The DA concentration was higher during coincident release than without any ADO and there is correlation between the concentration of the ADO transient and the concentration of the DA transient. A_1 receptors affect the frequency of ADO transients and the DA transients, but A_{2A} receptors influence the concentration of those DA transients and the incident rate at which coincident release occurs. Thus, spontaneous adenosine and dopamine events occur simultaneously and their interactions may be important for pharmacological treatments that depend on DA and ADO system interaction.

3.5 Methods

3.5.1 Animals & Surgery

Male and Female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and A_1 receptor knockout mice, and A_{2A} receptor knockout mice were obtained from Dr. S. Jamal Mustafa, West Virginia University,[40,41] and housed on a 12:12-h light/dark cycle with food and water provided *ad libitum*. Six to eight week old mice were anesthetized with 4% isoflurane in 100% oxygen for induction and anesthesia, and maintained with 1.5–3% in 100% oxygen delivered via a facemask

(Stoelting, Wood Dale, IL, USA). A heating pad was used to sustain mouse body temperature around 37 °C. The surgical site was shaved and bupivacaine (0.10 mL, APP Pharmaceuticals, Schaumburg, IL, USA) was administered under the skin for local anesthesia. In a stereotaxic frame, the skull was exposed and holes were drilled to allow the placement of the electrode in the caudate putamen (AP +1.1 mm, ML + 1.5 mm, and DV -3.0 mm). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia.

3.5.2 Chemicals

Electrodes were calibrated in phosphate-buffered saline (PBS) solution (3.0 mM KCl, 10.0 mM NaH_2PO_4, 2.0 mM $MgCl_2$, 131.25 mM NaCl and 1.2 $CaCl_2$, all from Fisher, Fair Lawn, NJ) with pH adjusted to 7.4. A 10.0 mM stock solution of adenosine (Sigma Aldrich, Milwaukee, WI, USA) and a 10.0 mM stock solution of dopamine (Sigma Aldrich) were prepared separately in 0.1 mM $HClO_4$ and these were diluted daily in PBS solution to 1 μM for calibration of the electrodes.

3.5.3 Electrochemistry

Fabrication of carbon-fiber microelectrode with T-650 carbon fiber was previously described[42]. Cylinder electrodes 150–200 μm long and 7 μm in diameter were used. Fast-scan cyclic voltammetry was used to detect adenosine and dopamine[42,43] and the electrode was scanned from −0.4 V to 1.45 V and back to −0.4 V at 400 V/s with a frequency of 10 Hz .

3.5.4 Data analysis and statistics

Electrodes were implanted and equilibrated for at least 30 min with the applied waveform prior to data collection. Data were excluded if fewer than 10 transients were observed within the initial 30 min. If robust transients were not found, a new electrode was inserted, up to five new electrodes for each animal. After initial adenosine transients were identified, the electrode placement was optimized and data were collected for 4 hr. Some of the data analyzed here was collected for previously published studies, specifically the data in A_1KO and A_{2A}KO mice.[7] However, this is the first time the data has been analyzed to look for dopamine, and new analysis techniques based on structural similarity algorithms were used.[44]

Transient adenosine and dopamine events were identified and characterized using structural similarity image analysis[44] and adenosine, dopamine, and co-release events were confirmed by an analyst to exclude any signals that were erroneous. The primary oxidation peaks of adenosine and oxidation peaks of dopamine were identified using a library of 15 verified adenosine and dopamine peaks. Co-release transients were identified and defined as transients in which an adenosine and a dopamine transient occurred within 5 s of each other. All statistics were performed in GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). All data are shown as mean ± SEM. Statistical significance was designated at $p < 0.05$.

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Chapter 4

Cocaine-Haloperidol Reduces Spontaneous Transient Adenosine Frequency in the Caudate

""You may encounter many defeats, but you must not be defeated. In fact, it may be necessary to encounter the defeats, so you can know who you are, what you can rise from, how you can still come out of it."
— Maya Angelou

Chapter 4: Cocaine-Haloperidol Reduces Spontaneous Transient Adenosine Frequency in the Caudate

Abstract

The relationship between adenosine and dopamine signaling has been investigated in depth over the last 20 years, but mainly from the perspective of how adenosine affects dopamine. In this study, we use the uptake inhibitor cocaine, and the D_2 antagonist haloperidol to investigate how spontaneous adenosine transients are affected by the increase in dopamine levels and signaling. We found a reduction of 20% in the number of spontaneous adenosine transients per hour following application of cocaine/haloperidol with a similar 20% increases in the time interval between transients. There was no observable difference in adenosine transient concentration for the pre-drug, during cocaine administration alone, or after cocaine/haloperidol. These findings were surprising, as based on our previous research, we found that dopamine release is coincident with adenosine 86% of the time, and it was therefore thought that an increase in dopamine would lead to an increase in adenosine. This was also thought to be the case because the application of a D_2 receptor antagonist, like haloperidol, should prevent D_2 antagonism of excitatory A_{2A} receptors, which would increase adenosine transient frequency. This new discovery in discreet adenosine signaling and its relationship to dopamine signaling could provide insights into the pharmacodynamics of drugs of abuse and addiction and also advise caution in the development of adenosine and dopamine therapies.

4.1 Introduction

Adenosine-dopamine pharmacodynamics has been studied for the treatment of dopamine diseases for over 20 years. In that time, understanding of both the adenosine and dopamine systems has expanded significantly. Dopamine modulation by adenosine is likely through adenosine-dopamine receptor relationships, specifically A_1-D_1 and A_{2A}-D_2 receptor pairings in the brain. [1,2] Adenosine/dopamine receptor pairs have antagonistic relationships that allow adenosine to control the level of dopamine through activation of adenosine receptors. [3] Receptor activations cause allosteric changes in the receptor complex that alter affinity and G-protein coupling in the connected receptor. [4] Through these mechanisms, adenosine regulation of dopamine controls excitability of spinal motor neurons, [5] affects cocaine addiction behaviors, [6] and affects severity of symptoms in Huntington's disease. [7] Our lab has found that stimulated adenosine modulates electrically evoked dopamine transients in brain slices through A_1 receptors, [8] which prompted further investigation into the adenosine/dopamine interaction *in vivo* in real time. We then found that most (86%) of dopamine release measured was correlated with adenosine release and that changes to the adenosine system via A_1 and A_{2A} receptor global knockouts also affected the dopamine release (Chapter 3). What remained unanswered was how changes in the dopaminergic system would affect spontaneous adenosine transients.

