Investigating the Role of Transient Adenosine to Modulate Neurotransmitters through Electrochemical and Fluorescence Imaging Techniques

Kailash Shrestha

Baklauri, Nepal

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Abstract:

Adenosine is a ubiquitously present local neuromodulator in the brain, generated through the enzymatic hydrolysis of adenosine triphosphate (ATP), and it plays a dual role as a neuroprotector. It is crucial for maintaining neuronal homeostasis and rapidly responding to cellular stress. Adenosine's regulatory actions are mediated via GPCR receptors, primarily A1 and A2A receptors, which exert opposing effects on neurotransmitter release.

Fast scan cyclic voltammetry (FSCV) allows for sub-second monitoring of adenosine and its neuromodulatory effects on neurotransmitter release. However, while FSCV offers exceptional temporal resolution, it is limited by its lack of spatial resolution, selectivity, and inability to detect non-electroactive neurotransmitters. This dissertation investigates the integration of FSCV with fluorescence imaging (iGluSnFR3) to enable real-time analysis of adenosine's modulation of neurotransmitters.

Chapter 1 introduces adenosine, fast scan cyclic voltammetry (FSCV), and various methods for measuring adenosine levels. It highlights techniques such as micro-dialysis, biosensors, and FSCV, focusing on their ability to capture adenosine dynamics on a rapid time scale. The strengths and limitations of these approaches are analyzed, particularly in the context of fast-acting adenosine. Studies involving electrically stimulated, spontaneously released, and mechanically stimulated adenosine are reviewed.

Chapter 2 focuses on transient adenosine neuromodulation of serotonergic neurons in the dorsal raphe nuclei (DRN), using FSCV. Exogenous adenosine was found to inhibit serotonin release by more than 50% within the first 20 seconds, with recovery occurring alongside adenosine clearance in wild-type (WT) slices. In contrast, this inhibitory effect was

limited to the first 10 seconds in A1KO slices. Importantly, A1, A2A, and A3 receptors did not directly contribute to adenosine's inhibitory effect. Instead, adenosine inhibited serotonergic neurons via densely expressed 5HT1A autoreceptors.

Chapter 3 discusses the integration of multiplexing fast scan cyclic voltammetry (FSCV) and fluorescence imaging (iGluSnFR3) to enable simultaneous monitoring of multiple analytes and their interactions in real time. While FSCV is specifically designed to measure electroactive molecules, the addition of genetically encoded fluorescence sensors enhances the ability to monitor multiple electroactive and non-electroactive molecules simultaneously with improved specificity and spatial resolution. The study revealed an inverse correlation between electrically stimulated dopamine release and glutamate levels in the caudate putamen. Furthermore, adenosine was found to inhibit the release of both dopamine and glutamate, with this inhibition restricted to a 250 μm radius. Pharmacological studies using A1 receptor antagonists confirmed that this inhibitory effect of adenosine is mediated through A1 receptor activation.

Chapter 4 focuses on the application of multiplexing techniques to simultaneously study the effects of transient ischemia on neurotransmitters such as dopamine and glutamate. Deprivation of glucose and oxygen during oxygen-glucose deprivation (OGD) depletes cytosolic ATP, leading to a rapid rise in extracellular adenosine, which in turn modulates the release of other neurotransmitters. During OGD, both electrically stimulated dopamine and glutamate release were significantly reduced, and this suppression persisted for the first 30 minutes of reperfusion. While dopamine levels gradually recovered to baseline over the course of 120 minutes of reperfusion, glutamate levels remained at OGD-induced levels. Using transgenic mice (A2AKO) and pharmacological interventions (NMDA receptor antagonist and DPCPX), the study highlighted the critical neuroprotective roles of A1 and A2A receptors during OGD. Specifically, A1 receptor activation prevents excessive glutamate accumulation in extracellular spaces, thereby reducing excitotoxicity and minimizing neuronal damage.

Overall, this dissertation explored adenosine neuromodulation on other neurotransmitters by integrating fast scan cyclic voltammetry (FSCV) and fluorescence sensors. This integration allowed for simultaneous monitoring of both electroactive and non-electroactive molecules, along with their interactions, in real time with enhanced spatial resolution. Adenosine was shown to modulate dopamine and glutamate via A1 receptors in the striatum, with this effect being localized within a 250 μm range. Similarly, adenosine demonstrated neuromodulation of serotonergic neurons in the midbrain through densely expressed 5HT1A autoreceptors.

The combination of FSCV and fluorescence highlights the potential of multiplexing techniques to study complex neuromodulatory interactions, providing a powerful tool for understanding the dynamics of neurotransmitter systems in both normal and pathological conditions.

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Chapter 1: Adenosine modulation

The human brain possesses billions of neurons that form intricate connections and facilitate communication through the delicate interplay of regulated neurochemical release. Maintaining physiological levels of neurotransmitters and neuromodulators at the synapse is crucial for relaying and processing information necessary for healthy brain function. Adenosine, one of the most abundantly found non-classical neurotransmitters, modulates the release of other neurotransmitters through its G-protein-coupled receptors (GPCRs).[1] As a constituent of adenosine triphosphate (ATP)—the "energy currency of life"—adenosine is implicated in various neurological conditions, including Parkinson's disease, depression, and insomnia.[1] Despite extensive research on purinergic signaling, critical questions remain regarding the spatial and temporal dynamics of adenosine release under both physiological and pathological conditions. For instance, multiple neurotransmitters such as serotonin, glutamate, and GABA are associated with Parkinson's disease, highlighting the need to monitor their levels in real time with high spatial resolution.[2,3] A comprehensive understanding of adenosine's role in the brain requires tools that can simultaneously detect multiple neurotransmitters with high spatial and temporal resolution and molecular precision.

As adenosine is a crucial neuromodulator in the central nervous system, there is significant interest in understanding its dynamics in relation to other neurotransmitter releases. Although considerable progress has been made in developing sensitive techniques for measuring individual neurotransmitters, a notable gap remains in the ability to simultaneously monitor multiple neurotransmitters and their interactions in real time. Current methods for neurotransmitter detection include electrochemistry, enzyme-based biosensors, fluorescence imaging, and microdialysis coupled with high-performance liquid chromatography and mass

spectrometry.[4–7] However, each of these methods has limitations, such as selectivity to electroactive chemicals, larger probe sizes, longer detection times, and low spatial resolution.

This work aims to address these limitations by integrating electrochemical and fluorescent imaging techniques to develop a tool capable of monitoring multiple neurotransmitters and their dynamics with high spatial and temporal resolution.

1.1 Overview of Adenosine

Adenosine is an essential purine nucleotide involved in neurotransmission and neuromodulation. It is one of the most ubiquitous metabolic intermediates, primarily formed through the enzymatic metabolism of adenosine triphosphate (ATP).[8,9] Adenosine uniquely integrates both excitatory and inhibitory signaling pathways and regulates cellular functions by activating four different adenosine G-protein-coupled receptors (GPCRs): A1, A2A, A2B, and A3 receptors. Among these, A1 and A2A receptors are most abundantly expressed and have higher affinity, whereas A2B and A3 receptors are less expressed and have lower affinity. This unequal receptor distribution enables adenosine to modulate the release of other neurotransmitters effectively.[10,11]

Adenosine's neurophysiological actions are largely inhibitory, primarily suppressing the release of excitatory transmitter releases such as dopamine, glutamate, and serotonin.[6,11,12] Previous methods for adenosine monitoring relied on techniques with slower time resolution, such as microdialysis and biosensors, restricting their ability to capture rapid adenosine signaling in real-time.[13] Fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes overcomes these limitations by leveraging the redox properties of electroactive neurotransmitters, allowing for the detection of rapid extracellular concentration changes with sub-second resolution.[14] Adenosine signaling occurs through spontaneous release, lasting 2-3 seconds, and mechanically stimulated release, persisting for 25-30 seconds.[6,15]

Adenosine is an endogenous neuromodulator and a sensitive indicator of ischemic insults, exhibiting neuroprotective effects through the activation of its GPCR receptors..[16,17] During ischemic events, extracellular adenosine levels increase dramatically—by up to 100-fold—via the enzymatic hydrolysis of ATP, reflecting a corresponding depletion of ATP levels.[18,19] Hippocampal neurons and medium spiny neurons within the striatum are particularly vulnerable to ischemic damage. Adenosine acts as a retaliatory metabolite, modulating neuronal activity through GPCR receptors, with A1 receptors being the most abundantly expressed. Activation of A1 receptors inhibits calcium-dependent neurotransmitter release, including glutamate, dopamine, and serotonin.[17,20] A1 receptor activation counteracts excessive glutamate release in the extracellular space and limit tissue damage due to excitotoxicity. Adenosine antagonists, such as theophylline, have been shown to exacerbate hippocampal CA1 damage following ischemia..[21]

In contrast to A1 receptors, activation of A2A receptors promotes excitatory functions, stimulating neurotransmitter release. Pharmacological studies have demonstrated that A2A receptor antagonists, such as 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS15943) and 8-(3-chlorostyryl)caffeine (CSC), provide neuroprotective effects against forebrain ischemia, emphasizing their therapeutic potential.[22,23]

1.1.1 Adenosine regulation in central nervous system

Adenosine is a non-classical neurotransmitter formed through the enzymatic hydrolysis of ATP and released into the extracellular space via vesicular exocytosis and cell membrane fusion.[24] Its physiological effects are largely determined by its extracellular concentration. Additionally, ATP can be metabolized by ecto-nucleotidase enzymes at the outer cell membrane, leading to adenosine formation in the extracellular environment.[25]

Adenosine plays a multifaceted role in the brain, acting as a neurotransmitter, neuromodulator, neuroprotector, and regulator of cerebral blood flow. It exerts its effects through four G-protein-coupled receptors (A1, A2A, A2B, and A3), which modulate secondary messenger levels, as well as calcium and potassium ion channel activity. The spatial distribution and binding affinities of these receptors enable complex adenosine signaling in the brain, with A1 and A2A receptors predominantly expressed at pre- and postsynaptic synapses.[1,11]

Extensive research has focused on adenosine receptors, metabolic pathways, and membrane transporters to better understand adenosine signaling. Adenosine-targeted treatments for Parkinson's disease, chronic pain, cerebral ischemia, and neuroprotection involve adenosine receptor agonists and antagonists. Notably, the A1 receptor-mediated inhibitory role of endogenous adenosine has demonstrated neuroprotective effects in in-vivo and brain slice studies, particularly in hypoxia/ischemia models.[22,26,27]

1.1.2 Intra and extracellular Adenosine formation

Adenosine is generated through multiple pathways, both intracellularly and extracellularly. Intracellularly, it arises primarily from the enzymatic hydrolysis of ATP and, to a lesser extent, via the S-adenosylhomocysteine (SAH) pathway. Once synthesized, adenosine is transported across the cell membrane through bidirectional equilibrative nucleotide transporters (ENTs), facilitating its movement between intracellular and extracellular spaces.[8,9]

In the extracellular space, adenosine is primarily derived from ATP metabolism, catalyzed by ectoenzymes on the cell membrane. Cytosolic ATP can be packaged into vesicles by the vesicular nucleotide transporter (VNUT) and released via exocytosis in response to action potentials. Additionally, membrane channels such as pannexin and connexin contribute to ATP release.[28–30]

Once in the extracellular space, ATP undergoes rapid enzymatic hydrolysis by ectonucleotidases, particularly CD39 and CD73, converting it into adenosine. The basal extracellular adenosine concentration typically ranges from 100 to 200 nM, while its intracellular concentration is approximately 50 nM.[31,32]

Extracellular adenosine binds to adenosine receptors located on pre- and post-synaptic neurons, modulating neuronal activity under both physiological and pathological conditions. When extracellular adenosine levels rise, it can diffuse out of the synaptic cleft. Adenosine inactivation occurs through cellular uptake via equilibrative nucleotide transporters (ENTs), followed by its conversion to AMP by adenosine kinase or deamination to inosine by adenosine deaminase.[33,34]

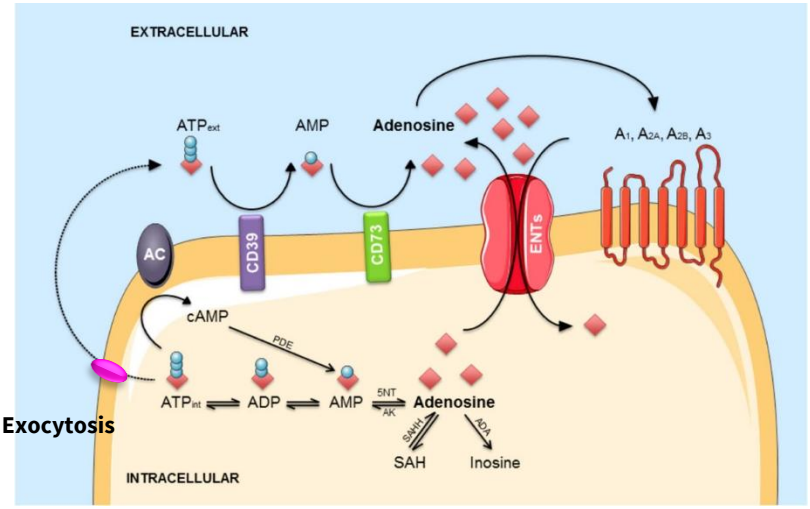


Figure 1: Adenosine Formation Scheme: Intracellular adenosine is produced through enzymatic metabolism of AMP and SAH present in the cytoplasm. ATP released at the extracellular spaces undergoes hydrolysis in the presence of ectonucleotidase enzymes (CD39 and CD73) forming adenosine at the extracellular space.

1.1.3 Adenosine receptors:

Extracellular adenosine mediates intercellular signaling via G-protein-coupled adenosine receptors (P1 purinoreceptors), subclassified as A1, A2A, A2B, and A3.[10,32,35] These receptors, located pre- and post-synaptically, regulate neuronal activity through pathways involving

adenylate cyclase (AC), phospholipase C (PLC), and G-protein-coupled inwardly rectifying potassium channels (GIRK). While adenosine receptors are widely expressed in the brain, their distribution and binding affinities vary, influencing both physiological and pathological processes.[36]

Adenosine primarily modulates neurotransmission by engaging the high-affinity A1 (70 nM) and A2A (150 nM) receptors, which are abundant and crucial for neuromodulation. In contrast, A2B (5100 nM) and A3 (6500 nM) receptors require higher adenosine concentrations for activation. Functionally, A1 and A3 receptors are inhibitory, while A2A and A2B receptors are excitatory, modulating adenylate cyclase activity via Gi or Gs proteins.[36,37]

Additionally, GPCR dimerization allows adenosine receptors to interact with other GPCRs, forming heterodimers like A1/A2A, A2A/D2, and A2A/mGlu5, which contribute to receptor crosstalk in normal and pathological conditions.[38–42]

1.1.3.1 A1 receptors:

Adenosine A1 receptors are highly expressed in the hippocampus, cerebellum, and cerebral cortex, making them the most abundant adenosine receptors in the brain.[27,36] These transmembrane GPCRs are located pre-synaptically, post-synaptically, and non-synaptically, where they modulate neuronal activity.

Upon activation, A1 receptors couple with Gi/o proteins, leading to adenylate cyclase inhibition, reduced cyclic AMP (cAMP) levels, and neuronal hyperpolarization.[43] This suppresses excitatory signaling, reducing glutamate release and excitotoxicity. A1 receptors also regulate the release of dopamine, serotonin, GABA, glutamate, and acetylcholine, playing a crucial role in neuroprotection and synaptic modulation.[44,45]

1.1.3.2 A2A receptors:

Adenosine A2A receptors are densely expressed in the olfactory bulb and striatum, regions critical for motor control. These G-protein-coupled receptors (GPCRs) are located at the

synaptic membrane and, when activated, stimulate adenylate cyclase, increasing cAMP levels and activating protein kinase A (PKA). This cascade facilitates synaptic neurotransmitter release, particularly influencing dopaminergic signaling involved in motor function and reward pathways.[37,38,46]

Dysregulation of A2A receptors disrupts neuro-glial crosstalk and is implicated in neurodegenerative disorders such as Alzheimer's, Huntington's, and Parkinson's disease.[46,47]

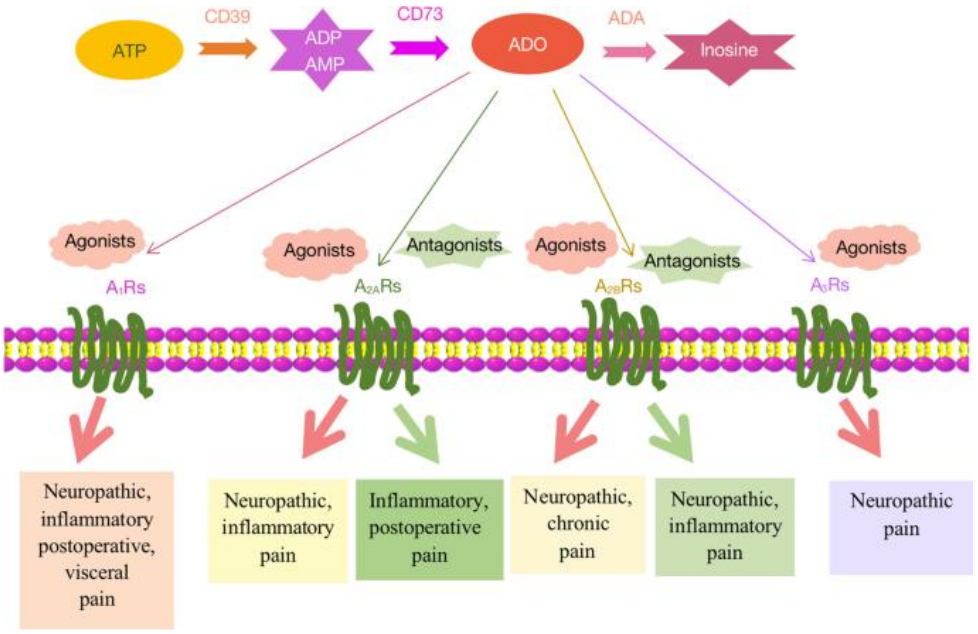


Figure 2: Scheme of adenosine formation and adenosine receptors role during pain[48]

1.1.3.3 A2B and A3 receptors:

These receptors are low-affinity GPCRs expressed in the brain and are activated by micromolar concentrations of adenosine.[49] Although fewer studies have focused on these receptors, they are known to be involved in anti-inflammatory and neuroprotective responses.

1.1.4 Adenosine transporter:

Maintaining balanced extracellular adenosine levels is essential for normal physiological function. These concentrations are primarily regulated by nucleoside transporters located on the cell membrane, which allow adenosine to move in and out of the cell. There are two main types of nucleoside transporters:

1. **Concentrative Nucleoside Transporters (CNTs)**: These are active transporters that rely on the transmembrane sodium gradient to facilitate the influx of nucleosides into cells.[50]

2. **Equilibrative Nucleoside Transporters (ENTs)**: These operate by passive diffusion, allowing the movement of nucleosides in both directions according to the concentration gradient. ENTs play a dominant role in managing extracellular adenosine concentrations. Inhibition of ENTs has been shown to decrease stimulated adenosine levels in the extracellular space by about 40%, underscoring their crucial role in extracellular adenosine regulation.[50]

1.1.5 Rapid Adenosine Signaling:

Adenosine consistently present both intracellularly and extracellularly, playing a crucial role as a neuromodulator and neuroprotector in the central nervous system (CNS).[33] Under normal physiological conditions, adenosine levels are maintained at a few hundred nanomolar. However, during cellular distress events such as hypoxia or cell damage, adenosine concentrations can rise significantly, acting to suppress excitatory neurotransmitter release and prevent excessive neuronal activity.[20,27]

Unlike classical neurotransmitters, which are released in direct response to action potentials, adenosine functions primarily as a neuromodulator. Once neurotransmitters are released into the synaptic cleft, they bind to G-protein-coupled receptors (GPCRs) on pre- or post-synaptic neurons, triggering a cascade of intracellular reactions that propagate signals,

encode memories, or initiate movement. In contrast, adenosine modulates neurotransmission by either enhancing or dampening the signal, thus fine-tuning neuronal communication. Adenosine signaling can be categorized into three different types of rapid adenosine release, as described below.