Adenosine, as a neuromodulator, has roles in immune response, [9] cancer detection, [10–12] and modulation of dopamine. [8,13–15] Adenosine has two modes of signaling, fast and slow, with more contemporary research focusing on spontaneous transient adenosine. [16–19] Spontaneous adenosine transients last only a few seconds

having concentrations in the hundreds of nM. [19] The transients are random in frequency, but are modulated by A_1 receptors [20,21] and are not activity dependent. [22] Though the release mechanism of these spontaneous transients is not fully understood, evidence suggests the largest contributor is exocytosis of ATP, [22–24] which is then rapidly broken down to adenosine in the extracellular space. [25] Adenosine is also released vesicularly [26] and to some extent through active and concentrative membrane transport. [23,26,27] While our lab has built a picture of adenosine regulation, how dopamine might regulate adenosine has not been addressed.

The monoamine neurotransmitter, dopamine, is one of the most studied molecules in the brain and plays a key role in addiction, [28–30] memory, [31,32] Parkinson's disease, [33,34] and schizophrenia. [35,36] In order to study the role of dopamine in these functions and disorders, it has been necessary to utilize drugs to manipulate the dopaminergic system and observe the changes that were evoked. The two main classes of drugs used for this purpose are monoamine uptake inhibitors [37,38] and dopamine receptor agonist/antagonists. [39,40] Uptake inhibitors block the dopamine transporter protein from removing dopamine from the extracellular space by competitive inhibition. [41] Dopamine receptor drugs work by activating (agonists) or blocking activation (antagonists) of dopamine receptors. [42] By using these drugs together, such as the uptake inhibitor cocaine and the D_2 receptor antagonist haloperidol, it is possible to reach a state of increased dopamine activity that is more readily measured in the extracellular space. [40,43] Dopamine has three modes of release: tonic, phasic without burst-fire, and phasic with burst-fire. [36] The most relevant mode is burst fire, since the increase in dopamine transients after addition of cocaine/haloperidol is due to burst

firing, and cocaine/haloperidol is what will be used to alter the dopaminergic system in this instance. [44] With volume transmission of dopamine in the striatum correlated with the release of adenosine, and altered by adenosine receptors, (Chapter 3) the unanswered question is: How do dopamine drugs like cocaine and haloperidol, and these transients affect spontaneous transient adenosine? To investigate this, we administered first cocaine, then haloperidol via i.p. injection and observed adenosine transients in the caudate putamen of anaesthetized wild type (WT) mice. What we found was that the frequency of spontaneous adenosine transients decreases under cocaine/haloperidol as compared to in animal control. This find is important because it is contrary to our current understanding of how the adenosine and dopamine systems interact and previous findings of studies of long-term exposure to cocaine or haloperidol.

4.2 Results

4.2.1 Co-Detection of Spontaneous Adenosine and Dopamine *In Vivo*

Adenosine and dopamine were simultaneously measured using fast-scan voltammetry (FSCV) at a carbon-fiber microelectrode, which allows real-time measurement of discrete concentration changes of both analytes with subsecond temporal resolution. This technique is ideal for observing rapid changes in analyte concentration, but is unable to measure basal level concentrations due to background subtraction.[45] Simultaneous detection of dopamine and adenosine with FSCV was demonstrated previously by our lab [46,47](Chapter 3) and by Kendall Lee's lab.[48] Measurements were made in the caudate putamen because it is rich in dopaminergic

neurons, has been the target of dopamine research [8,49,50], and also exhibits spontaneous adenosine release and high expression of adenosine receptors [8,20,47,51].

The carbon-fiber microelectrode was scanned from -0.4 V to 1.45 V and back at 400 V/s and 10 Hz. Adenosine undergoes two oxidation reactions to produce the characteristic two peaks at 1.3 and 1.0 V. Dopamine undergoes a redox reaction where dopamine is oxidized to dopamine-o-quinone, with a peak around 0.6 V, and DOQ is reduced back to dopamine with a peak around -0.2 V. Figure 4.1 shows a representative false color plot of a typical three-minute file of adenosine/dopamine measurement. The false color plot displays all the data, allowing changes in current due to the applied voltage to be visualized over time. Peaks for both DA and ADO are evident at different voltages. The ADO primary oxidation peak appears around 1.3 V on the back scan. The color plot at the top (Fig. 4.1A) is typical of the raw data that is exported by the HDCV program, while the bottom color plot (Fig. 4.1B) shows the color plot following being run through our image similarity search index program in

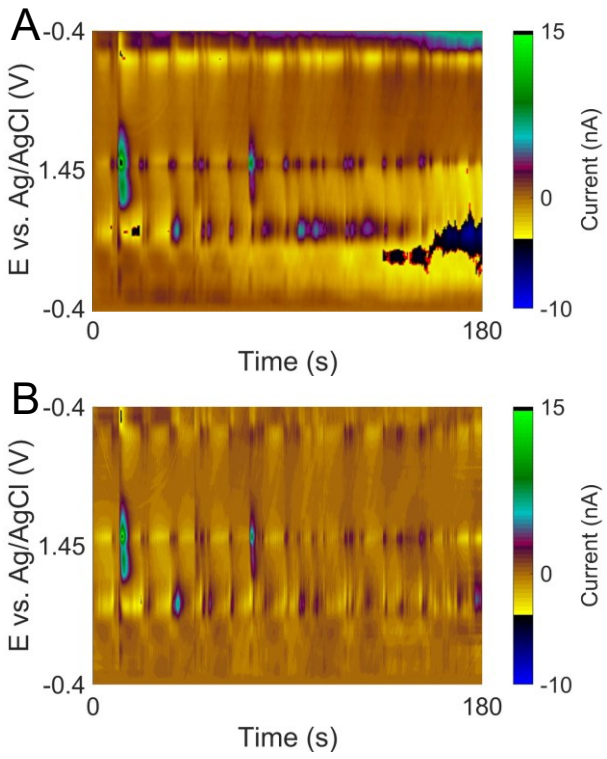

Figure 4.1. Comparison of fast-scan cyclic voltammetry color plot data from before and after application of Butterworth filter. (A) Representative false color plot data of spontaneous adenosine and dopamine transients following cocaine and haloperidol (10 mg/kg, 0.05 m/mL i. p.) injection. The y-axis is applied voltage, the x-axis is time, and the current in represented in false color. (B) The same false color plot data as in A, after being run through the Butterworth filter, showing the signal loss for the observed dopamine transients.