1.1.5.1 Electrical Stimulation of Adenosine:

Our lab was the first to develop a method for directly detecting electrically stimulated adenosine release in vivo using carbon-fiber microelectrodes combined with fast-scan cyclic voltammetry (FSCV).[14] We characterized electrically evoked adenosine release across different brain regions, including the dorsal caudate-putamen, nucleus accumbens, and hippocampus (CA1 region), through both in-vivo and brain slice experiments.[6,14]

A short train of electrical stimulation (60 pulses at 60 Hz) applied to the substantia nigra induced a 1 μM adenosine release in the caudate-putamen within 2–3 seconds, with the effect persisting for approximately 15 seconds.[51] Pharmacological studies using N6-cyclopentyladenosine (CPA, an A1 receptor agonist) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, an A1 receptor antagonist) confirmed that A1 receptors regulate electrically stimulated adenosine release.[51–53] Specifically, CPA inhibited evoked adenosine release, while DPCPX reversed this effect, highlighting the key role of A1 receptors in adenosine modulation within the caudate putamen.

Mechanistically, electrically stimulated adenosine release is activity-dependent, originating from the intracellular breakdown of ATP, with its release mediated by bi-directional nucleoside transporters.[54] The regional expression of A1 receptors influences the magnitude of evoked adenosine release, contributing to variations in adenosine levels across different brain regions.

1.1.5.2 Spontaneous Transient Adenosine:

The Zylka group was the first to discover spontaneous adenosine release in spinal cord slices using the FSCV technique.[55] Our lab further extensively characterized spontaneous transient adenosine signaling in the brain.[6,14,56,57] The transient adenosine concentration was 0.1 μM and lasted only 2-3 seconds, suggesting rapid enzymatic formation and clearance of adenosine in the extracellular space.[6,56,58] These findings indicate that adenosine may play a local and rapid neuromodulatory role.

Calcium ions (Ca^{2+}) also play a significant role in the release of spontaneous adenosine.[59] In our lab, pharmacological studies demonstrated that these pulsatile adenosine releases are modulated by A1 receptors and depend on ecto-nucleotidase membrane enzymes (CD39 and CD73), which metabolize ATP to adenosine.[60] Furthermore, studies have shown that other neurotransmitters, such as glutamate, and its receptors are crucial in regulating spontaneous adenosine release.[61] For instance, NMDA receptor antagonists increased the frequency and concentration of spontaneous adenosine release, while GABAB receptor activation reversed these effects.[57]

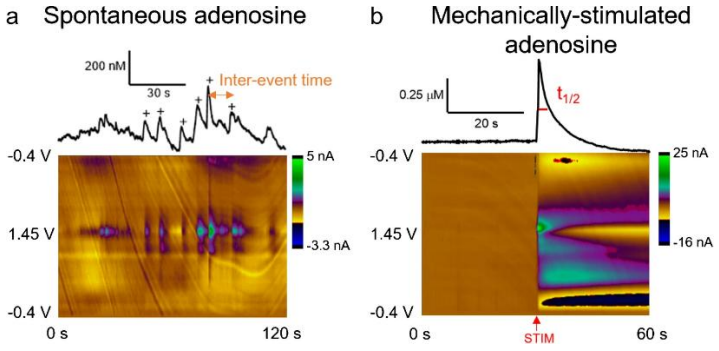


Figure 3: Rapid adenosine signaling examples in WT brain slice. Example FSCV pseudo-color plot of (A) spontaneous adenosine release and (B) mechanically stimulated adenosine in hippocampus CA1 region. Each spike in the concentration vs time trace (above color plot) corresponds to spontaneous adenosine release, green/purple spots in color plot.[15]

1.1.5.3 Mechanically stimulated adenosine

Mechanically stimulated adenosine is produced through the shear stress applied by lowering a capillary pipette or working electrode into the tissue. The shear stress generated by the movement of the electrode induces minor tissue damage, resulting in the release of adenosine.[15,62] In the rat prefrontal cortex, mechanically stimulated adenosine generates a relatively larger concentration (3.4 μM in vivo, 0.8 μM in brain slices) compared to spontaneous adenosine (0.19 μM) and persists for about 30-40 seconds before being cleared from the system (Figure 3).[15]

Studies using tetrodotoxin (TTX) and calcium chelation with EDTA, which reduced adenosine levels, indicate that mechanically stimulated adenosine release is activity-dependent and primarily formed through ATP metabolism.[24,63] However, the precise mechanism behind mechanically stimulated adenosine release, as well as its effects on receptors and transporters, remains unclear. This type of adenosine release can be mimicked by puffing adenosine next to the working electrode, where it lasts around 40 seconds after puffing (Figure 4).

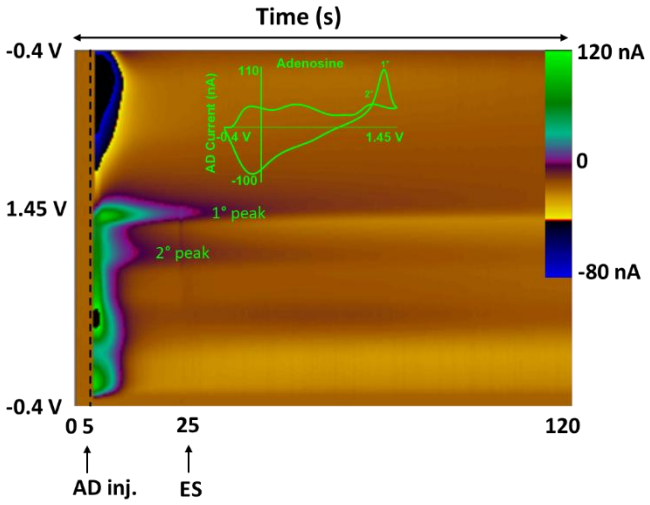


Figure 4: Adenosine puffing in dorsal raphe. Adenosine was puffed 20 seconds before electrically stimulating serotonin in WT and monitored with CFMEs using FSCV technique. Background subtracted adenosine CV inset in color plot with both primary and secondary adenosine oxidation peaks.

Our lab has demonstrated the inhibitory effects of mechanically stimulated adenosine on dopamine, serotonin, and glutamate in the caudate putamen.[24,56,57] However, the concentration of mechanically stimulated adenosine does not appear to be influenced by ATP degradation enzymes such as CD39 and CD73.[60]

1.2 Functions of adenosine in the central nervous system

Adenosine primarily acts as inhibitory neuromodulator regulating the sleep-wake cycle, cerebral blood flow, and inhibiting the release of excitatory neurotransmitters such as dopamine and glutamate to prevent excitotoxicity.[25,45] Extracellular adenosine binds to A2A receptors on blood vessels, causing vasodilation and increasing the flow of oxygenated blood to the brain. Studies have also shown that adenosine receptors can form dimers with other receptors, such as dopamine receptors.[38,42] Therefore, to understand the role of adenosine in neuromodulation, it is essential to analyze the interactions between multiple neurotransmitters.

Adenosine provides neuroprotective effects by activating A1 receptors, which are expressed presynaptically or by hyperpolarizing postsynaptic neurons. This leads to the inhibition of adenylate cyclase and a reduction in cAMP formation, ultimately decreasing neurotransmitter release at the synapse. Adenosine significantly influences the release of other neurotransmitters, particularly through its A1 and A2A receptors.[34,64] Some of the neuromodulatory and neuroprotective effects of adenosine are as follows:

- **Glutamate Release**: Adenosine A1 receptors inhibit excitatory glutamate release in regions such as the hippocampus and nucleus accumbens. During ethanol exposure, increased extracellular adenosine inhibits glutamate release via A1 receptors. Adenosine also exerts a neuroprotective response to transient ischemia by inhibiting glutamate release in the hippocampus via A1 receptors.[39,59]

- **Dopamine Release**: In the striatum, A1 receptors inhibit the release of dopamine as well as glutamate. Our lab demonstrated that transient adenosine decreased dopamine levels by more than 50% in the caudate-putamen. Conversely, A2A receptors stimulate dopamine release, which plays a key role in regulating motor control and reward pathways.[24,42,54]

- **Adenylate Cyclase Pathway**: This pathway is a significant signaling mechanism for GPCR-mediated transduction, leading to diverse downstream effects within the cell. Depending on the receptor it binds to, adenosine can exert both inhibitory and excitatory effects by modulating adenylate cyclase. Binding to A1 receptors slows adenylate cyclase activity, reducing cAMP formation and decreasing PKA activity.[65,66] In contrast, binding to A2A receptors activates adenylate cyclase, increases cAMP production, and enhances PKA activity, resulting in more robust signal propagation.[22,46]

- **Ion Channel Modulation**: When adenosine binds to A1 receptors, it activates potassium channels, which hyperpolarize neurons and prevent them from firing. On the other hand, activation of A2A receptors increases calcium influx through voltage-gated calcium channels, leading to neuronal firing.[67]

- **Ischemia and Hypoxia**: Adenosine exhibits neuroprotective functions by inhibiting or slowing the release of other excitatory neurotransmitters, such as glutamate. Under pathological conditions such as hypoxia or ischemia, adenosine levels rise to protect the brain from further tissue damage by inhibiting glutamate release via A1 receptors.[18,20]

- **Inflammation**: Adenosine modulates immune cell activity, reducing inflammation and protecting against inflammatory damage in the brain.[68,69]

- **Parkinson's Disease**: In the striatum, A2A receptors interact with dopamine receptors. Their activation can counteract the dopamine deficit seen in Parkinson's disease, offering potential therapeutic benefits.[38,46]
- **Crosstalk with Other Systems**: Adenosine receptors are G-protein-coupled receptors that can form both homomers and heteromers with other GPCRs through receptor-receptor interactions. Known heteromeric complexes include A2A/D2 and A1/mGlu5 receptors, which involve dopamine and glutamate signaling, respectively.[38,42]

1.3 Adenosine detection techniques

1.3.1 Microdialysis

Detecting tissue adenosine concentrations accurately is crucial for understanding its role in both normal and pathological conditions. Over the years, extracellular adenosine has been studied using biochemical and physiological methods, with precautions taken to minimize post-mortem ATP breakdown and to rapidly inactivate enzymes responsible for adenosine production and degradation. In early studies, radiolabeled adenine was used to trace cytosolic ATP.[53] Released radiolabeled adenosine, along with other ATP-derived metabolites, was then monitored using HPLC techniques. The current HPLC method is coupled with a microdialysis probe (~300 μm in diameter) that collects samples every 5-10 minutes.[70,71] The microdialysis probe contains a semi-permeable membrane that allows the exchange of cerebrospinal fluid with buffer, and the collected fluid samples are analyzed using HPLC coupled with detection techniques like mass spectrometry and capillary electrophoresis (Figure 5). While this method can analyze and quantify multiple analytes with great precision, it lacks sufficient spatiotemporal resolution to monitor the fast dynamics of adenosine in extracellular space. Additionally, the large size of the probe increases the likelihood of tissue damage, leading to inflammation around the probe, which can induce further neurotransmitter release.[72] For this reason, microdialysis sample collection is typically performed 24 hours after probe implantation.

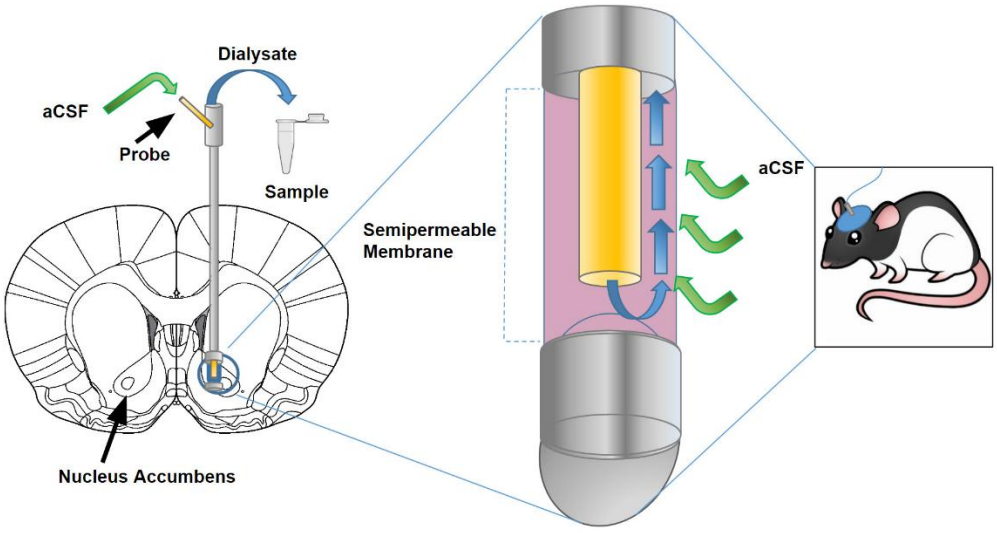


Figure 5: Microdialysis coupled with HPLC/MS to collect and measure neurotransmitter and other metabolites. Probe collect sample through semi-permeable membrane and transfer it to external detector to identify and measure absolute concentration however with poor spatial and slow temporal resolution. (From Sombers lab website)

1.3.2 Amperometry

Electrochemical techniques, which take advantage of the redox properties of analytes like adenosine, provide better spatiotemporal resolution and allow real-time measurement of current proportional to analyte concentration at the electrode surface. In an amperometry biosensor, enzymes such as adenosine deaminase, nucleotide phosphorylase, and xanthine oxidase are immobilized at the electrode surface, where they break down adenosine to release hydrogen peroxide, an electroactive analyte (Figure 6).[13,20,73] The resulting hydrogen peroxide is detected at the electrode surface using amperometry. The enzyme coating improves selectivity for the target analytes, enabling the detection of non-electroactive analytes like glutamate. However, the electrode size and slow electron transfer limit the temporal resolution of this biosensor.[13]

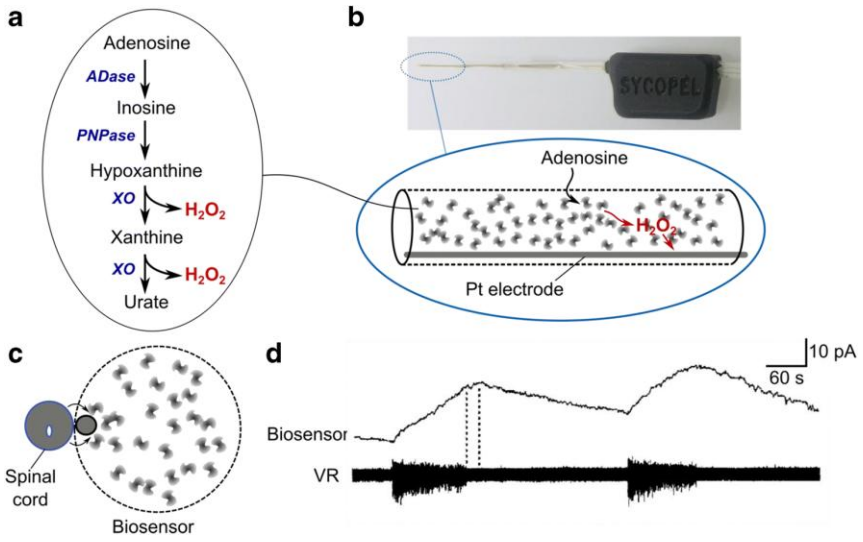


Figure 6: First enzymatic biosensor to record adenosine in real time using modified microdialysis probe with cascade of enzymes that metabolizes adenosine to produce H2O2 and detected by platinum electrodes. (a) enzyme cascade coated on the surface of (b) microdialysis probe with Pt electrode inside it. (c) Schematic diagram of modified electrode placement with respect to Pt electrode to detect adenosine (d) real-time i v t trace of adenosine detection[13]

1.3.3 Fast Scan Cyclic Voltammetry (FSCV)

Fast Scan Cyclic Voltammetry (FSCV) is another electrochemical technique used to monitor rapidly release and uptake of electroactive neurotransmitter such as dopamine, serotonin and adenosine.[74] It offers a better spatiotemporal resolution than microdialysis in the range of milliseconds.[75] This background subtraction technique employs a small carbon fiber (7 µm diameter) probe to detect rapid and spontaneous adenosine release, both in vivo and in vitro, as demonstrated by our lab.[74] The smaller diameter of the fiber reduces tissue damage and inflammation, making it less toxic to tissue. Carbon fibers surface contains oxide groups that enable adsorption for cationic neurotransmitters.[76]

The rapid change in potential at the carbon fiber and records resulting oxidation and reduction current from neurotransmitters. A triangular waveform (-0.4 V; +1.45 V; 400 V/s; 10 Hz) versus a Ag/AgCl reference electrode is applied at the carbon fiber to monitor adenosine,

and the fast scan rate allows sub-second temporal resolution (Figure 7A).[24] However, rapid potential changes at the carbon fiber can generate significant background charging currents due to double layer charging at the electrodes that overwhelm the small faradic signal from adenosine (Figure 7B and 7C).[77,78] Therefore, background subtraction is required to obtain a signature cyclic voltammogram specific to the analyte Figure 7D.[74]

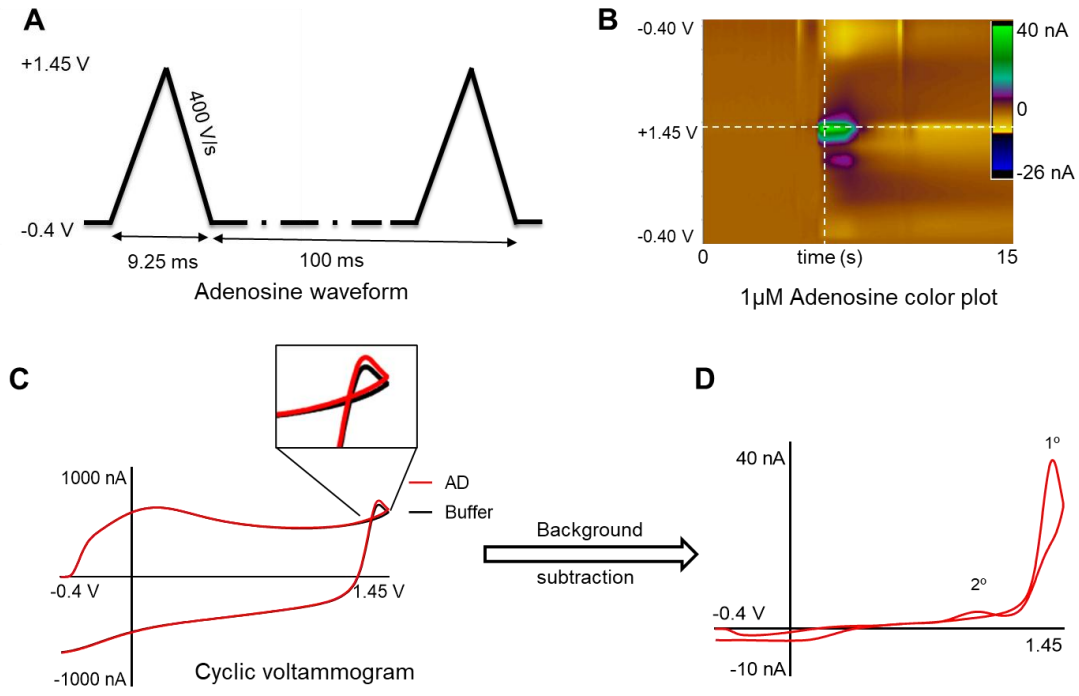
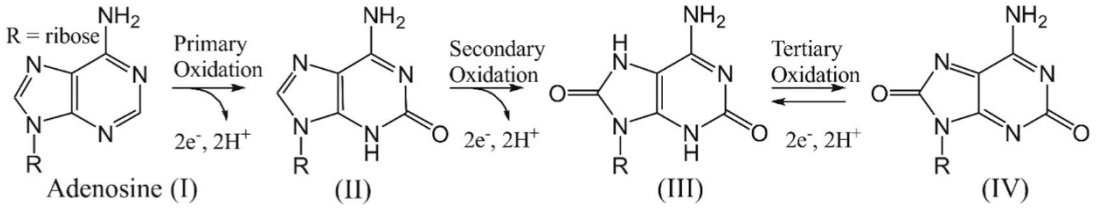


Figure 7: Example of adenosine detection via FSCV. (A) Adenosine waveform: A triangular waveform is applied to a carbon fiber microelectrode (CFME), starting at a holding potential of -0.4 V, increasing to a switching potential of +1.45 V, and returning to -0.4 V. This scan is performed at a rate of 400 V/s with a frequency of 10 Hz. (B) False color plot: A conventional false color plot for 1 μM adenosine in PBS (pH 7.4) with oxidation peaks. Green represents the primary oxidation peak, purple indicates the secondary oxidation peak, and brown indicates the background charging current. The color intensity index is displayed in the upper-right corner of the plot. (C) Cyclic voltammogram; The black trace represents the background charging current in the PBS buffer, while the red trace shows 1 μM adenosine in the PBS buffer. The top inset highlights a small difference between the adenosine and buffer traces. (D) Background-Subtracted Cyclic Voltammogram: The signature cyclic voltammogram of adenosine shows a primary oxidation peak at +1.45 V and a secondary oxidation peak at +1.10 V after subtracting the background current.

Adenosine is an electroactive molecule that undergoes a series of three oxidation steps, each involving two electron transfers, as outlined in Scheme 1. The primary oxidation of adenosine involves two irreversible electron transfers, forming product II, which is observed at +1.45 V in the false color plot. The secondary oxidation, transitioning product II to product III, similarly involves two irreversible electron transfers, detected at +1.1 V in the false color plot.[79] Finally, the tertiary oxidation of product III to product IV involves two reversible electron transfers. However, FSCV using carbon fiber microelectrodes (CFMEs) can only detect the first two irreversible oxidation products and are unable to measure the tertiary oxidation product due to potential limit using carbon fiber. As a result, the background-subtracted cyclic voltammogram for adenosine contains two distinct oxidation peaks, as shown in Figure 7D.



Scheme 1: Adenosine oxidation mechanism. Adenosine undergoes a series of three oxidation steps, with each step involving a two-electron transfer. The first two oxidation steps are irreversible and the final oxidation step, however, is reversible.

One drawback of FSCV is analyte selectivity compounds with similar structures or that oxidize/reduce at the same potential can produce similar cyclic voltammograms, making them difficult to distinguish. Additionally, FSCV can only measure rapid changes in analyte concentration, not basal levels.[74,76]

1.3.4 Genetically encoded fluorescence sensor

Genetically encoded sensors have become popular due to their better selectivity and high spatial and temporal resolution. Recently, the Yulong Li lab developed a G-protein-coupled receptor-based adenosine (GRAB) fluorescence sensor to monitor rapid changes in

extracellular adenosine concentration.[80] Once it binds endogenous adenosine, it undergoes conformational changes emitting fluorescent change. They were able to detect electrically stimulated adenosine release in prefrontal cortex as well as hippocampal CA1 region with varying affinities ranging from nanomolar to micromolar range. Although, these genetically encoded sensors can specifically monitor neurotransmitter dynamic changes at the synapse, it cannot quantify concentration at the synapse and prone to photobleaching if exposed for long time recordings.

1.4 Glutamate

Glutamate is the primary excitatory neurotransmitter in the brain, playing a crucial role in cognition, memory, learning, synaptic transmission, plasticity, and neurodegenerative disorders.[2,7,81,82] Glutamate receptors are widely distributed in both neurons and glial cells. Although glutamate concentrations are high in plasma blood, the blood-brain barrier (BBB) prevents its direct entry into the brain.[81] Instead, astrocytes play a key role in glutamate synthesis, storing it in synaptic vesicles within presynaptic glutamatergic neurons. Each vesicle contains over a thousand glutamate molecules, which are released into the synapse through a Ca^{2+}-dependent mechanism upon neuronal activation. Once in the synaptic cleft, glutamate binds to distinct receptor families on postsynaptic membranes, mediating excitatory signaling.[39]

Glutamate pathways are interconnected with other neurotransmitter systems, including dopaminergic and GABAergic pathways, playing a key role in modulating neurotransmission.[5,40,83] Glutamate levels are 1,000 times higher than those of other neurotransmitters and are tightly regulated through an efficient reuptake system. Maintaining glutamatergic neurotransmission is an energy-intensive process, requiring multiple regulatory mechanisms and high glucose and oxygen consumption.[81] Once released into the extracellular space, glutamate acts on ionotropic and metabotropic receptors to mediate excitatory signaling. After signal transmission, glutamate transporters on postsynaptic neurons and glial cells clear

glutamate from the synaptic cleft, resetting the system for subsequent action potential generation. Disruptions in this regulatory system can lead to excess glutamate release by 55-fold, causing hyperexcitability in postsynaptic neurons and, in severe cases, excitotoxicity and cell death (cytotoxicity).[84] To prevent excessive tonic receptor activation, extracellular concentrations of glutamate and other excitatory amino acids must be tightly controlled. Dysfunction in glutamatergic neurons has been implicated in neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease and epilepsy.[2,85] A deeper understanding of glutamate's role in the pathogenesis and pathophysiology of neuropsychiatric disorders could lead to more rational approaches to drug development.

Electrophysiological recording, fluorescent glutamate nanosensors, microdialysis and amperometric methods are some of the analytical techniques to monitor dopamine release in brain slice.

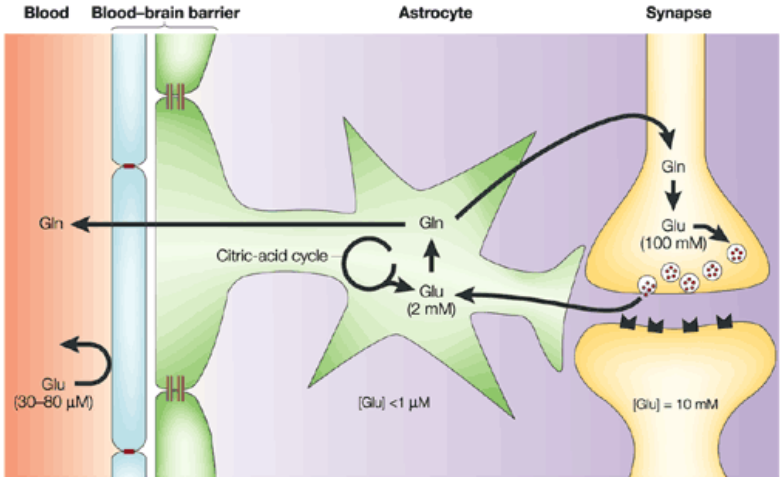


Figure 8: Glutamate synthesis and its movement in the brain. Glial cells release glutamine in extracellular spaces. Presynaptic neurons uptake glutamine to convert it into glutamate and package into synaptic vesicles. Excess glutamate molecules are recycled into neurons through astrocytes.[81]

1.5 Dopamine

Dopamine constitutes approximately 80% of catecholamine in the mammalian brain and serves as a key neurotransmitter involved in neuromodulations such as motor control, locomotion, cognition, emotion and positive reinforcement.[86] Dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra (SN), located in the ventral midbrain regions, are primary source of dopamine in the brain. Major dopaminergic pathways include the nigrostriatal, mesolimbic and mesocortical pathways each projected project to dorsal striatum, nucleus accumbens and cortex respectively.[86–88]

Dopamine is synthesized, packaged into vesicles by vesicular monoamine transporter 2 (VMAT2), and released into the synaptic cleft upon presynaptic neuron activation.[86] It then interacts with five dopamine receptors (D1-D5), classified into two families: D1 like receptors (D1R and D5R) and D2 like receptors (D2R, D3R and D4R).[86] These G-protein coupled receptors (GPCRs) regulate adenylyl cyclase and cAMP levels, either excitatory or inhibitory, depending on the receptor type.[89] As key pharmacological targets, they play a crucial role in treating several neurological and psychiatric disorders. Excess dopamine in the synapse is primarily cleared via high-affinity dopamine transporters (DAT) through reuptake, aided by monoamine transporters with lower efficacy.[86]

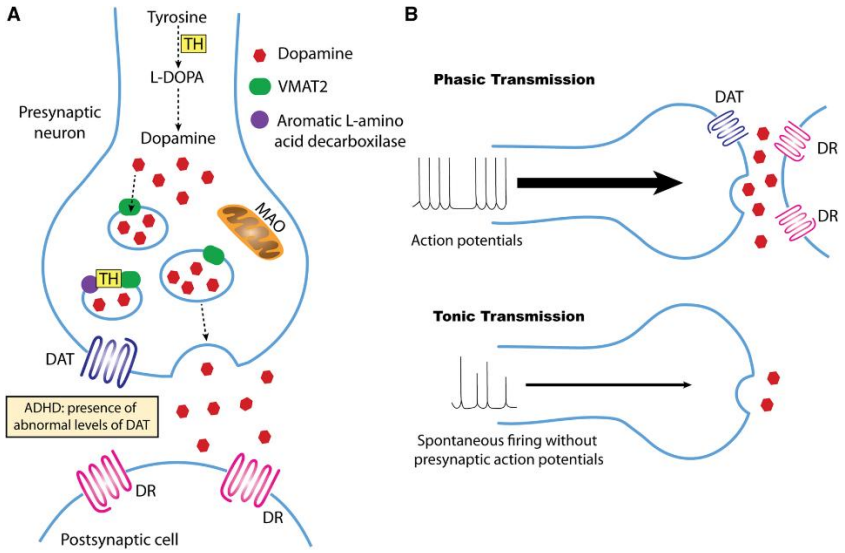


Figure 9: Dopamine synthesis and signaling (a) Synthesis route of dopamine from tyrosine, packaged and transportation to synaptic vesicle via VMAT2 transporter (b) Action potential triggers dopamine release during phasic transmission resulting huge surge of dopamine in extracellular spaces. Whereas tonic neurotransmission is independent of action potential and regulates the basal level of dopamine in extracellular spaces[88]

Phasic and tonic signaling regulates extracellular dopamine concentrations. Phasic signaling occurs when presynaptic neurons release dopamine in response to action potentials, leading to rapid fluctuations in extracellular dopamine levels, typically in the micromolar range. This transient dopamine surge activates D1 receptors (D1Rs), promoting synaptic plasticity and facilitating reward-based learning by altering neuronal excitability. [86,88] Tonic signaling, in contrast, is regulated by ongoing neuronal activity along with dopamine reuptake and clearance mechanisms. It maintains basal dopamine levels in the nanomolar range, selectively activating D2 receptors (D2Rs) without reaching concentrations high enough to stimulate D1Rs.

Dopaminergic neurons project widely throughout the brain, supporting functions such as motor control, learning, rewards, memory formations. Studies have shown interactions between dopaminergic system and other neurotransmitter systems, including glutamatergic, GABAergic and serotonergic pathways, through receptor-receptor interactions.[38,39,41] Examples of receptor

heteromerization include NMDA-D1R in rat hippocampus, A1-D1R in fibroblast cells, A2A-D2 in the basal ganglia, which enables cross-talk between signaling pathways via G-protein interactions, contributing to complex neuromodulation.[38–40,42]

The widespread distribution of dopaminergic neurons also contributes to a broad spectrum of neurological and psychiatric disorders associated with dopamine dysfunction or degeneration. Parkinson's disease (PD), schizophrenia, and addiction are among the key conditions linked to dysregulated dopaminergic neurotransmission. Due to its critical role in brain function, the dopaminergic system has been a major focus of research. Pharmacological interventions targeting dopamine receptors, synthesis, and reuptake are widely used in the clinical treatment of these disorders, aiming to restore dopamine balance and mitigate symptoms.

1.6 Oxygen/Glucose deprivation (OGD)

The central nervous system (CNS) heavily relies on continuous supply of oxygen and glucose from the blood to function properly and needed for neuronal activity. During transient ischemia, blood flow is temporarily occluded and resolved before causing any permanent damage to the brain.[84,90] Compared to other cell types present in CNS, neurons are particularly prone towards ischemia and rapidly compromise nerve cell functions.[82] Ischemic insult drastically reduces ATP formation within a minute of oxygen and glucose deprivation, and leads to an imbalance of neuronal transmembrane gradient, thus impairing neuronal signaling.[91]

Additionally, anoxic depolarization at presynaptic terminals leads to neurotransmitter release such as glutamate. Excitatory neurotransmitter clearance from the synaptic cleft is an active process.[91] Due to inadequate ATP supply, clearing process is hindered, thus neurotransmitter concentration is elevated during ischemic condition and results excitotoxicity. Removal of extracellular magnesium from the NMDA receptors substantially increase conductance, consequently, increases intracellular calcium concentration. This leads to

activation of several calcium-dependent processes such as excess neurotransmitter release, free radical formation, and the initiation of cell death process including apoptosis and autophagy.[82,92]

The main pathogenesis of ischemic insult induces significant oxidative injury to surrounding neurons, which can be mimicked in by an oxygen and glucose deprivation (OGD) procedure applied to brain slices. The cellular consequences of altered oxygen and glucose supply in rodent are widely studied and simulated using OGD model.[93] Studies show that hippocampal CA1 pyramidal cells and Purkinje cells followed by striatal medium-sized neurons are most highly susceptible to brief ischemic injury.[92,93] During transient ischemic injury, glutamate is significantly affected and excessively released from presynaptic neurons. Similarly, extracellular adenosine level rises due to net breakdown of intracellular ATP and depresses excitatory synaptic transmission.[92] Although OGD studies focuses on glutamatergic signaling, monoamine neurotransmitters such as dopamine and serotonin are also released immediately after the onset of ischemic conditions.[19]

1.7 Overview of the dissertation

FSCV is a powerful method where potential is applied at CFMEs to investigate electroactive neurotransmitters like adenosine, dopamine, and serotonin in real-time. Although FSCV has high temporal resolution, its limitations include poor spatial resolution and selectivity, restricting its scope to electroactive molecules. This dissertation bridges this gap by combining FSCV with fluorescence techniques, enabling simultaneous monitoring of electroactive and non-electroactive neurotransmitters. This approach provides insights into adenosine's neuromodulatory role, neurotransmitter interactions, and regional effects.

In Chapter 2, we investigated the neuromodulatory effect of transient adenosine on serotonin release in the dorsal raphe nuclei (DRN), rapid adenosine (50 pmol) significantly inhibited serotonin release within 20 seconds, with recovery once adenosine cleared from the

slice. By utilizing transgenic mice (A1KO, A2AKO) and pharmacological treatments (DPCPX, MRS 1220), it was determined that adenosine receptors A1 and A3 were not directly involved. Instead, 5-HT1A autoreceptors play a critical role regulating serotonergic neurons, as revealed using the antagonist (S)-WAY 100135 dihydrochloride. This highlights an indirect pathway for adenosine's regulation of serotonin.

In Chapter 3, we multiplexed FSCV and genetically encoded fluorescence sensors (e.g., iGluSnFR3.v857) to simultaneously measure adenosine, dopamine, and glutamate in real-time. Adenosine showed inhibitory actions on both dopamine and glutamate in the caudate putamen, with an inhibitory range of 250 μm. This effect was confirmed as A1 receptor-mediated, as shown by the reversal with the antagonist DPCPX. These findings emphasize adenosine's spatial and temporal neuromodulatory profiles.

In Chapter 4, transient oxygen-glucose deprivation (OGD) insult and subsequent reperfusion caused significant reductions in dopamine and glutamate release over time. Mixed recovery responses were observed during extended reperfusion periods (up to 120 minutes). Through transgenic models (A2AKO) and pharmacological studies (NMDA receptor antagonist, DPCPX), adenosine receptors A1 and A2A were shown to play neuroprotective roles during OGD and reperfusion, demonstrating their therapeutic potential in ischemic pathology.

Overall, this dissertation integrated FSCV and fluorescence techniques to uncover adenosine's neuromodulatory interactions with neurotransmitters under both normal and pathological conditions, such as ischemia. These innovative methodologies open new scope for understanding neurotransmitter interactions in real-time during diseases like Parkinson’s, paving the way for translational neuroprotective strategies.

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Chapter 2: Transient adenosine modulates serotonin release indirectly in the DRN

Kailash Shrestha, B. Jill Venton*

Abstract

Rapid adenosine transiently regulates dopamine and glutamate via A_1 receptors, but other neurotransmitters such as serotonin have not been studied. In this study, we examined the rapid modulatory effect of adenosine on serotonin release in the dorsal raphe nuclei (DRN) of mouse brain slices by using fast-scan cyclic voltammetry (FSCV). To mimic adenosine release during damage, a rapid microinjection of adenosine 50 pmol was applied before electrical stimulation of serotonin release. Transient adenosine significantly reduced electrically-evoked serotonin release in the first 20 seconds after application, but serotonin release recovered to baseline as adenosine was cleared from the slice. The continuous perfusion of adenosine did not change evoked serotonin release. Surprisingly, the modulatory effects of adenosine were not regulated by A_1 receptors, as adenosine still inhibited serotonin release in A_1KO mice and also after perfusion of an A_1 antagonist (8-Cyclopentyl-1,3-dipropyl xanthine, DPCPX). The inhibition was also not regulated by A_3 receptors, as perfusion of the A_3 antagonist (MRS 1220) in A_1KO brain slices did not eliminate the inhibitory effects of transient adenosine. In addition, adenosine also inhibited serotonin release in A_{2A}KO mice, showing A_{2A} did not modulate serotonin. However, perfusion of a selective $5HT_{1A}$ autoreceptor antagonist drug ((S)-WAY 100135 dihydrochloride) abolished the inhibitory effect of transient adenosine on serotonin release. Thus, the transient neuromodulatory effect of adenosine on DRN serotonin release is regulated by serotonin autoreceptors and not adenosine receptors. Rapid, transient adenosine modulation of neurotransmitters such as serotonin may have important implications for diseases such as depression and brain injury.

Keywords: Neuromodulation, Adenosine, Serotonin, Transient, FSCV, dorsal raphe nuclei (DRN), $5HT_{1A}$ antagonist

2.1 Introduction

Adenosine is a widely distributed neuromodulator in the central nervous system that modulates neurotransmission and plays a neuroprotective role via adenosinergic, G protein-coupled receptors.[1,2] Four sub-types of membrane-bound adenosine receptors exhibit inhibitory (A_1 and A_3) and excitatory effects (A_{2A} and A_{2B}) by altering adenylyl cyclase activity at the synaptic terminal.[1,3–6] During pathological conditions, extracellular adenosine accumulates at different rates. Slower accumulation during damage or stroke can last for hours, and prevents further tissue damage by reducing excitatory neuronal firing at the synapse.[6–9] However, there are rapid modes of adenosine release which last for just a few seconds.[10,11] Dunwiddie et. al. discovered transient adenosine signaling that ranged from milliseconds to seconds, with rapid neuromodulatory effects in the hippocampus.[12] Similarly, using fast-scan cyclic voltammetry (FSCV), our lab characterized spontaneous transient adenosine, lasting 2-3 seconds, that modulates neurotransmitter release and is released in response to ischemia.[9,11,13,14] Mechanical perturbation of tissue also generates transient adenosine, but mechanically-stimulated adenosine is higher in concentration and lasts around 20-30 seconds with a broader effective area.[4,9] This mode of rapid adenosine release may serve a neuroprotective role during physical injuries and trauma. Adenosine, a well-known retaliatory metabolite, modulates the synaptic release of other neurotransmitters such as dopamine (DA), glutamate, and GABA through its presynaptic and postsynaptic receptors.[1,11,15] Basal changes in adenosine have been reported to modulate serotonin release in the hippocampus, but there have been no investigations of the effect of rapid changes in adenosine to modulate serotonin release in the DRN, which is responsible for cognition, sleep, and locomotion.[16–18]

The dorsal raphe nuclei (DRN) and medial raphe nuclei are the largest serotonergic cluster in the brain.[19,20] A wide distribution of serotonin neurons and abundance of serotonin receptors contribute to serotonin's broad modulatory roles and diverse physiological functions, such as regulating cognition, sleep-wake cycle, aggression, locomotion, and mood.[21] These excitatory serotonin neurons are closely regulated by the abundant somatodendritic 5-HT_{1A} autoreceptor, a G-protein coupled receptor.[18,20,21] In addition, inhibitory GABAergic neurons, glutamatergic and cholinergic neurons alter neuronal firing through change in K^+ and Ca^{2+} channel permeability.[16,18,22] However, the direct modulatory role of adenosine on the serotonin release has not been well understood particularly in the DRN.