MATLAB. This program also includes smoothing and removal of background shifts through the utilization of a Butterworth filter.[52] Due to the nature of this filtering, all of the longer (> 8.5 s) dopamine transients in the data are filtered, making it hard to choose dopamine transients after the pharmacology because the dopamine release was longer. Since the program also does signal smoothing, some of the dopamine transients were also not chosen by the program due to the loss in amplitude moving the transients below the threshold for automatic detection. Therefore, dopamine transient characterization will not be within the scope of this study and the reaction of the adenosine system to cocaine and haloperidol will be the main focus.

4.2.2 Spontaneous Adenosine Transient Characterization After Administration of Cocaine and Haloperidol

The purpose of this experiment was to observe and characterize how spontaneous adenosine transients are affected by changes in the dopamine system. Previous studies have examined the effect that changes in the adenosine system had on dopamine, [3,8,53](Chapter 3) but no previous study has shown how spontaneous

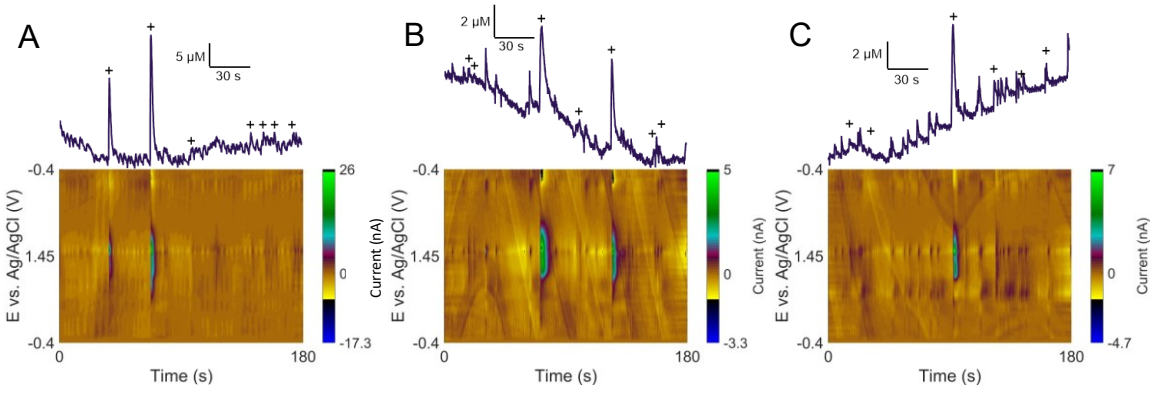

Figure 4.2. Example data from the caudate putamen for pre-drug, cocaine, and cocaine/haloperidol. Concentration traces for adenosine (top) and a 3D color plot (bottom) compare ADO release throughout drug application. ADO transients are marked with plus (+) signs in the concentration traces.

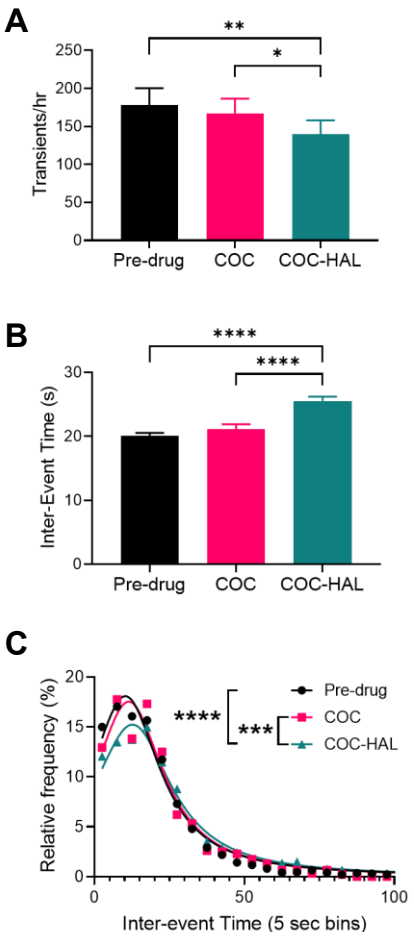

Figure 4.3. Comparison of spontaneous adenosine transient frequency in the caudate putamen between control, 10 mg/kg cocaine, and 10 mg/kg cocaine/0.5 mg/kg haloperidol. (A) Number of transients per hour during control, under cocaine, and under cocaine/haloperidol. There was a significant overall effect on the number of transients per hour (RM one way ANOVA, Tukey multiple comparisons, p=0.0042, n=11), with cocaine/haloperidol having fewer transients than both control and cocaine. (B) Mean inter-event times. There was an overall main effect of the drugs on inter-event time (Brown-Forsythe ANOVA, Games-Howell's multiple comparisons, p<0.0001, n=1961 pre-drug, n=919 COC, n=1537 COC-HAL). (C) Inter-event time distributions. There is an overall main effect of drugs on inter-event time distribution (Kruskal-Wallis test, p<0.0001, n=1961 pre-drug, n=919 COC, n=1537 COC-HAL). The distributions of the IET show a significantly wider distribution of inter-event times for COC-HAL, than either COC or pre-drug. The pre-drug and COC inter-event time distributions are not significantly different. Graphs are marked *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001.

adenosine transients *in vivo* are changed by the addition of monoamine reuptake inhibitors and D_2 receptor antagonists. Figure 4.2 shows typical three-minute false color plot data for the control (Fig. 4.2A), during administration of 10 mg/kg cocaine (Fig. 4.2B), and after cocaine and 0.5 mg/kg haloperidol (Fig. 4.2C). Current vs time traces are above the color plot for the primary oxidation peak potential of adenosine (top) and the oxidation peak of dopamine (middle). Transients identified by the automated algorithm are marked with + for ADO. Spontaneous ADO transients were less frequent in mice under cocaine/haloperidol than in the pre-drug control.

To better understand the effect of dopamine pharmaceuticals on spontaneous adenosine transients, we first analyzed changes in transient

frequency. Absence of A_{2A} receptors increased adenosine transient frequency (Chapter 3) and haloperidol antagonizes D_2 receptors, which in turn antagonize A_{2A} receptors [54]. This led to our hypothesis that spontaneous adenosine transient frequency would increase under cocaine/haloperidol. For data analysis purposes, we use two measures of frequency, inter-event time (IET) and transients per hour. Figure 4.3 shows the effect of drug administration on the frequency of adenosine transients. There was a significant main effect of drugs on the number of adenosine transients per hour (RM one way ANOVA, Tukey multiple comparisons, p=0.0042, *n*=11), individually, with cocaine (167 ± 20 transients/hr) there was no significant difference in the number of spontaneous adenosine transients per hour compared to that of the pre-drug period (178 ± 22 transients/hr). However, after the administration of haloperidol, there were significantly fewer transients (140 ± 18 transients/ hr) than during both pre-drug or cocaine (Fig. 4.3A). When mean IET was compared, the same trends were observed. There was a significant overall main effect of drugs on IET (Brown-Forsythe ANOVA, Games-Howell's multiple comparisons, p<0.0001, *n*=1961 pre-drug, *n*=919 COC, *n*=1537 COC-HAL) with COC-HAL (26 ± 0.7 s) having a significantly longer mean IET than pre-drug (20 ± 0.5 s) (Games-Howell post-test, p<0.0001, *n*=1961 pre-drug, *n*=1537 COC-HAL). COC-HAL was also significantly different from COC (21 ± 0.7 s) (Games-Howell post-test, p<0.0001, *n*=919 COC, *n*=1537 COC-HAL), though the mean IET was not significantly different between COC and pre-drug (Fig 4.3B).

We next compared the concentration of these spontaneous adenosine transients, which can be seen in Figure 4.4. First, we examined the mean concentration per animal

and there was no significant main effect between pre-drug (0.05 ± 0.01 μM) and COC (0.04 ± 0.01 μM), or under COC-HAL (0.06 ± 0.02 μM) (RM one way ANOVA, p=0.1845, *n*=11) (Fig. 4.4A). When examining the concentration data by using every transient individually, there was also no significant difference between pre-drug (0.04 ± 0.002 μM), COC (0.04 ± 0.002 μM), and COC-HAL (0.05 ± 0.003 μM) (Brown-Forsythe ANOVA, p=>0.9999, *n*=1961 pre-drug, *n*=919 COC, *n*=1537 COC-HAL) (Fig. 4.4B). The concentration distributions were also not significantly different (Kruskal-Wallis test, p=0.9573, *n*=1961 pre-drug, *n*=919 COC, *n*=1537 COC-HAL) (Fig. 4.4C). Thus, spontaneous adenosine transient frequency was reduced with dopamine uptake inhibitor and D_2 autoreceptor antagonists, but the concentration was unchanged.

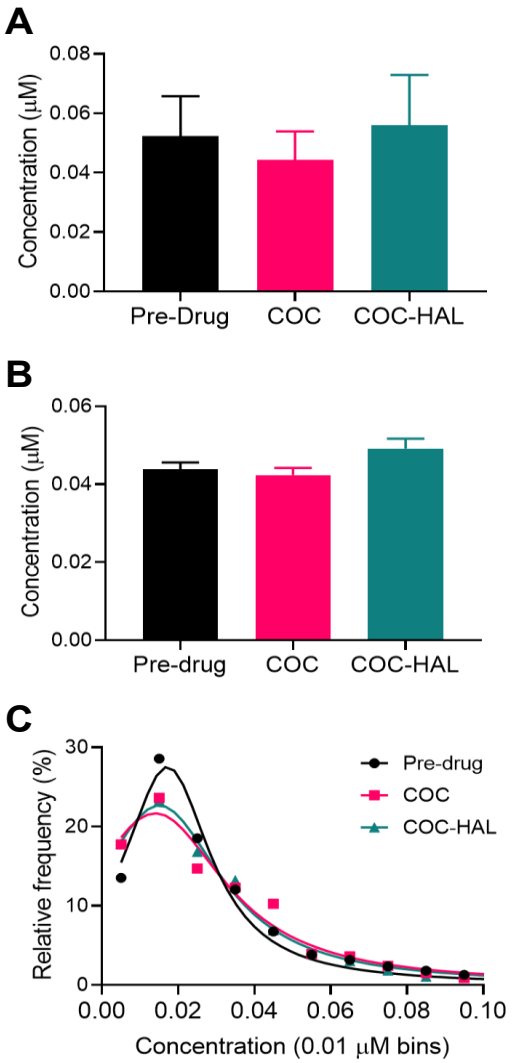

Figure 4.4. Comparison of spontaneous adenosine transient concentration in the caudate putamen between control, 10 mg/kg cocaine, and 10 mg/kg cocaine/0.5 mg/kg haloperidol. (A) Adenosine transient concentration during control, under cocaine, and under cocaine/haloperidol averaged by animal. The mean adenosine transient concentration was not significantly different under drugs than it was during control (RM one way ANOVA, p=0.1845, n=11). (B) Mean adenosine transient concentration by individual transient. There was no significant difference in mean adenosine transient concentration between pre-drug, COC, and COC-HAL (Brown-Forsythe ANOVA, p>0.9999, n=1961 pre-drug, n=919 COC, n=1537 COC-HAL). (C) Adenosine transient concentration distributions. There was no significant difference in adenosine transient concentration distribution (Kruskal-Wallis test, p=0.9573, n=1961 pre-drug, n=919 COC, n=1537 COC-HAL).