Adenosine has a modulatory effect on neurotransmitters and direct measurements of these neurotransmitters help elucidate these effects. For example, Ross et al. demonstrated inhibitory action of transient adenosine on dopamine by 50% via A_1 receptors in the caudate putamen.[11] Similarly using super-fusion techniques, Feuerstein et al. found that both theophylline and 8-phenyl theophylline, A_1 receptor antagonists, abolished the inhibitory effect of endogenous adenosine via A_1 receptors on stimulated serotonin release in the rabbit hippocampus slice.[23,24] Thus, A_1 receptors may regulate serotonin release.[25,26] Furthermore, adenosine deaminase, an enzyme responsible for adenosine metabolism, is present in the DRN and thus adenosine may regulate serotonin in the DRN.[27] In the previous study by Feuerstein et al., the modulatory effect of adenosine was studied in slower time scale using superfusion technique in which isotopic serotonin samples were collected every 60 minutes and analyzed.[28] Although 5-HT_{1A} autoreceptors and GABAergic receptors tightly regulate

serotonin concentrations, there are no studies that address the modulation of transient adenosine on serotonin release in the DRN.[29,30]

The goal of this study was to test the modulatory effect of transient adenosine on serotonin release in the DRN in real-time. Rapid adenosine and serotonin release were measured using FSCV, a highly sensitive real-time detection technique with sub-second temporal resolution[31,32] after puffing adenosine, which lasted 25 seconds, mimicking mechanosensitive release. Transient adenosine inhibited serotonin by 40% in WT in the first 20 seconds of adenosine application, but this same effect was not observed during adenosine perfusion. A_1KO mice and an A_1 antagonist were used to investigate the role of inhibitory A_1 adenosine receptors, but there was no change in adenosine modulation of serotonin in either model. Similarly, inhibition of serotonin was not abolished in A_{2A}KO mice or using a selective A_3 antagonist in A_1KO mice. However, adenosine modulation of serotonin was eliminated during the perfusion of a selective $5HT_{1A}$ antagonist. Thus, adenosine modulates serotonin dynamics in the DRN, not through adenosine receptors, but indirectly through $5HT_{1A}$ autoreceptors.

2.2 Methods

2.2.1 Chemicals

All chemical reagents were purchased from Sigma Aldrich. The artificial cerebral spinal fluid (aCSF) buffer was composed of 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM $MgCl_2{\cdot}6H_2O$, 25 mM $NaHCO_3$, 11 mM glucose, and 15 mM tris(hydroxymethyl) aminomethane dissolved in deionized water (Milli-Q Biocel; Millipore, USA). The aCSF was freshly prepared before experiments and adjusted to pH 7.4 for brain slice experiments. Adenosine was purchased from Acros Organics (Morris Plains, NJ, USA).

Adenosine stock solution (10 mM) was prepared in 0.1 M perchloric acid and diluted to 1 mM concentration with aCSF.

8-Cyclopentyl-1,3-dipropyl xanthine (DPCPX, A_1 adenosine receptor antagonist) and MRS 1220 (A_3 adenosine receptor antagonist) were purchased from Tocris Bioscience (Minneapolis, USA). 10 μM of DPCPX and 5 μM of MRS 1220 were freshly prepared by dissolving in 1 ml of dimethylsulfoxide (DMSO) through sonication and diluted with aCSF buffer.

2.2.2 Electrochemistry

Cylinder carbon-fiber microelectrodes (CFMEs) were fabricated by aspirating a T-650 carbon fiber (7 μm diameter, Cytec Engineering Materials, West Patterson, NJ) into a glass capillary (1.2 mm OD × 0.68 mm ID, 4″, A-M systems) using a vacuum pump. The glass capillary was pulled via a horizontal electrode puller (model PE-21, Narishige, Tokyo). The exposed carbon fiber was cut approximately 70−100 μm long using a scalpel under a microscope.

A CFME was dipped into isopropanol alcohol (IPA) for 10 minutes to remove any impurities from its surface and backfilled with 1 mM KCl solution before its use. Adenosine waveform (-0.4 to +1.45 and back, scan rate: 400 V/s and 10 Hz vs Ag/AgCl reference electrode) was applied at the CFME and electrochemical data collected using a Chem-Clamp (Dagan, Minneapolis, MN, U.S.A.). A dopamine waveform with a switching potential has previously been used for serotonin detection,[34] and here extending the potential to 1.45 V allowed both adenosine and serotonin to be detected simultaneously with minimal fouling. All data were analyzed using HDCV analysis software (UNC Chemistry, Chapel Hill, NC, U.S.A.).

2.2.3 Brain slice experiments

The Animal Care and Use Committee (ACUC) of the University of Virginia approved all animal work. 6-8 weeks old wild-type C57BL/6 mice (Jackson Lab), A_1KO mice, and A_{2A}KO mice

were anesthetized with Isoflurane. Following quick decapitation, the brain was removed from the skull and transferred into 0−5 °C oxygenated aCSF (95% O_2, 5% CO_2) for recovery. The brain was quickly mounted on the cold slicing stage and coronal section slices (400 μm) prepared via a vibratome (Leica VT1000S, Bannockburn, IL, USA). Slices were transferred into a chamber containing oxygenated aCSF (32°C) and kept for 30-45 minutes to recover. Once the slice was moved to the recording chamber, an electrical stim electrode was placed in the DRN. The carbon-fiber electrode was inserted approximately 75 μm deep in the dorsal raphe and equilibrated for 10-15 minutes with waveform applied. Electrically stimulated serotonin release was measured with or without puffed adenosine (50 pmol) while applying the adenosine waveform at the CFME. After each stimulation, 10 minutes was used as a recovery time before another stimulation. Throughout the experiment, aCSF was continuously perfused over the brain slice by a perfusion pump (Watson-Marlow 205U, Wilmington, MA, USA) at 2 mL/min.

2.2.4 Pharmacology experiments

Pharmacological experiments were performed in a 10 s time frame as we saw significant effect of transient adenosine over serotonin release in both WT and genetically A_1, A_2 and A_3 knocked out mice. An A_1 antagonist (DPCPX, 10 μM, K_i = 3.9 nM) or A_3 antagonist (MRS 1220, 5 μM, K_i > 1 μM) was perfused continuously for 30 minutes to study the role of A_1 and A_3 receptors respectively.[51,52] Both antagonists were dissolved in 1 ml of dimethysulfoxide (DMSO) and diluted with aCSF. Electrically stimulated serotonin and pre-drug scans with adenosine (10 seconds after exogenously puffed adenosine) were collected perfusing aCSF. Next, the drug was perfused for 30 minutes. During drug perfusion, serotonin was stimulated after adenosine application and recorded every 10 minutes. And finally, post-drug stimulated serotonin (with adenosine) was recorded for recovery. A_1 antagonist was used in WT and A_{2A}KO mice whereas A_3 antagonist was in A_1KO mice. Genetically altered A_1 receptor knock-out and A_{2A} receptor

knock-out mice were made by the Mustafa lab (Dr. S. Jamal Mustafa, West Virginia University).[53,54]

Selective $5HT_{1A}$ antagonist ((S)-WAY 100135 dihydrochloride, 5μM) was perfused to examine the role of $5HT_{1A}$ autoreceptor on DRN serotonergic neurons by adenosine. Initially evoked serotonin was recorded and the $5HT_{1A}$ perfused for 30 minutes. During this perfusion, serotonin was stimulated at 20th minute without adenosine and at the 30th minute with adenosine 10 s before. Finally, the perfusion buffer was switched back to aCSF and a stimulation applied for the recovery measurement. All serotonin release was normalized and compared with initial electrically stimulated serotonin release without adenosine injection.

2.2.5 Statistics

All the statistics were performed by GraphPad 9.0 (GraphPad Software Inc., San Diego, USA), and all data are reported as mean ± standard error of the mean (SEM) for “n” number of mice. One-way ANOVA test with Dunnett’s post-test was performed to analyze initial serotonin to the serotonin with puffed adenosine with or without perfused drugs on the brain slice.

2.3 Results and Discussion

This study tests the hypothesis that rapid adenosine transiently downregulates DRN serotonergic release. To assess how adenosine modulates serotonin release, we measured stimulated serotonin in a DRN brain slice by implanting a CFME and using FSCV. Twenty-five pmol of adenosine was locally microinjected via pico-spritzer at different time intervals (10, 20, 40 and 80 seconds) before serotonin stimulation. Exogenously puffed adenosine lasts ~25 seconds, which is similar to mechanically-stimulated adenosine release.[11]

2.3.1 Rapid changes in local adenosine modulate DRN serotonergic neurons in WT mice

First, we measured the effect of transient adenosine on stimulated serotonin in WT mice. Figure 1A shows the experimental set up for this study. The working electrode (CFME) and the capillary with adenosine were inserted into the tissue about 50 µm apart. Although many other waveforms such as the Jackson waveform and extended serotonin waveform have been developed to more selectively monitor serotonin, we used the adenosine waveform (-0.4 V, 1.45 V; Scan rate: 400 V/s) to monitor both adenosine and serotonin in the same scan.[33–35] The main interferent for serotonin with that waveform would be dopamine, but there are not dopaminergic neurons in the DRN, so dopamine is not an interferent for serotonin detection. Figure 1B summarizes the experimental design, where serotonin was electrically stimulated at different time intervals (10, 20, 40 and 80 s) after adenosine injection. Stimulations were repeated every 10 minutes to allow recovery of release, and this time interval produces the same release for all stimulations.[36] Figure 1C shows stimulated serotonin 80 seconds after adenosine application. Adenosine is cleared from DRN around 20 s after injection, as seen in *i-t* curve (top inset, red trace). This *i-t* curve mimics mechanically stimulated adenosine, which is quickly released and elevated for about 20-30 s before it is fully cleared from the extracellular space.[9] FSCV is a background subtracted method that monitors rapid change in electroactive analyte concentration. The background subtracted CV, inset, is a signature CV for serotonin that confirms the identity of the release of serotonin release. Supplementary Figure 1 shows an example color plot of electrically evoked serotonin 20 seconds after adenosine injection, at two different color contrasts to show the adenosine (which is higher concentration and produces larger oxidation currents) and serotonin. We measured serotonin levels from the i vs t curve at the oxidation potential for serotonin.

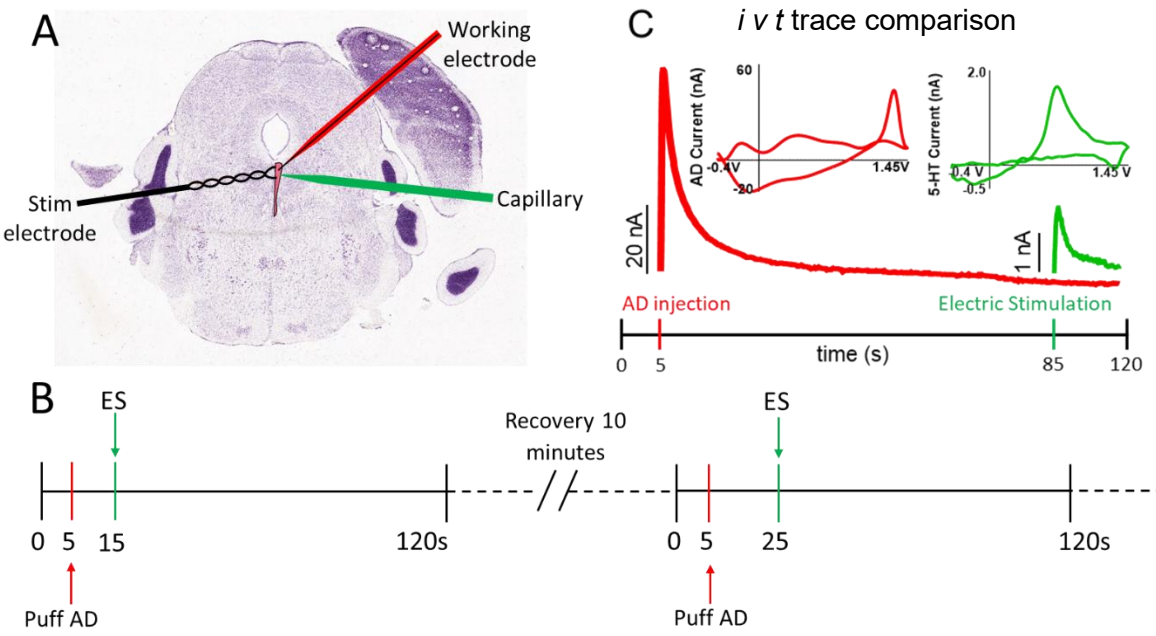

Figure 10. Experimental Design. (A) Experimental setup showing CFME, stimulating electrode, and glass capillary for adenosine injection using pico-spritzer in the DRN. (B) An experimental timeline shows adenosine injection at 5 seconds (red arrow) followed by stimulation (green arrow) after 10 seconds. The interval between adenosine application and stimulation is increased i.e., 10, 20, 40, and 80 seconds with the following scans, and finally recovery scan was performed without adenosine (not shown in the timeline). There was 10 minutes between experiments. (C) Representative example, 1 mM adenosine (50 pmol) is administered via pico-spritzer (10 psi, 10 ms, 100 nL) 80 seconds prior to serotonin stimulation as shown with the i vs t curve for adenosine (red trace) and serotonin (green trace). The inset contains an adenosine CV (red) with two oxidation peaks at +1.4 V (primary) and +1.0 V (secondary), and a serotonin CV with an oxidization potential at 0.7 V (green), indicating that both analytes can be detected using adenosine waveform.

Figure 2A shows overlapped serotonin release traces before and after adenosine puffed at different time intervals. Adenosine suppresses electrically evoked serotonin release in the DRN, particularly when the time interval between the adenosine application and stimulation is small. As the time interval of adenosine injection and serotonin stimulation increases, serotonin release gradually increases back to baseline. The recovery curve (grey trace) shows that serotonin release returns to normal when no adenosine is applied to the slice at the end of the experiment, showing the tissue is viable. Thus, when adenosine is present, stimulated serotonin is inhibited.

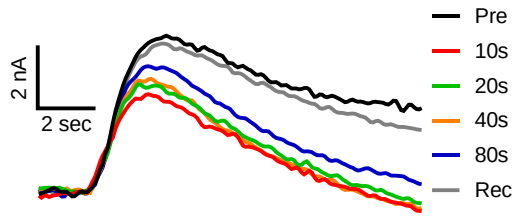

Figure 11. Serotonin (5-HT) release i-t curve with adenosine at different time intervals (10s, 20s, 40s, and 80s) and without adenosine ES (pre-AD) and Rec is after the AD trials. The y-axis is serotonin current (nA) detected after electrically evoked serotonergic release in the DRN. The x-axis is time (s).

Figure 3A compares averaged data for serotonin stimulations at various time intervals after adenosine application. The y-axis is current normalized to the initial, pre-adenosine stimulated serotonin and the x-axis is the time interval between the adenosine administration and electrical stimulation. The grey bar is a final recovery stimulation without any adenosine, showing the tissue is still viable and stimulated serotonin release has not changed over time. Adenosine decreased for the 10 and 20 s intervals, and then gradually returns to its initial serotonin level with 40 s and 80 s. and recovery (Figure 3A). There is a significant main effect of the time interval of adenosine on serotonin release (One-way ANOVA; n=7; p=0.0291) and post-tests show that transient adenosine significantly reduced stimulated serotonin for the 10 and 20 s adenosine groups (Dunnett's post-test: 10s: Red, p= 0.0195; 20s: Green: p=0.0231). The 40 s, 80 s, and recovery groups are not significantly different than the initial stimulation. (One-way ANOVA; n=7; 40s: Orange, p=0.1605; 80s: Blue, p=0.4138; Recovery: Grey, p=0.8638). Serotonin was suppressed by 40% for the 10 and 20 s group, showing that transient adenosine decreased release by almost half. The adenosine i vs t trace (Fig. 1C) reveals that adenosine is elevated in the tissue for about 20 s, so these data show that adenosine modulates serotonin only when it is transiently elevated. Thus, adenosine transiently modulates DRN serotonergic release when adenosine is elevated.

As a control, we replaced the adenosine injection with aCSF buffer injection and recorded stimulated serotonin (Figure 3B). aCSF was injected at the same time intervals as in

the adenosine experiment and there was no significant effect of aCSF injection on the serotonin release (One-way ANOVA, n= 4; p=0.8221). Finally, to make sure that the effect was due to adenosine and not its common downstream metabolite, we injected inosine, the major metabolite of adenosine which is formed from adenosine deaminase breakdown. There is no significant effect of puffing on inosine at the various time intervals before stimulating serotonin (Fig. 3C, One-way ANOVA, n=3, p=0.8888). This further confirms that adenosine is causing the neuromodulatory effects on serotonin release, and the decrease is not just an effect of puffing on a reagent or a metabolite.

After testing the effects of rapid adenosine neuromodulation, we tested the effects of bath application of adenosine, simulating increase of basal adenosine levels. Figure 3D shows perfusion of 10 μM adenosine for 30 minutes, while stimulating serotonin every 10 minutes, and a recovery stimulation after perfusion was switched to normal aCSF. The bath perfusion did not suppress stimulated serotonin at 10, 20, or 30 minutes. Stimulated serotonin levels were not significantly different between pre- and post-adenosine perfusion in the DRN region (One-way ANOVA; n= 4, p= 0.9621). Thus, these data confirm that the inhibition of serotonin release is mediated by transient adenosine but not with changes in basal adenosine levels.

Adenosine is a ubiquitous neuromodulator, predominantly known for its inhibitory function via $G_{i/o}$ proteins on neurotransmitters such as dopamine, GABA, and glutamate.[7,23,24,37] Here, examined the extent to which adenosine regulates phasic serotonin release by mimicking mechanosensitive adenosine release, which elevates adenosine for about 20-30 s in the extracellular space.[9] The main finding is that adenosine inhibits serotonin release only when it is transiently elevated. After adenosine is cleared, in approximately 20 s, stimulated serotonin release is not significantly inhibited. The maximum inhibition is about 40%, which is similar to the transient inhibition of dopamine by adenosine in the caudate putamen.[11] Similarly, dopamine was only inhibited when adenosine was transiently elevated, and not after adenosine had been

cleared. Thus, there is a significant, but partial depression of serotonin release in the presence of transient adenosine.

Early studies examined the effects of adenosine to regulate serotonin release and found that A_1 receptors mediate adenosine inhibition of evoked serotonin in the hippocampus, but not the caudate putamen.[23,24] However, those studies did not examine transient adenosine or the modulation of adenosine on DRN serotonergic neurons. Here, we found that mimicking increases in basal levels of adenosine by perfusing adenosine did not have any inhibitory effect on phasic serotonin release. Thus, adenosine acts transiently to rapidly activate receptors and downregulate serotonin release, and not through constant action of basal receptor occupancy. The main finding here is that adenosine inhibits serotonin release in the DRN only while it is transiently elevated. This rapid effect could be very important in temporarily suppressing neurotransmission during tissue damage or other trauma conditions.

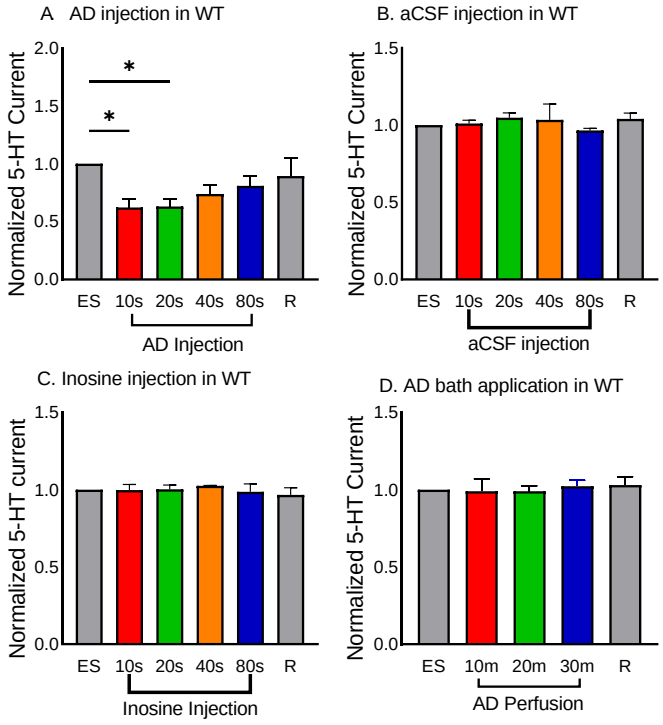

Figure 12. Neuromodulatory effect of adenosine on stimulated serotonin. (A) Effect of transient adenosine. 50 pmol of adenosine was exogenously puffed after an electrical stimulation at different time intervals (10s, 20s, 40s, and 80s) in the DRN of a WT brain slice. Each bar is serotonin current normalized to the first stimulation without adenosine. (Black: Initial electric stim, Red: Adenosine applied 10 s before, Green: 20 s, Orange: 40 s, Blue: 80 s, Grey: Recovery (no adenosine at the end)). There is a significant effect of the time interval for adenosine on serotonin release (One-way ANOVA, n=7; p= 0.0291). (B) Transient aCSF control. 0.1M $HClO_4$ was diluted in aCSF as adenosine stock was, and then applied instead of adenosine and no significant effect was observed on serotonin (One-way ANOVA, n= 4; p=0.8221). (C) Inosine (10 μM) injection in the DRN at different time interval to test the effect of an adenosine metabolite. There was no different in serotonin release with or without inosine injection (One-way ANOVA, n=3, p=0.8888). (D) Bath application of adenosine (10 μM) for 30 minutes. Serotonin was stimulated every 10 minutes and normalized to the initial stimulated serotonin. Serotonin release did not differ with adenosine perfusion (One-way ANOVA, n=3, p=0.9621). Error bar*s* are SEM. ES: Electric stim

2.3.2 Transient adenosine suppression of serotonin release is not regulated by A_1 receptors

Adenosine typically exhibits an inhibitory role predominantly via A_1 receptors, which are located presynaptically.[7,27,38] A_1 receptors regulated the transient modulation by adenosine of dopamine[11] and here we hypothesized A_1 receptors would have the same role for serotonin. To examine inhibitory effect of adenosine via A_1 receptors, adenosine modulation of serotonin

release was monitored in genetically modified A_1KO mice and in WT mice slices perfused with DPCPX, an A_1 inhibitor.

Figure 4A shows serotonin release after adenosine application in A_1KO mice, with the same protocol used for WT mice (10 s, 20 s, 40 s and 80 s intervals followed by recovery without adenosine). There was a significant main effect of adenosine on serotonin release in A_1KO mice (One-way ANOVA, n=7, $p<0.0001$) and stimulated serotonin release was significantly inhibited by transient adenosine in the first 10 seconds of application (Dunnett's post-test, n=7, $p<0.0001$). The decrease of serotonin to 45 % of the initially stimulated serotonin was similar to WT. However, serotonin suppression was not significantly different than initial stimulation for the 20 second time interval in A_1KO mice, unlike WT (Dunnett's post-test; 20s: Green: $p=0.1373$, n=7). This slight increase in serotonin release after 20 s of adenosine application in A_1 knocked-out mice could be due to compensatory overexpression of another inhibitory receptor, such as A_3 adenosine receptors or GABA receptors, that have a different timeframe of interaction.[39] Similar to WT, stimulated serotonin release gradually recovers to baseline with no significant differences from initial stimulation for transient adenosine with 40 s, 80 s or recovery. The large inhibition that occurs at 10 s indicates that A_1 receptors are not primarily responsible for the inhibition of serotonin in the DRN.

To further ensure A_1 receptors have no role in this inhibition by transient adenosine, we perfused the A_1 antagonist drug DPCPX (10 μM) in WT mice for 30 minutes and stimulated serotonin release every 10 minutes with adenosine applied 10 s before (Figure 4B). All serotonin currents were normalized to the initial, stimulation with no adenosine. There was a significant effect of adenosine on stimulated serotonin release (One-way ANOVA, n=3, $p=0.0016$) and serotonin release was inhibited by adenosine even during perfusion of DPCPX. Thus, A_1 receptors are not responsible for mediating the inhibitory effect of transient adenosine on serotonin.

The vast majority of studies demonstrating inhibitory effects of adenosine have shown A_1 receptors that mediate these effects; thus our results are surprising.[4,7,24] For example, the inhibition of serotonin release in the hippocampus by adenosine was blocked by an A_1 antagonist, 8-Phenyltheophylline, indicating A_1 receptors mediate the inhibitory effect.[23] Adenosine modulation of dopamine release was also mediated by A_1 receptors in caudate putamen and hippocampus.[5,11,15] In the DRN, A_1 receptors are expressed and adenosine deaminase is present, an enzyme responsible for adenosine metabolism, suggesting adenosine plays a neuromodulatory role there.[25–27] In A_1KO mice, there is still a strong inhibitory effect of adenosine applied 10 s before serotonin stimulation, indicating A_1 receptors are not primarily mediating the inhibition. There were some slight variations in the 20 s data, which are not significant in A_1KO mice but were in the WT mice, but the overall inhibition with 10 s is just as strong as the WT mice. Pharmacological experiments with DPCPX, an A_1 antagonist, in WT mice also verify that A_1 receptors do not mediate inhibition of serotonin release. These data are surprising because of the evidence of A_1 inhibition in other studies, but here A_1 receptor blockade is not sufficient to remove the inhibition of serotonin by adenosine. Thus, A_1 receptors are not the primary receptor mediating the inhibitory action of adenosine on DRN serotonergic release.[11,24]

2.3.3 The inhibitory effect of adenosine on serotonin release is not mediated by A_3 or A_{2A} receptors.

Since A_1 receptors are not primarily responsible for the suppression of DRN serotonin release, we tested the effects of A_3 receptors, which are also inhibitory and might be upregulated in A_1KO mice.[39] A_3 receptors are also inhibitory with lower affinity, although they are more sparsely located than A_1 receptors.[1,4,7,37] An A_3 antagonist (MRS 1220, 5 μM), was perfused in A_1 knock-out slice. The A_1KO was used to eliminate both of the possible inhibitory receptors at once when the antagonist was perfused. We perfused MRS 1220 for 30 minutes,

while stimulating serotonin 10 seconds after adenosine application every 10 minutes (Figure 4C), before washing out drug for a recovery stimulation with adenosine application. There is a still a significant effect of adenosine on serotonin release (One-way ANOVA, n=3, p=0.0014) and no differences with the A_3 receptor antagonist. Thus, adenosine still clearly inhibited serotonin release, even in an A_1KO slice in the presence of an A_3 antagonist. These results show that neither of the two major inhibitory receptors, A_1 or A_3 receptors, are directly mediating the inhibition of serotonin by transient adenosine.

A_{2A} receptors are the main excitatory receptors for adenosine, and they are the second most abundantly distributed receptors in the CNS.[2,4,5,7] The activation of these excitatory receptors, coupled with $G_{s/olf}$, activates presynaptic receptors to stimulate neurotransmitter release. However, these excitatory receptors can form heteromers with inhibitory A_1 adenosine receptors at the same terminal and they modulate glutamate neurotransmitter release.[40] Thus, although A_{2A} receptors are excitatory, we tested their effects. We used A_{2A} knock-out mice along with DPCPX to block both A_1 and A_{2A} receptors, to test if heteromers were responsible for the inhibition. A similar experimental set-up was used as with the A_3 antagonist data. There was a main effect of adenosine in regulating serotonin release (Fig. 4D, One-way ANOVA, n=3, p=0.0127). Serotonin release was significantly suppressed by adenosine in the A_{2A} knock-out slice when adenosine was injected 10 seconds before electric stimulation (One-way ANOVA, n=3, p=0.0257). When 10 μM of DPCPX was perfused, stimulated serotonin after adenosine was still significantly inhibited compared to initial stimulation (One-way ANOVA, n=3, p=0.0311). Thus, in an A_{2A}KO mice, serotonin release is still inhibited by transient adenosine applied 10 s before and when perfusing 10 μM of DPCPX in the A_{2A} knockout mice, adenosine still inhibits serotonin release. This data shows that A_{2A} receptors are not responsible for the inhibitory effects of adenosine and that heteromers are also likely not the source of adenosine

modulation. Thus, surprisingly, no adenosine receptors were identified that mediate the inhibitory effect of transient adenosine on serotonin release.

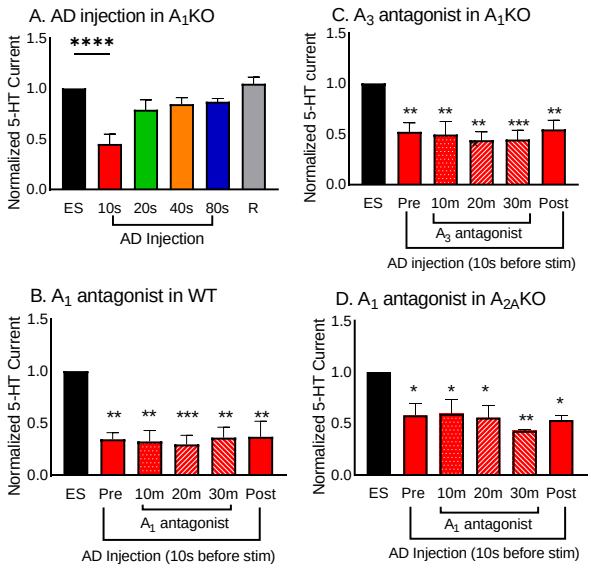

Figure 13. Effect of adenosine receptors to regulate the effects of transient adenosine on serotonin release. (A.) AD injection in A_1KO mice. The time interval was varied for adenosine application before serotonin stimulation and serotonin is still suppressed by adenosine with the 10 s interval (One-way ANOVA, n=7; ES; 10s: Red, $p<0.0001$) (B.) A_1 antagonist (10 μM DPCPX) perfused for 30 minutes in WT mice. Serotonin was stimulated 10 s after adenosine application and DPCPX did not stop the inhibition of adenosine (One-way ANOVA, n=3, $p=0.0016$). (C.) Effect of A_3 antagonist (5 μM of MRS 1220) on inhibition of adenosine on serotonin release in A_1KO mice. The drug had no effect as adenosine still inhibited serotonin (One-way ANOVA, n=5; $p= 0.0014$) (D.) A_1 antagonist (10 μM DPCPX) perfused in A_{2A}KO slice with adenosine applied 10 s before serotonin stimulation. The A_1 antagonist did not change the inhibitory effect of transient (One-way ANOVA, n=3, $p=0.0127$). The error bar represents SEM. For B, C, D asterisks mark significant differences from pre-drug ES. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (AD: Adenosine; ES: Electric Stim pre-AD)

2.3.4 A selective $5HT_{1A}$ antagonist blocks the effects of adenosine on serotonin release

Our data showed that transient adenosine still inhibits electrically evoked serotonin release while blocking A_1, A_{2A}, or A3 receptors. Since no adenosine receptors were responsible for the inhibition by transient adenosine, we tested if $5HT_{1A}$ autoreceptors, most abundantly found in the DRN, regulate the inhibition of serotonin by adenosine.[21] These presynaptic $5HT_{1A}$

autoreceptors are densely packed at DRN and regulate serotonergic release through a negative feedback loop.[18,21]

The selective $5HT_{1A}$ antagonist (S)-WAY 100135 dihydrochloride (5 μM, $5HT_{1A}$ IC_{50}=34 nM) was perfused over a WT slice for 30 minutes.[41] Figure 5 shows the data. An electrical stimulation was performed without drug, then WAY 100135, a $5HT_{1A}$ antagonist, perfused and an electrical stimulation performed with no adenosine. The $5HT_{1A}$ antagonist did not alter electrically stimulated serotonin levels, similar to previous microdialysis studies that 5-HT_{1A} antagonists alone do not increase serotonin.[42] Similar effects have been observed in previous studies using electrophysiology, where the 5-HT_{1A} agonist 8-OH-DPAT (7-(dipropylamino)-5,6,7,8-tetrahydronapthalen-1-ol) also didn't change serotonin levels.[41,43] Then, in the presence of $5HT_{1A}$ antagonist an electrical stimulation was performed with adenosine applied 10 s before, and finally a recovery stimulation with no adenosine and no drug was performed. There was no effect of adenosine on stimulated serotonin release in the presence of a $5HT_{1A}$ antagonist (One-way ANOVA, n=3; p=0.3623). Pre-drug stimulated serotonin was similar to stimulated serotonin release in the presence of the 5-HT_{1A} antagonist and with adenosine puffed 10 s before serotonin stimulation. With perfusion of $5HT_{1A}$ antagonist drug, the inhibitory effect of transient adenosine was eliminated, indicating $5HT_{1A}$ autoreceptors tightly regulate DRN serotonergic release and are at least indirectly mediating the inhibitory effect of adenosine. Future studies could also use different 5-HT_{1A} autoreceptor drugs to confirm the specificity of the drug, but this data suggests that serotonin autoreceptors are regulating the effects of adenosine to modulate serotonin.

Serotonin receptors tightly regulate its own local vesicular release through inhibitory 5-HT_{1A} autoreceptors.[44] These inhibitory somatodendritic autoreceptors are associated with K^+ channels via G protein-coupled inwardly rectifying K^+ channels (GiRK), which closely regulate basal serotonin levels via negative feedback loop.[16,18] Activation of $G\alpha_{i/o}$-coupled serotonin

autoreceptors upregulates K^+ conductance, hyperpolarizes neuronal membranes, and generates fewer action potentials that suppresses neurotransmitter release in the extracellular space.[45] A_1 adenosine receptors, GABA receptors, 5-HT_{1A} receptors and D_1/D_2 dopamine receptors are some of the common receptors coupled with GiRK channel activation via GPCRs in the brain.[46] A potent $5HT_{1A}$ antagonist ((S)-WAY 100135 dihydrochloride, 5 μM), which blocks both pre and postsynaptic $5HT_{1A}$ autoreceptors, was perfused to examine if autoreceptors regulated the inhibition of serotonin release by adenosine.[41] Stimulated serotonin levels do not change in the presence of the $5HT_{1A}$ antagonist, similar to previous studies.[42] Interestingly, when transient adenosine was applied 10 s before serotonin stimulation in the presence of a $5HT_{1A}$ antagonist, there was no suppression of serotonin release. Thus, $5HT_{1A}$ inhibition blocked the inhibition of serotonin by transient adenosine.

The exact mechanism of how adenosine interacts with the serotonin autoreceptors is unknown. It is doubtful that adenosine acts directly at the serotonin autoreceptors, as no affinity for adenosine is known at serotonin receptors. Activation of 5-HT_{1A} leads to decreased cAMP concentrations; thus, adenosine may activate this pathway as well, by inhibition of adenylate cyclase producing cAMP.[47] There is a possibility of indirect inhibition pathway or cross coupled with other channels such as GiRK mediated by G- βγ subunits and Ca^{2+} channels that adenosine activates that feedback on serotonin levels or serotonin autoreceptors.[48] Adenosine increases GiRK channel conductance via A_1 receptors, which further depolarizes neuronal action potential and suppresses stimulated release in hippocampus.[46,49] Similarly, changes in GiRK channel permeability are also associated with inhibitory $GABA_B$ and auto-inhibitory $5HT_{1A}$ receptors, another possible inhibitory route to suppress DRN serotonin release.[46,50] Further studies are needed to establish the direct inhibitory role of adenosine as our technique, FSCV, is limited to detecting rapid change in electroactive neurotransmitter release and cannot measure changes in basal levels of serotonin.

These studies establish that adenosine is a transient inhibitory signal in the brain and that for serotonin, its own autoreceptors are more important for mediating the effects of adenosine than adenosine receptors. More work is needed to figure out the exact mechanisms of adenosine regulation of DRN serotonin release, and how adenosine may interact with other possible inhibitory pathways. However, this study establishes that adenosine is a transient important modulator of serotonin. Mechanical stimulation or shear stress, caused by injuries such as traumatic brain injury, cause transient adenosine release and may suppress serotonin release during transient injury events.[4,14] Thus, adenosine is an important modulator of serotonin.

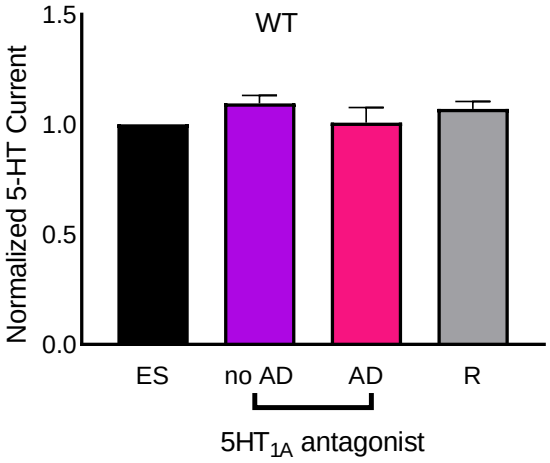

Figure 14. Adenosine modulation of serotonin release in the presence of selective $5HT_{1A}$ antagonist perfusion ((S)-WAY 100135 dihydrochloride, 5 μM). Bars are normalized to the first serotonin stimulation (black). In the presence of WAY 100135, serotonin was stimulated without and then with adenosine applied 10 s before. The recovery is stimulated serotonin with normal aCSF. There is no significant difference in the stimulated serotonin (One-way ANOVA, n=3; p=0.3623).

2.4 Conclusions

In this study, we investigated modulation of adenosine on DRN serotonin release and tested the effects of adenosine receptors (A_1, A_{2A} and A_3) and $5HT_{1A}$ autoregulatory receptors to mediate these effects in real-time using FSCV. Transient adenosine significantly inhibited serotonergic release in the first 20 seconds of adenosine injection, when adenosine was

elevated, and recovered to baseline as the adenosine was cleared from the extracellular space. There were no changes in stimulated serotonin release during continuous adenosine perfusion. Surprisingly, inhibitory effects were still observed in A_1 knock out mice in the first 10 seconds of adenosine injection. Moreover, perfusion of an A_1 antagonist or an A_3 antagonist drug did not abolish adenosine inhibition of serotonin release. Similarly, there were no changes in adenosine modulation of serotonin release in A_{2A} knock out mice with or without an A_1 antagonist. Thus, adenosine inhibited serotonin release in all cases blocking adenosine receptors, indicating adenosine receptors do not directly regulate the inhibition of serotonin release by adenosine. However, perfusing the $5HT_{1A}$ antagonist (S)-WAY 100135 dihydrochloride abolished the inhibitory effect of transient adenosine on serotonin release. Thus, transient adenosine transiently inhibits serotonin release, but does not directly act through adenosine receptors, but acts indirectly through $5HT_{1A}$ autoreceptors, which tightly control serotonin release at DRN. Understanding the neuromodulatory role of adenosine on release of serotonin helps to investigate and develop new therapeutic approach associated with injuries in the brain and other diseases of neuromodulation.