4.3 Discussion

In this study, we characterized spontaneous adenosine transients in the caudate putamen after cocaine and haloperidol. The combination of these drugs has been previously shown to increase phasic dopamine release in the caudate. [40] Our research goal was to understand how altering dopamine release changes the frequency or concentration of adenosine transients. Cocaine, a monoamine reuptake inhibitor, did not significantly change the number of transients per hour or the amount of time between transients. Haloperidol, a D_2 receptor antagonist, when combined with cocaine, reduced the number of transients per hour by 20%, which increased the time between transients by 5 s on average. For mean concentration, however, there were no significant difference found between the pre-drug (52 ± 14 nM) and cocaine (44 ± 10 nM), nor after the addition of haloperidol (56 ± 17 nM). This study shows that the frequency of spontaneous adenosine transients is affected by a combination of cocaine and haloperidol, but that spontaneous adenosine transient concentration is not.

These data show that an increase in extracellular dopamine levels is accompanied by a reduction in the number of spontaneous adenosine transients, which was surprising given the findings of possible co-release in the last chapter. Control of transient adenosine frequency is typically associated with A_1 receptors in the caudate [21](Chapter 3), and D_2 receptors are more closely associated with A_{2A} receptors. [55] Our hypothesis was that A_{2A} receptors counteract the inhibitory effects of A_1 receptors [56]; therefore when D_2 receptors antagonistically paired with A_{2A} receptors are suppressed, increased activity of the A_{2A} receptors should reduce the inhibitory A_1 receptors and increase adenosine transient frequency. Also, from previous findings, we knew that

cocaine and haloperidol would increase the frequency of dopamine transients, [40] and if dopamine and adenosine were co-released, we expected that adenosine frequency would also increase. However, the results were the complete opposite, as the frequency of adenosine transients goes down with cocaine and haloperidol. This implies that there is another pathway through which dopamine is regulating spontaneous adenosine transient frequency.

4.3.1 Spontaneous Transient Adenosine Frequency Regulation is Complex

Spontaneous dopamine transient signaling increases after of COC-HAL. [40,57] The monoamine reuptake inhibitor serves to block dopamine reuptake through the dopamine transporter, allowing more dopamine to be present in the extracellular space where it can be detected electrochemically. [58] The D_2 antagonist prevents the D_2 receptor inhibition of dopamine release and increases overall dopamine activity. [39,59] Not only does the frequency of transients increase, but the average amplitude and duration of these events increased as well in past work. [40] While we did not quantitatively analyze dopamine here, we qualitatively observed longer and more dopamine transients in the color plots.

Dopamine transients are typically accompanied by adenosine release when anesthetized animals are not given any drugs (86% of spontaneous dopamine occurs with a coincident adenosine event). (Chapter 3) Therefore, we expected that increasing spontaneous dopamine frequency through the use of cocaine/haloperidol would also increase adenosine frequency. Previously, we speculated that ATP or adenosine may be packaged vesicularly with dopamine, due to its coincident release. (Chapter 3) If that is

the case, then an increase in vesicular dopamine release events should also lead to an increase in the coincident ATP/adenosine release events, causing transient frequency to increase. The results however, showed the opposite, that adenosine frequency decreased with COC-HAL instead of increasing. The addition of cocaine increases extracellular basal levels of dopamine, and the concentration, duration of dopamine transients, and increases rate of release. [38,57] Haloperidol, does not change dopamine frequency by itself, but will in the presence of a reuptake inhibitor, [40] though its effect on adenosine was unknown. As we observed, the increase in dopamine due to the cocaine/haloperidol did not increase the adenosine frequency or concentration. This means that if ATP or adenosine is packaged into vesicles with dopamine for release, that either there are not more vesicles being released under cocaine/haloperidol or that the vesicles that are being released do not contain ATP/adenosine. We know from previous studies that the dopamine events stimulated by cocaine originate from a separate reserve pool of dopamine vesicles, and not from the readily releasable pool. [44,60] It is possible that this reserve pool is not packaged with ATP/adenosine as the readily releasable pool is, since it is not advantageous to store away the ATP/adenosine when it could be readily utilized. Additionally, with an increase of vesicle release frequency, ATP/adenosine may be used up and not packaged into vesicles at the same rate, thereby reducing the available ATP/adenosine released the extracellular space. This level of depletion would account for the reduction, but not elimination of adenosine transients, since we already know that this coincident release is not the only mode of adenosine signaling. (Chapter 3) Thus, this study provides interesting information that increasing the rate of dopamine transients does not necessarily increase adenosine, and

that adenosine might not be co-released with DA when it is released at higher rates or from a different pool.

Although unexpected, the diminished adenosine signaling observed here in response to amplified dopamine activity does have important implications. Adenosine is a modulator of dopamine and adenosine and dopamine receptors are co-localized in the brain, where adenosine can reduce some of dopamine's excitatory effects and secondary messengers through receptor interactions. [53,55,61] Here, we observe that when the dopaminergic system is pushed beyond its normal limits, that the modulation normally provided by fast adenosine is diminished. This could cause an even greater effect to dopamine signaling as a result of the reduced modulation and inhibition of adenosine through A_1 receptors. Previous studies have found that psychostimulants increased extracellular adenosine levels overall, but did not measure discreet adenosine signaling, instead focusing on long term effects and contributions to addiction. [62] Though the investigation of these longer-term effects is important, it is equally important to understand what is happening in the microenvironment upon initial exposure to these drugs. A reduction in adenosine modulation of dopamine upon an initial spike in the dopaminergic system might account for some of the increased feeling of reward upon first use of substances experienced by drug users. Additionally, there are implications that the increased adenosine response in chronic users may be due to compensation for adenosine depletion that occurs during drug use. This discovery could advise caution when developing adenosine and dopamine therapies and could provide insight for adenosine dysfunction in individuals under the influence of drugs that manipulate the dopaminergic system.

4.4 Methods

4.4.1 Animals & Surgery

Male and Female C57BL/6J mice (6 males, 5 females) were purchased from Jackson Laboratory (Bar Harbor, ME) and housed on a 12:12-h light/dark cycle with food and water provided *ad libitum*. Six to eight week old mice were anesthetized with 4% isoflurane in 100% oxygen for induction and anesthesia, and maintained with 1.5–3% in 100% oxygen delivered via a facemask (Stoelting, Wood Dale, IL, USA). A heating pad was used to sustain mouse body temperature around 37 °C. The surgical site was shaved and bupivacaine (0.10 mL, APP Pharmaceuticals, Schaumburg, IL, USA) was administered under the skin for local anesthesia. In a stereotaxic frame, the skull was exposed and holes were drilled to allow the placement of the electrode in the caudate putamen (AP +1.1 mm, ML + 1.5 mm, and DV -3.0 mm). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia.

4.4.2 Chemicals

Electrodes were calibrated in phosphate-buffered saline (PBS) solution (3.0 mM KCl, 10.0 mM NaH_2PO_4, 2.0 mM $MgCl_2$, 131.25 mM NaCl and 1.2 $CaCl_2$, all from Fisher, Fair Lawn, NJ) with pH adjusted to 7.4. A 10.0 mM stock solution of adenosine (Sigma Aldrich, Milwaukee, WI, USA) and a 10.0 mM stock solution of dopamine (Sigma Aldrich) were prepared separately in 0.1 mM $HClO_4$ and these were diluted daily in PBS solution to 1 μM for calibration of the electrodes.

4.4.3 Electrochemistry

Fabrication of carbon-fiber microelectrode with T-650 carbon fiber was previously described[63]. Cylinder electrodes 150–200 μm long and 7 μm in diameter were used.

Fast-scan cyclic voltammetry was used to detect adenosine and dopamine[50,63] and the electrode was scanned from −0.4 V to 1.45 V and back to −0.4 V at 400 V/s with a frequency of 10 Hz .

4.4.4 Data analysis and statistics

Electrodes were implanted and equilibrated for at least 30 min with the applied waveform prior to data collection. Data were excluded if fewer than 10 transients were observed within the initial 30 min. If robust transients were not found, a new electrode was inserted, up to five new electrodes for each animal. After initial adenosine transients were identified, the electrode placement was optimized and data were collected for 1 hr. Cocaine (0.25 mL 10 mg/kg i. p.) was then injected and data was collected for 30 min, at which point haloperidol (0.25 mL 0.5 mg/kg i. p.) was injected and data was collected for another 1 hr.

Transient adenosine and dopamine events were identified and characterized using structural similarity image analysis[64] events were confirmed by an analyst to exclude any signals that were erroneous. The primary oxidation peaks of adenosine and oxidation peaks of dopamine were identified using a library of 15 verified adenosine and dopamine peaks. All statistics were performed in GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). All data are shown as mean ± SEM. Statistical significance was designated at $p < 0.05$.

4.5 References

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Chapter 5
Conclusions and Future Directions

“You only grow by coming to the end of something and by beginning something else.”

— John Irving

5.1 Contributions of the dissertation to the field

In this thesis, I have focused on expanding our understanding of adenosine signaling and adenosine's role as a neuromodulator in the brain using FSCV. I have been the first to explore sex differences in spontaneous transient adenosine *in vivo*, to characterize adenosine and dopamine coincident release *in vivo*, and to characterize the effect of dopaminergic changes on adenosine in real time. In this final chapter, I will briefly summarize my findings and discuss possible directions for future research.

5.2 The role of spontaneous adenosine signaling in the brain

There have been many studies of spontaneous adenosine signaling in the brain, however those studies have focused on elucidating the mechanism of spontaneous adenosine release. [1–4] These studies have brought us much information about the characteristics of these transients and their origins, [5–7] but left many unanswered questions regarding spontaneous adenosine's role in signaling and its relationship with other neurochemicals. In **Chapter 2**, we began to investigate these relationships by characterizing sex differences in the CA1 region of the hippocampus, the medial prefrontal cortex, and basolateral amygdala. Sex differences were observed in both concentration and frequency of adenosine transients and they varied by brain region. Females displayed larger, less frequent adenosine transients in the hippocampus and amygdala, while in the prefrontal cortex, the relationship was reversed. Adenosine also varies with estrous cycle, showing higher concentrations during the proestrus stage when estrogen and progesterone levels are high. This suggests a link between

spontaneous adenosine transients and sex hormone concentrations in females, making it important to include both male and females in further studies going forward.

Adenosine is known to modulate dopamine in the central nervous system, and so **Chapter 3** focused on the relationship between coincidentally released spontaneous adenosine and dopamine and characterized these events *in vivo* for the first time. We also examined how these coincident transients were altered by global knockout of A_1 and A_{2A} adenosine receptors. Most (86%) of the dopamine events observed happened within 5 s of an adenosine event, though only about 30% of adenosine events had a coincident dopamine event. Though adenosine concentration was 10-fold higher than dopamine concentration, the concentration of dopamine transients was higher during coincident release events than without any adenosine present. This resulted in a positive correlation between the concentrations of the adenosine and dopamine transients. A_1 receptors affected transient frequency for adenosine and dopamine, while A_{2A} receptors affected dopamine transient concentration and the incident rate of coincident release. A_{2A} receptors also increased the rate of coincidence between adenosine and dopamine transients These findings suggest that a portion of spontaneous adenosine release is due to ATP/adenosine packaged into dopamine vesicles and are regulated by A_{2A} receptors, which can be utilized as an avenue for targeted drug therapies.