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Chapter 3: Multiplexing FSCV and iGluSnFR3 sensors reveals adenosine transiently inhibits stimulated dopamine and glutamate release

Kailash Shrestha[1*], Yuanyu Chang[1*], Yajun Zhang[2], B. Jill Venton[1]

Abstract

Simultaneous measurements of different neurotransmitters are challenging, but necessary, to understand neurotransmitter interactions in the brain. Genetically-encoded sensors have a high spatial resolution, but there are still limited colors, while electrochemistry provides high time resolution but only for a limited number of electroactive analytes. Here, we multiplexed fast-scan cyclic voltammetry (FSCV) and genetically-encoded fluorescence sensors to simultaneously measure adenosine, dopamine, and glutamate to investigate the spatial and temporal profiles of adenosine neuromodulation. A genetically-encoded glutamate sensor (iGluSnFR3.v857) was expressed in the caudate-putamen region and then in a brain slice, a carbon-fiber microelectrode (CFME) was implanted to monitor electrically-stimulated dopamine release near cells expressing the glutamate sensor. Glutamate and dopamine release were inversely correlated, with areas with high stimulated glutamate release displaying low stimulated dopamine release and vice versa. Exogenous adenosine was applied locally to the brain slice, lasting 30 s, resulting in a transient inhibitory effect on both electrically-stimulated dopamine and glutamate release. Inhibition by adenosine was observed only within a 250 μm distance, showing regional inhibition effects. Dopamine and glutamate release recovered 10 minutes after adenosine injection. The A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) blocked the adenosine inhibition of both glutamate and dopamine release, indicating adenosine has a global transient inhibition effect on dopamine and glutamate release via A_1 receptor modulation. This study shows that multiplexing FSCV and fluorescence sensors (iGluSnFR3.v857) allows simultaneous monitoring of multiple neurotransmitters and reveals an overall transient inhibition by adenosine in the caudate.

Keywords: Multiplexing, Adenosine, Fast-scan cyclic voltammetry, Neuromodulator, Glutamate, Caudate-Putamen, Dopamine, A_1 receptors

3.1 Introduction

The brain uses chemical signals to transmit information after neuronal firing and neuromodulators can modulate that signaling through receptors to regulate neurotransmission.[1,2] Adenosine is a ubiquitous neuromodulator in the brain and regulates functions such as blood flow and the sleep-wake cycle through rapid adenosine signaling via G protein-coupled receptors (GPCRs).[3–7] Adenosine regulates neurotransmitters such as dopamine and glutamate,[8,9] specifically through abundant A_1 receptors by limiting adenyl cyclase formation and hyperpolarization of neurons.[7,10] For example, transient adenosine inhibits phasic dopamine release in brain slices via A_1 receptors.[11–13] Similarly, extracellular adenosine inhibits excitatory glutamate release via presynaptic A_1 receptors.[14–16] Most studies have examined adenosine modulation of one neuromodulator at a time, but it is crucial to monitor multiple neurotransmitters simultaneously to understand the dynamics and interactions between neurotransmitters in the complex system. Techniques such as microdialysis can monitor many neurotransmitters but have limitations of large probe size and low temporal resolution.[17] Although neural circuits are well defined, the spatial and temporal dynamics of adenosine neuromodulation are not well defined because we have not had the tools to examine multiple neuromodulators simultaneously with high temporal and spatial resolution.

One of the most common electrochemical techniques for detecting electroactive neurotransmitters is carbon-fiber microelectrodes (CFMEs) coupled with fast-scan cyclic voltammetry (FSCV). FSCV is capable of monitoring multiple electroactive analytes with sub-second temporal resolution.[18] FSCV has been used for multiplexed measurements of dopamine and adenosine co-transmission,[19] and is best for understanding the rapid neuromodulatory effects of adenosine.[20–22] Currents measured are proportional to extracellular neurotransmitter concentrations.[23,24] FSCV has a high temporal resolution (100 ms) but FSCV is limited in spatial resolution and only detects electroactive neurochemicals released into the extracellular

space.[25,26] Improvements are being made such as arrays for multiple-channel FSCV[27,28] and enzyme and aptamer-based biosensors to expand electrochemical detection to non-electroactive analytes.[29–31] FSCV has been used to study adenosine modulation of dopamine,[19,21] but multiplexing FSCV with other techniques would allow a greater array of neuromodulators to be studied simultaneously.

The toolkit for monitoring neuromodulators recently expanded with the introduction of genetically-encoded fluorescent sensors for directly monitoring neuromodulators *in vivo*.[32] Some genetically-encoded sensors are constructed using bacterial periplasmic binding proteins (PBP), such as iGluSnFR3 and iSeroSnFR.[33,34] Others are based on GPCRs tagged with a fluorophore that turns on after the neuromodulator binds to the modified GPCR,[35–37] and sensors have been developed for a variety of neuromodulators including dopamine, acetylcholine, norepinephrine, serotonin, and adenosine.[35–38] These sensors are compatible with live tissues with high molecular specificity and affinity similar to endogenous receptors with excellent spatial resolution.[33] Combined with advanced fluorescence microscopy, genetically-encoded sensors monitor neuromodulators with single-cell spatial resolution and millisecond temporal resolution.[33,39] However, genetically encoded sensors do not provide concentration profiles and are limited to short-term measurements due to photobleaching. While there are genetically-encoded sensors for adenosine, glutamate, and dopamine and colors are expanding,[36,40,41] multiplexing fluorescence and electrochemistry will enable the simultaneous detection of multiple analytes in real-time without interferences.

The goal of this study is to multiplex FSCV and genetically-encoded fluorescent sensors to monitor dopamine, adenosine, and glutamate to understand the range and temporal dynamics of adenosine neuromodulation on other neurotransmitters in the brain slice. We expressed iGluSnFR3.v857 in the caudate[42,43] to record glutamate release and implant a carbon-fiber microelectrode (CFME) to monitor dopamine and adenosine in real-time after

adenosine application mimicking mechanically-induced release.[21,44] iGluSnFR3.v857 has increased sensitivity, high specificity towards synaptic glutamate along with fast binding and decay time constat with respect to other iGluSnFR variants.[33] These sensors were expressed via sindbis viral vector in caudate putamen. Sindbis viral vector enables quick expression of sensor within 18-24 hours due to its inherent characteristics of rapid expression of the transferred gene in mammalian cells.[45] Combining two techniques, we observed inhibitory action of adenosine on stimulated dopamine and glutamate release in the caudate within a 250 µm range. Continuous perfusion of an A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) blocked adenosine modulation of both glutamate and dopamine release, indicating the inhibition is modulated by A_1 receptors. This is the first experiment to demonstrate and simultaneously characterize adenosine, dopamine, and glutamate in real-time detection through multiplexing FSCV and genetically-encoded sensors (iGluSnFR3.v857). The study shows the utility of high spatial and temporal techniques for understanding the range and time course of adenosine neuromodulation of multiple electroactive and non-electroactive neuromodulators.

3.2 Methods:

3.2.1 Experimental design:

The experimental workflow for this project is shown in Figure 1A. No blinding or randomization was done to the allocated mice.

3.2.2 *Sindbis* injection and Brain slice preparation:

Wild-type mice (C57BL/6J male, RRID: IMSR_JAX:000664, 4-6 weeks, 22-24 g, Jackson lab) were used for multiplexing experiments. All experiments were approved by the Animal Care and Use Committee of the University of Virginia (Protocols number: 3517). A total of 40 mice were used for this project. All mice were group-housed, with 4 mice in each cage and maintaining a 12-12-hour day-night cycle with ad libitum access to food and water. The mouse

was deeply anesthetized by injecting Xylene (2 mg/kg, CAS # 23076-35-9; Bayer Healthcare) - ketamine (10 mg/kg, CAS # 1867-66-9, Zoetis Inc.) via intraperitoneal (i.p.) and positioned in a stereotaxic frame for Sindbis virus injection. Bupivacaine (0.05 ml, SKU 70069-0752-01; Somerset Therapeutics) was administered subcutaneously on the scalp to alleviate surgery pain. Using a hand driller, a small hole was drilled into the skull at the mentioned coordinates (bregma: anterior-posterior (AP): +1.0 mm; medial-lateral (ML): +1.8 mm). Each 1µL vial of Sindbis virus (titer volume: 2 x 10^8 viral particles/ml) was diluted into three vials. A borosilicate glass capillary containing filament (1.2 mm OD and 0.94 mm ID) was loaded with 1.5 µL of Sindbis virus expressing iGluSnFR3.v857. The capillary was lowered (Dorsoventral) 3.5 mm deep from the dura and 1.0 µL (100 nL/min.) was injected into the caudate putamen region with the help of a nanoliter injector. Ketoprofen (2-5 mg/kg S.C, CAS # 22071-15-4) was injected intraperitonially to relieve pain due to surgery. 18 hours were given for viral vector expression and recovery, maintaining a 12-12-hour day-night cycle with ad libitum access to food and water.[46]

Eighteen hours after injection, the mouse was anesthetized using isoflurane (0.5 ml, each ml contains 99.9% of isoflurane, Cat #: 26675-46-7; Covetrus) in the desiccator and decapitated. Skull was quickly removed and brain immediately transferred into a chilled oxygenated slicing buffer (0−5 °C, 95% O_2, 5% CO_2) containing (in mM) 2.5 KCl, 0.5 $CaCl_2$, 25 $NaHCO_3$, 1 NaH_2PO_4, 7 $MgCl_2.6H_2O$, 110 Choline Chloride, and 25 Dextrose to maintain brain integrity. The brain was mounted on a stage with the support of agarose gel and sliced into 400 □M thickness using a vibratome (Leica VT, IL, USA). Slices of the caudate region were transferred to oxygen-saturated Ringer's buffer containing (in mM) 125 NaCl, 1 NaH_2PO_4, 1.3 $MgCl_2.6H_2O$, 2 $CaCl_2$, 25 Dextrose, 2.5 KCl, 25 $NaHCO_3$ maintained at 35°C and kept 40-45 minutes to recover.

3.2.3 Electrochemistry and *iGluSnFR3.v857* sensor imaging:

A single strand of T-650 carbon fiber (7 □m diameter, Cytec Engineering Materials, New Jersey) was aspirated into the glass capillary (1.2 mm O.D and 0.68 mm I.D) with the help of a vacuum pump and pulled into two carbon fiber microelectrodes (CFME) using a horizontal electrode puller (PE-21, Narishige, Japan). The exposed carbon fiber lengths were maintained within the range of 70-100 □m. CFMEs surfaces were cleaned by dipping into isopropanol for 15 minutes and backfilled with 1 mM of KCl solution. Electrodes were equilibrated in aCSF buffer by applying an adenosine waveform (-0.4 V, 1.45 V, 400 V/s scan rate at 10 Hz) at the CFMEs using a WaveNeuro FSCV potentiostat and HDCV software (Pine Research Instrument, NC). Electrodes were inserted 50-75 □m into the slice tissue to measure electrically-stimulated dopamine release, an electroactive neurotransmitter.

Brain slices expressing iGluSnFR3.v857 (Cat. # 175186, Addgene) were analyzed using wide-field epifluorescence imaging (Olympus) equipped with Hamamatsu ORCA-Flash 4.0 camera. After 45 minutes of recovery, the brain slice was transferred into the recording chamber with continuous perfusion of oxygen-saturated Ringer's buffer maintained at 35°C temperature. A homemade bipolar stimulation electrode (Biphasic Stimulus Isolator, Microprobes) was placed on the slice. iGluSnFR3.v857 expressed cells at the caudate were excited with a wavelength of 488 nm (Lumencor Light Engine, Sola) and recorded using 530 nm emission filter. Images were collected for 100 seconds at a 10 Hz frame rate, during which FSCV data were collected for the final 60 seconds. In each run, the slice was electrically stimulated at the 50-second mark to observe both dopamine and glutamate release through FSCV and iGluSnFR3.v857 respectively. Adenosine was puffed close to CFMEs, 5 seconds prior to electric stimulation. Stimulations were repeated at 10-minute time intervals.

3.2.4 Pharmacology:

A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, CAS # 102146-07-6, Tocris) was dissolved in dimethylsulfoxide (DMSO, CAS # 67-68-5, Sigma-Aldrich) and diluted in Ringer's buffer. After initial scans using a normal buffer, DPCPX (500 nM) was perfused for 30 minutes to analyze if adenosine mediates an inhibitory role on dopamine and glutamate release via A_1 receptors at the caudate putamen. During DPCPX perfusion, at the 20^{th} minute, electrically stimulated dopamine and glutamate responses were collected. Next, (1 mM) adenosine was puffed locally 5 seconds before electric stimulation. Finally, a recovery scan was collected 10 minutes later without adenosine in the absence of DPCPX. Animals were assigned to groups and not randomized.

3.2.5 Statistics

Sample sizes were not predetermined using statistical methods, however, the sample size used in this study is similar to previously reported publications (For example, in Figure 3D, Dopamine (nA) = 16 nA, given alpha = 0.05, power =0.9, constant (C) = 10.51, 20% differences = 3.2 nA).[27,47] The Kolmogorov-Smirnov test was carried out for normality testing. Statistical analyses were performed using PRISM 9 (GraphPad Software Inc., USA) and presented as "mean ± standard error" (SEM) for the provided "n" number of slices for each group. Outlier test was not performed. An un-paired t-test was performed to analyze correlation of electrically stimulated dopamine and glutamate release. Similarly, an un-paired t-test was performed to compare the rise time and decay time of both stimulate dopamine and glutamate release. One-way analysis of variance (ANOVA) test was applied at 95 percentage confidence intervals to analyze the main significant effect of exogenous adenosine on dopamine and glutamate release in the absence or presence of DPCPX.

3.3 Results

Here, we multiplexed electrochemistry (FSCV) and fluorescence imaging (iGluSnFR3.v857) to simultaneously monitor adenosine modulation of electrically-stimulated dopamine and glutamate release in brain slices. The genetically-encoded glutamate sensor (iGluSnFR3.v857) was expressed in the caudate putamen using Sindbis virus, and brain slices were prepared 18 hours after injection. Figure 1A shows the experimental set-up, with a CFME and a stimulating electrode implanted near a cell expressing the fluorescent sensor. Electrically-evoked dopamine and glutamate release were simultaneously measured with FSCV and iGluSnFR3.v857 sensors respectively.

Adenosine (20 pmol) was applied 5 seconds before electric stimulation with a nanoinjector from a capillary tip (Figure 1B) and data was collected for 100 s. Figure 1C shows example traces of simultaneous detection of adenosine, dopamine, and glutamate. When the light for exciting fluorescence is first turned on, there is a brief change in the background current at the CFME due to the photoelectric effect. Thus, the stimulation was applied after 50 seconds of light exposure to allow the background to stabilize and enable stable background subtraction with FSCV.[48] When adenosine was puffed 5 s before the stimulation, the CFME immediately detected the adenosine signal, and it lasted around 30 s (Figure 1C), similar to mechanically-stimulated adenosine release.[49] The color plot and the background-subtracted cyclic voltammogram in Figure S1 proved the signal is adenosine. Dopamine and glutamate release were electrically stimulated 5 s after the adenosine application and their traces (red and green, respectively) are overlaid with injected adenosine traces in Figure 1C. Dopamine and glutamate were released simultaneously after electrical stimulation and cleared to the baseline after 2 seconds and 0.5 seconds, respectively. While it is difficult to know the relative time responses of the sensors, both are real-time sensors, so the glutamate signaling appears faster than the

dopamine signaling. Glutamate signals from expressed cells were confirmed with TTX treatment, and TTX blocked the response, showing it is not a stimulation artifact (Figure S2).

One advantage of multiplexing FSCV and fluorescence is that data is collected simultaneously. Figure 2 shows matched data for the simultaneous detection of glutamate release in brain slices near the stimulating electrode (without any adenosine). The relative release is inversely correlated, as higher dopamine release is correlated with lower glutamate signals and vice versa. Thus, higher glutamate measurements were correlated with lower dopamine, even though they were elicited in the same area with the same electrical stimulation. Future studies could further understand the possible modulation of dopamine by glutamate and reciprocal interactions.

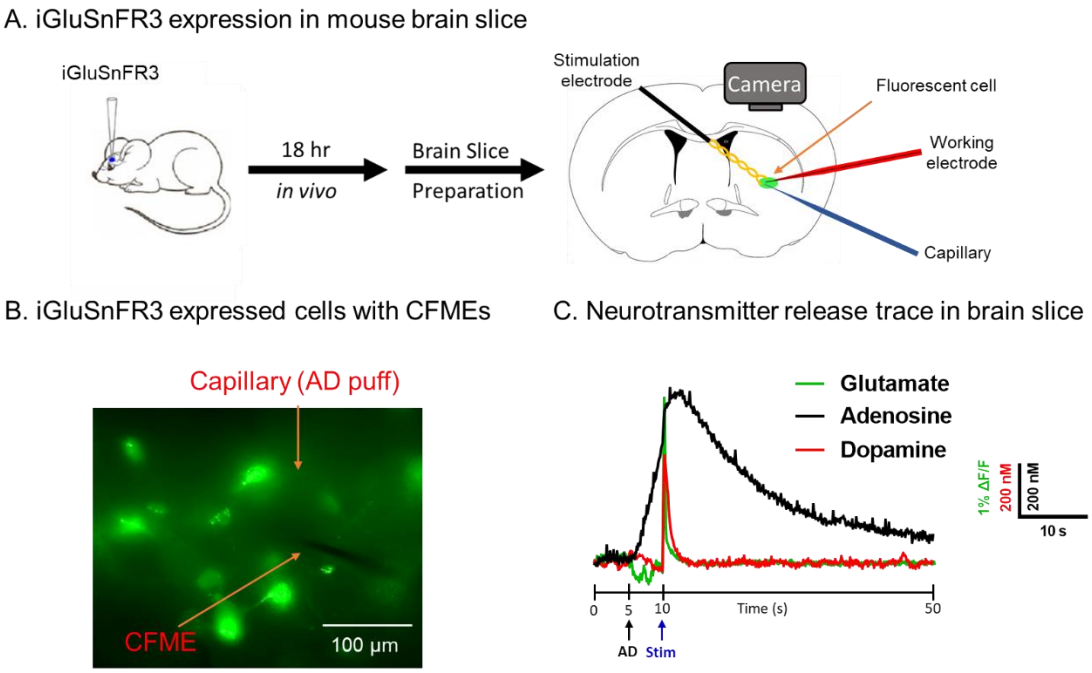

Figure 1. Multiplexing FSCV and the genetically-encoded fluorescent sensor (iGluSnFR3.v857) setup. A. Schematic of the design of imaging/FSCV experiments and slice preparation. CFME implanted near the glutamate-expressed sites. B. Fluorescent imaging of iGluSnFR3.v857, with arrows pointing to the CFME and capillary for adenosine injection. C. Example release traces of simultaneously detected adenosine, dopamine, and glutamate. Adenosine (20 *pmol*) was applied at 5 s by nanoinjector, and the electrical stimulation was applied at 10 s.