Dopamine influences brain chemistry through postsynaptic interaction with D_1 and D_2 receptors. [8,9] These postsynaptic receptors are co-localized in receptor complexes with A_1 and A_{2A} receptors, respectively. [10] Within these receptor complexes, adenosine and dopamine receptors display antagonistic actions on one another, and so dopaminergic system drugs can be used to manipulate the adenosine system as adenosine drugs do

for dopamine. In **Chapter 4**, we investigated factors affecting adenosine signaling by examining how spontaneous adenosine is changed by the administration of cocaine and haloperidol, two drugs commonly used to manipulate dopamine signaling. We found a 20% reduction in the number of adenosine transients per hour and 20% increase in the time interval between transients during addition of cocaine and haloperidol. There was however, no significant difference in the concentration of adenosine transients between pre-drug, under cocaine alone, nor under cocaine and haloperidol combined. These findings were contrary to our predictions that the increase in dopamine from the uptake inhibitor and D_2 receptor antagonist would result in increased adenosine activity through A_{2A} receptor activation. This discovery can provide insight into adenosine/dopamine pharmacodynamics for drugs of abuse and addiction and advise caution in the development of adenosine and dopamine therapies. Overall, we have explored spontaneous transient adenosine's role in extracellular signaling in real time, showing its connection to both biological sex and dopamine. These relationships and our findings regarding them are vital in the development of Parkinson's disease, schizophrenia, addiction, and cancer therapies based on adenosine, along with many others.

5.3 Future Directions

The purpose of this work was to investigate how rapid adenosine signaling related to sex and how spontaneous transient adenosine interacted with dopamine in real time. Though there is much regarding the interactions of spontaneous adenosine signaling that we have learned, there is still much to be discovered regarding adenosine interactions in the brain. Though sex differences have been observed in adenosine

research, more studies are needed to determine the source of these changes and the mechanics behind them. Future research to identify the mechanisms of action behind adenosine interactions utilizing FSCV, pharmacology, and other techniques will not only improve our understanding of this complex system, but also lead to better therapies for adenosine and dopamine dysfunction.

5.3.1 Spontaneous transient adenosine and sex hormones

In Chapter 2, we characterized differences in spontaneous adenosine transient concentration and frequency between the sexes, in different brain regions, and through all the different stages of estrous. However, in WT animals, sex hormones are constantly in flux and perfused throughout the brain, which makes attribution of evoked changes in adenosine to any one hormone impossible. [11] In fact, we already know that there are fundamental structural differences in adenosine signaling between males and females from the findings of Wang et al., where the observed differences in CD73KO mice is attributed to differences in tissue nonspecific alkaline phosphatase. [2] For these reasons, spontaneous adenosine transients should be observed in gonadectomized animals to first observe what differences are inherent to males and females without the influence of hormones. One reason for inherent differences in the sexes, void of sex hormones, may be in adenosine receptor density. Animals with different densities of adenosine receptors would have different levels of response to changes from stimuli, but the density of adenosine receptors in female rats, in particular, is unknown and unexplored. By observing adenosine in animals gonadectomized after maturation, we would be able to observe how spontaneous adenosine transients are affected by sexual differentiation alone. Once this has been accomplished, those same animals can be used to test the

effects of the addition of sex hormones, such as estradiol, progesterone, and androgen on adenosine transients. This would serve to further illuminate the effect of sex on adenosine signaling and better link adenosine's role to known sex differences in other neurochemicals, such as dopamine.

Dopamine signaling in the brain displays sex differences in larger transients, faster reuptake, [12] and lower extracellular concentrations in females. [13] These variances manifest themselves in a variety of different ways. When exposed to stress tests that induce a depressive-like state, males showed dopaminergic activity in the prefrontal cortex and hippocampus, but females did not. Further stress differences were noted during the application of chronic mild stress, which evoked behavioral changes in both sexes, but only elicited dopaminergic activity in the prefrontal cortex of females. [14] Females also show an increased propensity to self-administer cocaine in rats, with the addition of estradiol promoting even greater drug seeking behavior. [15,16] Though these previous examples show the down side of dopamine transmission in females, there are some benefits from these differences in severity of dopamine related diseases. Incidence of ischemia in women is much less frequent than men and the prevalence and severity of Parkinson's disease is also much less in women with estrogen showing protective effects against striatal dopamine loss. [17,18] Though both male and female animals were utilized to investigate coincident adenosine and dopamine interactions, a larger sample size would be needed to quantitatively evaluate any changes due to the inherent variance involved in these systems. However, more specific information into adenosine-dopamine interactions and how they differ between divergent levels of sex hormones leads to a better understanding of the underlying mechanics of both release

and sexual differentiation. Increased understanding of these relationships will lead to better targeted pharmacological options for patients based on gender or hormone levels, instead of utilizing the current “one size fits all” approach to treatment.

5.3.2 Adenosine-dopamine interactions in the brain

Adenosine modulation of dopamine signaling in the brain is a well-known and highly researched interaction. [19–23] Unfortunately most information we have on adenosine/dopamine interactions is based on adenosine receptor interactions with dopamine receptors and measured on a slow scale. This problem was addressed in Chapter 3, by using FSCV, a rapid technique with high temporal resolution, to measure and observe adenosine and dopamine simultaneously in real time however, questions still remain. Dopaminergic neurons in the brain project from the midbrain (substantia nigra, ventral tegmental area) into the striatum, the hypothalamus, the preoptic area, and the cortex [24] with heterogeneous dopamine signaling noted between regions. [25,26] Adenosine signaling is also heterogeneous between regions with changes in concentration and frequency of transients depending on the region of measure, along with differences in response to pharmacology. [6,7,27–29] (Chapter 2) By expanding our focus to include brain regions outside of the caudate, we can begin to assemble a better picture of the role of this coincident release mechanism in the overall role of adenosine and dopamine signaling.

Though identification of dopamine transients during the simultaneous measurement of adenosine and dopamine in Chapter 3 was accomplished through the development of image-based analysis software utilizing structural similarity image analysis, some difficulties persist. [30] The analysis program is adept at recognizing the

typical short burst type dopamine transients normally observed spontaneously in anesthetized animals, but because of the nature of the Butterworth filter [31] longer period transients are effectively removed from detection. The Butterworth filter employs a zero-phase high pass filter with very low cutoff frequency in order to remove baseline drift in the raw data. This method is very effective for removing drift. However, it also prevents the detection of transients more than around 8.5 seconds in length. When utilizing pharmacology to manipulate neurochemical systems, such as the use of cocaine and haloperidol in Chapter 4, the resulting signaling is often outside of the bounds of typical homeostatic signaling. As a result, in order to be able to accurately quantitate the change in dopaminergic signaling, the data must currently be manually analyzed, which increases analysis time significantly. To ensure maximum efficiency, elimination of bias, and accuracy, a new approach must be utilized in order to deconvolute and characterize these transients while still maintaining objectivity. In order to do so, the Butterworth filter portion of the program must be removed or bypassed and the dopamine reference transients, must be chosen manually depending upon what pharmacology is being employed. This would serve to increase productivity in these studies and reduce the length of time required for data analysis, thereby enabling an increase in total study throughput.

Another method that can be utilized to explore adenosine-dopamine interaction is fast scan controlled-adsorption voltammetry (FSCAV). Though FSCV is a vital technique in measuring neurotransmission with high spatial and temporal resolution, it does have its short comings. By employing a fast scan rate, a large background current is generated and the non-faradaic current must be subtracted from the signal in order to

analyze the faradaic current from the redox reaction of the analyte at the electrode surface. [32,33] The subtraction of this background current means that FSCV is incapable of measuring basal levels of analyte and is only able to measure discreet changes in analyte concentration. However, about 8 years ago FSCAV was developed, which allowed for measurement of analyte basal levels utilizing FSCV technology and principles. [34] This technique has previously been successful in studying tonic dopamine levels and adsorption [35] as well as having been employed in measuring basal serotonin levels. [36] By determining basal adenosine and dopamine levels during WT and control, the basal levels can then be determined again repeatedly over the course of the experiment. Basal levels in adenosine have previously been examined using microdialysis coupled to HPLC, [37–39] however the collection time for these experiments is over a period of 20 min, compared to the < 90 s analysis window of FSCAV. It would fill the gaps between FSCVs subsecond detection and the accurate, but slow analysis of microdialysis. FSCAV also has an advantage over microdialysis in that it utilizes the much smaller FSCV probe, which is less prone to cause tissue damage upon insertion. The information gleaned from these studies of background levels and the timing of shifts following the addition of drugs would be invaluable in the development of treatments and understanding of adenosine-dopamine dynamics.

One unexplored frontier of rapid adenosine signaling is that of spontaneous adenosine measurement in freely moving animals. To date all *in* vivo experiments in the measurement of spontaneous transient adenosine have been in anesthetized animals. With adenosine as one of the main neurochemicals involved in sleep, the size and frequency of the adenosine transients observed under anesthesia should be less than

those in awake freely moving animals. Previous studies have shown that extracellular levels of adenosine increase with increased metabolism. [40] In freely moving animals, basal adenosine levels increase throughout the course of wakefulness, until they reach a sleep-inducing threshold, and then slowly reduce over the period of sleep until wakefulness returns. [41] Previous measurements of adenosine in freely moving animals have been with microdialysis, the limitations of which have been previously discussed. By measuring spontaneous transient adenosine in freely moving animals, it could be observed how the adenosine system in different regions of the brain react to certain behaviors or stimuli. First, the brain regions of most interest to further experimentation could be observed for a full 24 hr dark/light cycle to see how wakefulness and transient characterization coincide. From there observations could be made regarding periods of vigorous activity vs. periods of rest to observe spontaneous adenosine's role in fatigue and/or stress response. It would also be possible to use Parkinson's disease model animals to study the effects of adenosine therapy drugs on rapid adenosine signaling in real time, while also observing physiological and behavioral symptoms. These experiments could lead to a better understanding of the role of rapid adenosine signaling and more effective adenosine therapies.

One problem with measurement of adenosine with freely moving animals however, is in regard to adenosine's two modes of rapid signaling, spontaneous release [42] and mechanosensitive release. [28] Though devices for FSCV monitoring of freely moving animals have been developed and utilized for decades, it is unclear if these devices would be sufficient to prevent and protect against repeated spurious mechanical stimulation of adenosine. [28] Fortunately, there have been recent developments in 3D

printed carbon electrode technology that may offer a solution to this problem. Recently, through direct laser writing, nano 3D printed carbon microelectrodes have been produced and successfully employed to detect dopamine in *Drosophila*. [43] This electrode would have a larger footprint than the typical 7 μM diameter carbon fiber electrodes, but the geometry is tunable. It is also able to be insulated to prevent spurious mechanically evoked transients from contaminating the measured spontaneous adenosine transients in the microenvironment. By manipulating the electrode geometry, it is possible to better specify where the electrode will measure from. Being built on a metal wire makes the electrode easier to mount without damaging the electrode and makes it more likely to move with the tissue should it be disturbed during measurement and would not resist as a rigid glass capillary would. It would also allow for alteration of the geometry based on what specifically was being examined. [44] These changes would allow monitoring of adenosine and dopamine transients throughout a full 24-hour day night cycle and also allow for behavioral testing and monitoring of neurochemical response. The gathering of this information would be vital to further adenosine therapeutic development and dopamine interaction.

5.4 Final Conclusions

In conclusion, detection of spontaneous transient adenosine using electrochemical methods has opened the doorway to a wealth of knowledge about the formation and function of adenosine in the brain. Spontaneous adenosine is heterogenous from brain region to brain region and is affected by sex overall and by fluctuations in gonadal hormones. The specifics of how much variance is due to sexual

differentiation and how much is due to extra- and intracellular hormone levels remains to be determined. We hypothesize that at least 30% of spontaneous adenosine release is due to vesicular co-release from ATP or adenosine packaged with dopamine, and that release is regulated by A_{2A} receptors. Though more investigation into how other neurochemicals might play a role in this release still remains to be found. Spontaneous transient adenosine frequency experiences a reduction in number and interval or 20% when under the influence of cocaine and haloperidol. This puzzling fact will move the field in new directions in order to explain the anomaly. Future studies can pick up where this research has left off and the new knowledge will allow the development of more effective and accurate therapeutic options for adenosine and dopamine related diseases and those that target specific adenosine signaling pathways.

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