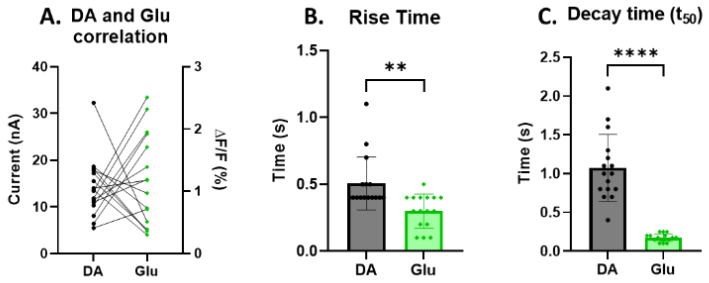

Figure 2: Paired results of electrically stimulated dopamine (DA) and glutamate (Glu) release before AD injection in the caudate-putamen region. A. Correlation of maximal signal for dopamine and glutamate (left panel). There is an inverse correlation. B. Rise time (s) for dopamine and glutamate measurements. (unpaired t-test; two-tailed test, t=3.509, df=30, p=0.0014; n=16) C. Decay time (t_{50}), which is the time to decay from the peak to 50% of the peak value (unpaired t-test; two-tailed, t=8.305, df=30, p<0.0001; n=16) Dopamine rise time and decay time are both larger than glutamate.

We characterized the regional effect of adenosine modulation on glutamate and dopamine release. Figure S3 shows the example image of the measurement, with multiple glutamate cells identified at different distances from the adenosine injection site. The top row of Figure 3 (A, C, E) shows the example traces of glutamate release from 3 different cells at different distances (<100 μm, 100-250 μm and >250 μm) from the adenosine injection site for 3 different conditions: pre-adenosine, 5 seconds after adenosine injection, and recovery (no adenosine). Stimulations were performed 10 min apart, an interval where release is typically constant. Adenosine inhibits glutamate release at close range (within 100 μm) and the inhibition effect decreases as the distance increases from the injection site. After 250 μm, there was no effect of adenosine, showing that 250 μm is its effective range in modulating glutamate. After the stimulation with adenosine was applied, we repeated another stimulation 10 minutes later without adenosine (termed recovery (R) stimulation) and there was no inhibition of glutamate release, indicating that the rapid adenosine transiently inhibited glutamate. The bottom of Figure 3 (B, D, F) shows the average data and there was a main effect of adenosine on glutamate release (One-way ANOVA, **p<0.0012, ****p<0.0001, n=8). Within a 100 μm distance, adenosine application significantly decreases glutamate release by around 50%. As the

distance increases from 100 to 250 µm, adenosine causes a 30% inhibition of glutamate release. At cells over 250 µm away from the adenosine application site, there is no significant inhibition of glutamate (Figure 3F).

The inhibitory action of transient adenosine on electrically stimulated dopamine was also monitored at a CFME implanted near the adenosine injection site. Figures 3G and 3H show the adenosine modulation effect on dopamine, which was collected simultaneously with the glutamate data. The electrode was placed less than 100 µm from the capillary filled with adenosine. Adenosine significantly transiently suppressed dopamine release by 50% after adenosine (One-way ANOVA, **p<0.0012, ****p<0.0001, n=8), matching previous findings.[12,50] Therefore, adenosine simultaneously inhibits glutamate and dopamine release by about 50%, within 100 µm of the adenosine release site. Moreover, the exogenous adenosine we applied modulates cells within a 250 µm distance, indicating adenosine diffusion in the tissue is similar to the mechanically-stimulated adenosine effects of previous reports.[12]

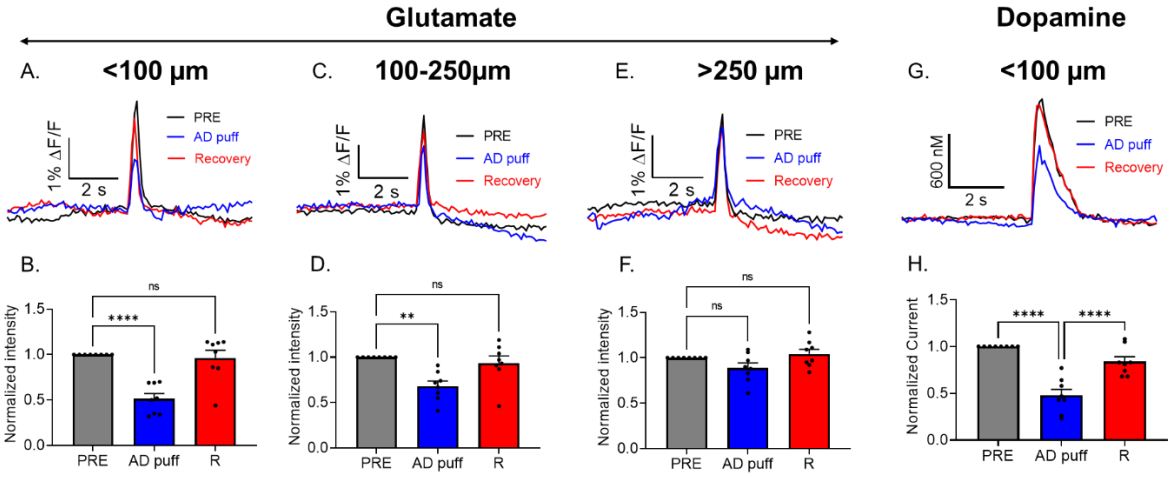

Figure 3. Exogenous adenosine inhibits glutamate and dopamine release transiently. (A, C, E) Examples of iGluSnFR3.v857 response vs Time traces at different distances. Adenosine was injected 5 s before the electric stimulation and glutamate was measured at distances from the injection site: A. <100 µm, C. 100-250 µm, E. >250 µm. (B, D, F). Average inhibition effect of adenosine on glutamate release at different distances from adenosine application B. <100 µm, D. 100-250 µm, F. >250 µm. G. Concentration vs time traces of dopamine release <100 µm. H. Averaged dopamine release with adenosine. Adenosine significantly inhibits dopamine release within <100 µm range (One-way ANOVA, **p<0.0012, $F_{2,21}$=9.221, ****p<0.0001, $F_{2,21}$=20.32, n=8)

To ensure the inhibitory effect was caused by adenosine, we applied PBS injection as a control experiment. The same volume of PBS buffer was injected into the tissue, approximately 100 µm from the iGluSnFR3.v857 expressed cells, 5 s before the stimulation. Figure 4A and B present example traces of stimulated glutamate and dopamine release after PBS injection, and there is no difference in the fluorescence or the electrochemical responses. The overall results in Figure 4C and D show that there is no significant effect of PBS injection on glutamate (One-way ANOVA, n=6, p=0.2760) and dopamine (One-way ANOVA, n=3, p=0.3042). Therefore, control data indicates that the decreased dopamine and glutamate signals are not due to the injection disturbance but because of the adenosine neuromodulation.

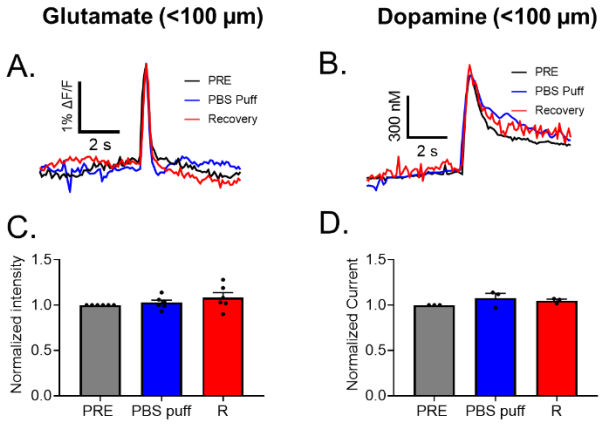

Figure 4. Control experiment by injecting PBS buffer. A) Example iGluSnFR3.v857 response vs time traces after injecting PBS at <100 µm B) Example concentration vs time traces of stimulated dopamine release at <100 µm from the site of injection. Overall effect of PBS injection on C) glutamate (One-way ANOVA, n=6, $F_{2,15}$=1.404, p=0.2760) and D) dopamine release (One-way ANOVA, n=3, F2,6=1.461, p=0.3042) at <100 µm.

Adenosine receptors are abundant in the brain and are important for both excitatory and inhibitory neurotransmission modulation at the synapse.[5,12,50] Previous studies show that perfusing A_1 receptor antagonist DPCPX eliminates adenosine inhibition of dopamine release.[12,50–52] Here, we tested the effect of DPCPX to block the inhibitory effect of adenosine on both dopamine and glutamate release. We perfused a high dose of DPCPX (500 nM) for 20

minutes, then applied electrical stimulation to see baseline glutamate and dopamine responses with DPCPX. Traces with DPCPX were not different from pre-drug (Figure 5A, B). Next, with DPCPX perfusion, adenosine was injected into the tissue 5 s before the electrical stimulation to test the effects of adenosine. The example traces of glutamate in Figure 5A and dopamine in Figure 5B indicate that DPCPX blocks the modulation of glutamate by adenosine and so there is no effect of adenosine application. Finally, normal aCSF buffer was perfused for 10 minutes to wash out the DPCPX, and electrical stimulation was performed to test recovery, and the same magnitude of response was observed for each analyte. The overall results in Figure 5C show that there is no significant difference in stimulated glutamate with DPCPX or adenosine with DPCPX (One-way ANOVA, n=4, p=0.4360). Similarly, the dopamine results in Figure 5D showed no significant effect of DPCPX or adenosine with DPCPX on dopamine release (One-way ANOVA, n=4, p=0.6585). Therefore, adenosine modulation of glutamate and dopamine are both regulated by A_1 receptors.[12,13,50]

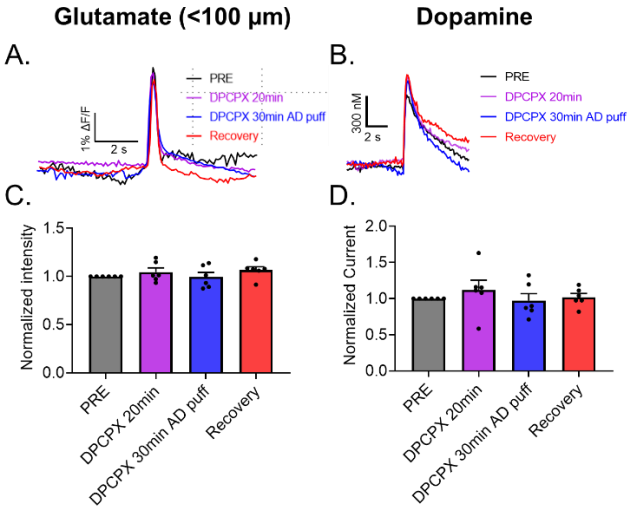

Figure 5. Adenosine modulation of glutamate and dopamine is blocked by A_1 inhibitor DPCPX. A. Example of iGluSnFR3.v857 response vs time traces. B. Example of concentration vs time traces of stimulated dopamine release. C-D. Overall effect of DPCPX in the absence and presence of adenosine injection on glutamate (One-way ANOVA, n=6, $F_{3,20}$=0.9485, p=0.4360) and dopamine release (One-way ANOVA, n=6, $F_{3,20}$=0.5429, p=0.6585).

3.4 Discussion

Neuromodulators regulate physiological processes, especially neurotransmitter release in the brain. Understanding the rapid signaling chemistry involved in neurotransmission and modulation at the cellular level requires techniques with precise spatiotemporal resolution. In this study, we multiplexed two different techniques: FSCV for dopamine and adenosine detection and genetically-encoded fluorescence sensor iGluSnFR3.v857 for glutamate. Combining techniques facilitated the monitoring of three neurochemicals simultaneously, including electroactive (adenosine and dopamine) and non-electroactive analytes (glutamate). The main result is that transient adenosine reversibly inhibited both dopamine and glutamate release near the release site. The range of adenosine inhibition was about 250 μm. This inhibition was blocked in the presence of A_1 antagonist drug (DPCPX) indicating both glutamate and dopamine inhibition were regulated via presynaptic A_1 receptors. Measuring dopamine and glutamate simultaneously allowed the correlation of the neurotransmitters and dopamine release was higher when glutamate release was lower and thus the stimulated release was inversely correlated. Overall, multiplexing FSCV and iGluSnFR3.v857 allows real-time measurements of adenosine neuromodulation of dopamine and glutamate, showing both are transiently inhibited.

3.4.1 Multiplexing FSCV and iGluSnFR3.v857 measurements.

FSCV is a background subtraction technique that utilizes fast potential sweeping at a CFME to monitor rapidly changing electroactive compounds such as adenosine and dopamine.[25] The small carbon fiber is implantable into tissue with minimal tissue damage and small electrodes allow rapid scanning to detect and quantitate analytes on the sub-second timescale.[53] Generally, FSCV detects electroactive molecules spilled over in the extracellular spaces from the synaptic cleft and it is difficult to detect other non-electroactive neurotransmitters such as glutamate and GABA. Our lab pioneered the detection of both

dopamine and adenosine with FSCV and dopamine and adenosine have different oxidation potentials so they can be co-detected.[12,54,55]

Genetically-encoded fluorescent proteins are expressed in the synapse on the membrane, and they are inherently highly sensitive and selective to specific molecules due to their protein engineering design.[56] Upon binding of the analyte, a fluorophore undergoes conformational changes resulting in fluorescence changes that are reversible when the analyte unbinds. In slices, a large area of fluorescence can be measured, so there is high spatiotemporal resolution. A variety of fluorophore probes are developed with different colors to monitor multiple analytes at the synaptic cleft. However, these fluorophore proteins are relatively large and can interfere with protein functionality, and undergo photobleaching when exposed to excitation light for a longer time.[56] In addition, nearly all genetically-encoded fluorescent sensors are used to measure changes in fluorescence over background, and thus they do not measure basal levels or absolute concentrations.[32,57] iGluSnFR3.v857 was developed from a bacterial periplasmic binding protein engineered to bind glutamate and has been extensively used in glutamate measurements in the synapse.[40,58]

In this study, we multiplexed FSCV and iGluSnFR3.v857 to monitor rapid changes of both dopamine and glutamate in the mouse brain slice. This is the first study to multiplex these techniques, although FSCV has been multiplexed with G-protein coupled fluorescent sensors for dopamine and serotonin.[59,60] Applying a light to an electrode can change the background current due to the photoelectric effect, so to perform simultaneous measurements, we turned on the light for fluorescence 50 s before we collected data with FSCV to allow the background to stabilize. The carbon-fiber electrode, at 7 μm in diameter, detects dopamine in the extracellular space, not the synaptic cleft, whereas iGluSnFR3.v857 is expressed at the synapse and detects glutamate localized at the synapse. One advantage of measuring dopamine and glutamate simultaneously is to understand their levels in the same experiment. In this study, electrically-

stimulated dopamine level was relatively higher when glutamate was lower and vice-versa, an inverse correlation. Previous studies have shown that dopamine and glutamate can modulate each other even in the absence of direct synaptic connections.[61] Usually, it's suggested that extracellular dopamine and glutamate in the extracellular space neuromodulate reciprocally.[1,62] Dopamine and glutamate have a complex relationship in the caudate and are associated with different functional, cognitive, and limbic roles.[62,63] For example, glutamate inhibits or excites dopamine depending on the receptors activated and the circuit.[63] While here we cannot say that dopamine and glutamate are modulating each other directly, we show that near the electrical stimulation electrode, there is an inverse correlation between dopamine and glutamate release that could be further investigated with multiplexed experiments.

We also studied the sensor time response for both dopamine and glutamate. Dopamine takes 0.5 s to reach the peak of its concentration whereas it takes around 0.3 s for glutamate to reach the peak concentration (Figure 2B). It is hard to directly compare the time responses of two such different techniques, but the slower rise in dopamine is likely due to the time needed to diffuse to the extracellular space for detection, while glutamate is measured in the synapse. Furthermore, we compared clearance times, or the time from reaching half of the maximum neurotransmitter concentration from the peak, for both neurotransmitters. On average, dopamine took more than 1 s to be cleared to half maximum concentration compared to 0.2 s for glutamate in the brain slice. Again, the site of measurement may affect the clearance, as the glutamate is likely rapidly cleared from the synapse to avoid excitotoxicity, while the dopamine in the extracellular space is volume transmission and likely diffuses longer before being cleared.[64,65] Glutamate released at the synapse diffuses away from the synaptic cleft towards the extracellular space and is cleared by the glutamate transporters while for dopamine, clearance is mainly by the dopamine transporter.[66] Overall, multiplexing two different techniques helps to monitor the completely different nature of neurotransmitters simultaneously.

3.4.2 Rapid adenosine inhibits both adenosine and glutamate.

Adenosine is predominantly known as an inhibitory modulator and acts as a neuroprotective agent in conditions such as stroke.[49,52] Previously our lab discovered two modes of transient adenosine release in multiple brain regions. Spontaneous release was about 2-3 s in duration and transiently modulates blood flow through A_{2A} receptors.[19,21] Mechanically-stimulated adenosine is longer, lasting 20-30 s, and occurs with damage such as microelectrode implantation and brain injuries.[27] Here, we injected adenosine 5 seconds before electric stimulation with a nanoliter injector to mimic mechanically-stimulated adenosine, and transient adenosine lasted approximately 30 seconds at the working electrode before it was cleared (Figure 1C). In previous studies, we have shown that adenosine only modulated dopamine or serotonin release when it is elevated, and 40 s after adenosine is cleared it does not affect dopamine release.[44 47]

Exogenously applied adenosine temporarily inhibited electrically-stimulated dopamine and glutamate release in the caudate putamen.[12,49,52] Adenosine inhibited glutamate and dopamine near the release site, when it was delivered 5 s before the stimulation, and during a recovery stimulation, 10 minutes later, there was no inhibition, indicating that the inhibition was transient. In previous studies, our lab demonstrated a regional effect of rapid adenosine using dual channel FSCV, where dopamine was inhibited to about 250 μm away from where it was applied, the same distance measured here with iGluSnFR3.v857 for glutamate.[27] Further from the adenosine application site, there is less inhibition because the amount of adenosine is lower due to clearance. Thus, adenosine only inhibits neurotransmission when it is elevated, and adenosine is cleared in about 250 μm.[27] Both dopamine and glutamate were inhibited by about 50% near the adenosine application site. Thus, adenosine provides a local, transient inhibition of both glutamate and dopamine, indicating that adenosine may act to cause transient neuromodulation to reduce neurotransmission during tissue damage. This inhibitory role of

transient adenosine can be an important neuroprotective mechanism in situations such as hypoxia and brain injury. Spontaneous adenosine transients increase in the caudate during stroke,[67] and may dampen excitotoxic damage, known to happen in ischemia due to glutamate signaling. Thus, transient adenosine is causing local neuromodulation and inhibition, but on a rapid time scale. Multiplexing FSCV and fluorescence techniques allow simultaneous monitoring the transient neuromodulatory effects of adenosine on multiple neurotransmitters.

The caudate-putamen has a high expression of A_1 inhibitory receptors, and these receptors also form heteroreceptors.[13,44,62] Adenosine A_1 receptors are abundantly present in the striatal neurons like dopaminergic and glutamatergic terminals. We hypothesized that adenosine acted at A_1 receptors to inhibit both stimulated dopamine and glutamate release. The inhibition of glutamate and dopamine release by adenosine was eliminated with the perfusion of DPCPX, an A_1 antagonist. Thus, adenosine acts through inhibitory A_1 receptors to dampen transient neuromodulation of adenosine on multiple neurotransmitters. Regulation of adenosine neuromodulation is the same for both dopamine and glutamate release, with receptors likely located on both neurons.

3.5 Conclusions

Overall, multiplexing of electrochemistry and fluorescence microscopy provides a better spatiotemporal resolution and overcomes the limitations of each technique. We demonstrated monitoring two different analytes by combining FSCV and fluorescence techniques simultaneously at the caudate region along with pharmacological studies. Adenosine transiently modulated both dopamine and glutamate release in the caudate putamen, with local effects limited to when and where adenosine is present. Thus, adenosine provides local, rapid neuromodulation of multiple neurotransmitters, which may lead to neuroprotective effects during brain injury.

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Chapter 4: Neuroprotective role of adenosine receptors on dopamine and glutamate release during transient oxygen glucose deprivation (OGD)

Kailash Shrestha, B. Jill Venton

Abstract

The striatum is one of the brain regions most sensitive to ischemia, triggering a massive release of neurotransmitters such as dopamine and glutamate into the extracellular space. This excessive neurotransmitter release causes excitotoxicity and leads to neuronal damage. Our understanding remains limited of how oxygen-glucose deprivation (OGD) affects the stimulated release and interactions of multiple neurotransmitters, so here we combined fast-scan cyclic voltammetry (FSCV) and fluorescence imaging of iGluSnFR3 to simultaneously monitor stimulated dopamine and glutamate release in real time during OGD and reperfusion. We investigate the neuroprotective role of adenosine A1 and A2A receptors during ischemia. Transient ischemia significantly reduces stimulated dopamine and glutamate release to about 50%, with this suppression persisting for an additional 30 minutes after reperfusion with oxygenated buffer. Dopamine release dipped lower during reperfusion, but recovered near to baseline 90 minutes after OGD, whereas glutamate was only at 50% of release even after 90 minutes. In A2AKO mice, while OGD still significantly decreased both dopamine and glutamate release, glutamate recovered more than in WT mice. In mice treated with the A1 antagonist DPCPX, there was no significant effect of OGD or reperfusion on glutamate release, showing A1 antagonist was neuroprotective. Dopamine still decreased 30 min after reperfusion but recovered more quickly in WT mice. Thus, both A1 and A2A adenosine receptors play a neuroprotective role against transient ischemic insults, with different time courses of effects for stimulated dopamine and glutamate.

4.1 Introduction

Adenosine is a non-classical neurotransmitter that is ubiquitously distributed in the brain and functions as a local neuromodulator maintaining homeostasis and synaptic functions.[1,2] Extracellular adenosine binds to and activates four G-protein-coupled receptors—A1, A2A, A2B, and A3—each of which mediates a variety of physiological effects.[3–6] Adenosine exhibits neuroprotective effects by rapid release in the extracellular spaces to micromolar range and activating corresponding adenosine receptors during events like ischemia and traumatic brain injury.[7]

According to the American Heart Association, ischemic stroke was the second leading cause of death globally in 2019 and remains a significant contributor to long-term disability.[8] Ischemic stroke occurs due to a transient or permanent reduction in blood flow to a brain region, typically caused by a thrombotic or embolic event in a cerebral artery.[9,10] One of the earliest pathological events following ischemia is a rapid increase in extracellular adenosine after ATP-metabolism. Reduced ATP production is followed by glutamate excitotoxicity, which is accompanied by acidosis, ionic imbalance, inflammation, oxidative stress, and peri-infarct depolarization, ultimately leading to cell death.[11–13] Oxygen-glucose deprivation (OGD) is a way to mimic ischemia in tissue slices, and it results in the release of adenosine.[14,15] Adenosine receptors play a neuroprotective role in this pathological response. The neuroprotective effects of adenosine are primarily attributed to the well-established inhibitory influence of A1 receptor activation on synaptic transmission.[16] Activation of A1 receptors plays a neuroprotective role by hyperpolarizing neurons, reducing glutamate release, and consequently minimizing neuronal and brain injuries, particularly during hypoxic and ischemic conditions.[6,14,17] Selectively blockade of A2A receptors also exhibited neuroprotective role against ischemic injury in the hippocampus and striatum.[18] However, the effect of these adenosine receptors on other neurotransmitter release is not known.

The striatum, along with the CA1 region of the hippocampus, is highly susceptible to ischemia.[19,20] Medium-spiny neurons in the striatum are particularly vulnerable to ischemic damage. Cerebral ischemia triggers the excessive release of neurotransmitters such as glutamate due to reduced cytosolic ATP level and plasma membrane NA+/K+-ATPase activity, disrupting ion gradients across the membrane, resulting in neuronal depolarization.[21] Thus, an abrupt imbalance of extracellular K+ level causes vesicular release of glutamate, activating AMPA and NMDA receptors.[11,12,21] Furthermore, NMDA receptor activation contributes to neuronal depolarization and Ca2+ influx, which further potentiates depolarization ultimately leading to neuronal injury.[12,22] During ischemic events, excess release of other neurotransmitters such as dopamine and serotonin causes depletion of neurotransmitter pools, excessive receptor activation, and neuronal death.[23,24]

The goal of this study is to determine how adenosine receptors exert neuroprotective effects over stimulated dopamine and glutamate release during transient OGD in the striatum. We used acute mouse brain slices, which preserve neuro-glial interactions and allow for pharmacological testing under controlled OGD conditions. Using a combination of fast-scan cyclic voltammetry (FSCV) to detect dopamine and fluorescence iGluSnFR3 to detect glutamate, we simultaneously monitored electrically evoked dopamine and glutamate release. The results showed that OGD significantly reduced stimulated dopamine and glutamate release, with more prolonged effects on glutamate. Glutamate recovered better in A2AKO mice while A1 antagonist DPCPX reduced inhibitory effect of OGD on stimulated dopamine and preserved stimulated glutamate release during OGD. Thus, adenosine receptors play a neuroprotective role during transient OGD and could be a target for treatment of ischemia.

4.2 Methods:

4.2.1 Chemicals:

All chemical reagents were purchased from Sigma-Aldrich. The artificial cerebrospinal fluid (aCSF) buffer was composed of 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 25 mM $NaHCO_3$, 11 mM glucose, and 15 mM tris(hydroxymethyl)aminomethane, all dissolved in deionized water (Milli-Q Biocel; Millipore, USA). The aCSF was freshly prepared each day, with the pH adjusted to 7.4. The slicing buffer contained (in mM): 2.5 KCl, 0.5 $CaCl_2$, 25 $NaHCO_3$, 1 NaH_2PO_4, 7 $MgCl_2 \cdot 6H_2O$, 110 choline chloride, and 25 dextrose with pH of 7.4. Glucose was replaced with equimolar concentration of sucrose for OGD buffer.

DPCPX (an A1 adenosine receptor antagonist) and D-AP5 (an NMDA receptor antagonist) were purchased from Tocris Bioscience (Minneapolis, USA). DPCPX (10 μM) and D-AP5 (50 μM) solutions were freshly prepared by dissolving the compounds in 1 mL of dimethyl sulfoxide (DMSO) using sonication, followed by dilution in aCSF buffer.

4.2.2 *AAV* injection and Brain slice preparation:

Wild-type male C57BL/6J mice (Jackson Lab, 6–8 weeks old, 22–24 g) and A2A receptor knockout (A2AKO) mice (Mustafa Lab, West Virginia University, 6–8 weeks old, 22–24 g) were used for oxygen-glucose deprivation (OGD) experiments.[25] Each cage housed four mice, maintained under a 12-hour light/dark cycle with ad libitum access to food and water. All animal experiments were approved by the Animal Care and Use Committee at the University of Virginia.

All surgical procedures were performed on anesthetized mice (xylazine, 2 mg/kg, and ketamine, 10 mg/kg, administered via intraperitoneal injection) fixed in a stereotaxic frame for

adeno-associated virus (AAV) injection (~750–1000 nL). To minimize surgical pain, bupivacaine (0.05 mL, 2.5 mg/mL) was administered subcutaneously to the scalp. A small hole was drilled into the skull at the specified coordinates (bregma: anterior-posterior (AP): +1.2 mm; medial-lateral (ML): +1.2 mm) using a microdrill. A Hamilton syringe loaded with AAV was lowered 3.0 mm dorsoventrally from the dura and injected into the caudate-putamen region using a nanoliter injector. Post-surgical pain was managed with ketoprofen (2–5 mg/kg, administered intramuscularly or subcutaneously). Mice were allowed to recover for three weeks to ensure viral vector expression, while maintaining a 12-hour light/dark cycle with free access to food and water.

For brain slice preparation, mice were anesthetized using isoflurane (Cat #: 26675-46-7; Covetrus; each mL contains 99.9% isoflurane) in a desiccator and then decapitated. The skull was rapidly removed, and the brain was immediately transferred to chilled, oxygenated slicing buffer (0–5°C, 95% O_2, 5% CO_2) to maintain tissue integrity. The brain was mounted on a vibratome stage with agarose gel support and sliced into 300-µm-thick sections using a vibratome (Leica VT, IL, USA). Slices of the caudate-putamen region were then transferred to oxygen-saturated aCSF buffer containing (in mM): 125 NaCl, 1 NaH_2PO_4, 1.3 $MgCl_2 \cdot 6H_2O$, 2 $CaCl_2$, 25 dextrose, 2.5 KCl, and 25 $NaHCO_3$. The buffer was maintained at 32°C, and slices were allowed to recover for 30 minutes before experimentation.

4.2.3 Electrochemistry and *iGluSnFR3* sensor imaging:

A single strand of T-650 carbon fiber (7 µm diameter, Cytec Engineering Materials, New Jersey) was aspirated into a glass capillary (1.2 mm outer diameter, 0.68 mm inner diameter) using a vacuum pump and pulled into two carbon fiber microelectrodes (CFMEs) with a horizontal electrode puller (PE-21, Narishige, Japan). The exposed carbon fiber lengths were maintained within a range of 70–100 µm. To ensure cleanliness, the CFMEs were soaked in isopropanol for 15 minutes and then backfilled with a 1 mM KCl solution. Electrodes were

equilibrated in aCSF buffer by applying an adenosine waveform (-0.4 V to 1.45 V, 400 V/s scan rate at 10 Hz) using a WaveNeuro FSCV potentiostat and HDCV software (Pine Research Instrument, NC). Electrodes were inserted 70–100 µm into the brain slice tissue to measure electrically stimulated (12 pulse, 300 µA) dopamine release, an electroactive neurotransmitter.

Brain slices expressing iGluSnFR3 (Addgene, Cat # 106174-AAV1) in the caudate-putamen were analyzed using wide-field epifluorescence imaging (Olympus) equipped with a Hamamatsu ORCA-Flash 4.0 camera. After 30 minutes of recovery at 32°C, the brain slice was transferred to a recording chamber with continuous perfusion of oxygen-saturated aCSF buffer maintained at 32°C. A custom-made bipolar stimulation electrode (Biphasic Stimulus Isolator, Microprobes) was placed on the slice. iGluSnFR3-expressing cells in the caudate were excited with a 488 nm wavelength light source (Lumencor Light Engine, Sola), and emissions were recorded using a 530 nm filter.

Images were acquired for 50 seconds at a 10 Hz frame rate, while FSCV data were collected for 25 seconds. Electrical stimulation was applied to measure both stimulated dopamine and glutamate release. Stimulations were repeated at 10-minute intervals. From each slice, three pre-OGD, one OGD, and 12 recovery stimulations were collected. During pharmacological studies, the first drug perfusion scan replaced the third pre-OGD stimulation.

4.2.4 OGD/Reperfusion model

Transient global ischemia was induced in brain slices by subjecting them to oxygen-glucose deprivation (OGD) for 10 minutes. The OGD buffer was prepared by replacing glucose with an equimolar concentration of sucrose and substituting the oxygen supply (95% O_2 and 5% CO_2) with nitrogen bubbling (95% N_2 and 5% CO_2).

Immediately after the completion of the third pre-OGD scan, oxygenated aCSF was replaced with N_2-bubbled OGD buffer for 10 minutes. Following this period, data was collected

under OGD conditions before quickly switching back to normal oxygenated aCSF for a 2-hour reperfusion phase.

4.2.5 Pharmacology:

The A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, CAS #102146-07-6, Tocris) was dissolved in dimethyl sulfoxide (DMSO, CAS #67-68-5, Sigma-Aldrich) and then diluted in aCSF buffer. After initial scans using normal aCSF, DPCPX (10 μM) in aCSF was perfused for 10 minutes before the onset of OGD and was maintained throughout the experiment.

During DPCPX perfusion, electrically stimulated dopamine and glutamate responses were recorded at the 30-minute mark. OGD was then induced by switching to an OGD buffer containing DPCPX (10 μM), and data were collected at the 40-minute mark. Following OGD, recovery scans were conducted in normal aCSF containing DPCPX (10 μM) for the next two hours.

A similar approach was applied for the NMDA receptor antagonist (DAP-5), except that the NMDA antagonist concentration in the perfusion buffer was maintained at 50 μM.

4.2.6 Statistics

All statistical analyses were performed using PRISM 9 (GraphPad Software Inc., USA) and are presented as mean ± standard error of the mean (SEM) for the given “n” number of slices in each group. A two-way ANOVA was used to assess the effect of 10 minutes of OGD on electrically stimulated dopamine and glutamate release. Additionally, a one-way ANOVA was conducted at a 95% confidence interval to evaluate the main effect of OGD on dopamine and glutamate release in the absence or presence of DPCPX.

4.3 Results:

This study investigated the hypothesis that adenosine receptors play a neuroprotective role under ischemic conditions. The well-established oxygen-glucose deprivation (OGD) model was employed to mimic transient ischemia in brain slices. To test this, stimulated dopamine and glutamate from striatal cells were simultaneously monitored using multiplexed FSCV and fluorescence across three conditions: pre-OGD, OGD, and post-OGD/reperfusion. Simultaneous measurements allow a better understanding of the time course and interactions of glutamate and dopamine release.

4.3.1 OGD effects stimulated dopamine and glutamate release in WT

To investigate the effect of OGD on stimulated dopamine and glutamate release in wild-type (WT) mice, we employed a carbon fiber microelectrode (CFME), which was inserted 70–100 μm deep into the tissue adjacent to glutamate-expressing cells (iGluSnFR3.v184). Each experimental run lasted 50 seconds, during which the brain slice was electrically stimulated at the 20-second mark, as shown in Figure 1A. An adenosine potential waveform (-0.4 V to 1.45 V, sweep rate: 400 V/s) was applied to monitor dopamine release, while fluorescence signals from iGluSnFR3 were simultaneously recorded to measure glutamate release. The light for the fluorescence was turned on for 15 s before the FSCV measurements were taken, because the light affects the background current of the electrode, and this time allows the background current to stabilize before the experiment. Electrical stimulation was repeated every 10 minutes to allow for the recovery of both dopamine and glutamate pools.

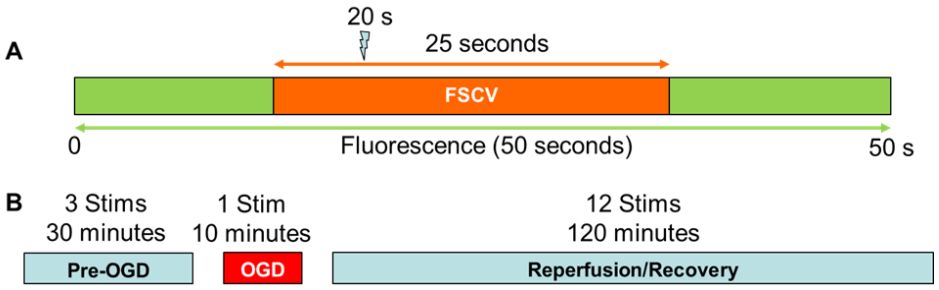


Figure 15: Illustration of stimulation protocols for the experiment. A. In each trial, changes in fluorescence are recorded continuously over 50 seconds, while FSCV data is collected for 25 seconds. Electrical stimulation is applied at 20 s. B. The full experimental scheme for OGD experiments. Each slice undergoes three distinct phases: pre-OGD, OGD, and reperfusion. Stimulations are performed every 10 minutes, so there are 3 pre-OGD stims, 1 during OGD, and 12 during the reperfusion. The experiment spans 160 minutes total.

Figure 1B outlines the experimental setup, which included three conditions—pre-OGD, OGD, and post-OGD/reperfusion—to examine stimulated dopamine and glutamate release. Prior to OGD, three scans were performed by perfusing oxygenated artificial cerebrospinal fluid (aCSF) every 10 minutes. The OGD buffer consisted of the same ingredients as normal aCSF, with the substitution of glucose by an equimolar concentration of sucrose, and it was bubbled with nitrogen instead of oxygen. Following the third pre-OGD scan, the oxygenated aCSF was replaced with a nitrogen-bubbled OGD buffer for 10 minutes. The buffer was then switched back to oxygenated aCSF for reperfusion studies. Stimulated dopamine and glutamate release were simultaneously monitored for 120 minutes post-OGD.

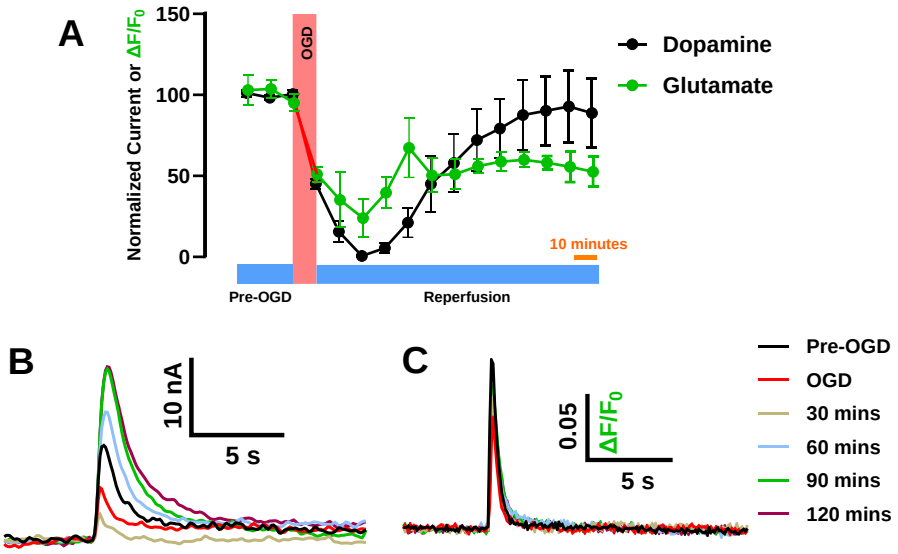


Figure 16: Simultaneous measurement of dopamine and glutamate response to OGD and reperfusion in WT (n=7 slices). Pre-OGD phase (indicated by the blue band) is first conducted to establish a baseline, followed by a 10-minute OGD phase (marked by the red bar) and then a 120-minute reperfusion phase (indicated by the blue band). Data points are at 10-minute intervals. The black dots are stimulated dopamine, while the green dots are changes in glutamate sensor fluorescence. The y-axis is normalized to the average of the pre-OGD values. (B) Dopamine i v t trace (C) iGluSnFR3 response towards electric stimulation during pre-OGD, OGD and reperfusion every 30 minutes as mentioned in legends. Both dopamine and glutamate are inhibited during OGD and the beginning of reperfusion.

Figures 2A shows the normalized stimulated dopamine and glutamate release measured every 10 minutes across all three conditions: pre-OGD, OGD, and post-OGD/reperfusion. During OGD, both dopamine and glutamate release decreased by more than 50% of initial stimulate release. This reduction persisted for 20 minutes after switching back to the normal aCSF buffer (reperfusion), with both having lower stimulated release in the first 20 minutes after OGD than during OGD. Recovery for both neurotransmitters began 30 minutes post-OGD. Glutamate levels never decreased as much as dopamine, but they also never recovered to pre-OGD values, remaining at about 50% of pre-OGD values 120 min after reperfusion. Stimulated dopamine release goes to nearly zero, before recovering to about 80-90% of pre-OGD levels. Thus, dopamine decreases more than glutamate but also recovers more in 120 minutes after OGD.

Figure 2B and 2C shows example dopamine i v t traces and iGluSnFr3 responses during pre-OGD, OGD and different times during reperfusion. Both electrically stimulated dopamine and glutamate were significantly depressed when exposed to OGD. For dopamine measurements, the duration is shorter with a smaller signal, but for glutamate, the duration is about the same no matter the size of the stimulation. Again, this difference in durations is likely due to glutamate being synaptic, while the extracellular dopamine is cleared faster when the signals are smaller. Overall, stimulated dopamine release lasts longer than glutamate release, which is expected because dopamine release is measured extracellularly while the glutamate release is synaptic.

To get a better picture of OGD insult on stimulated dopamine and glutamate release, we averaged the data over time into categories of pre-OGD, OGD, and reperfusion in 30 minutes bins (Figure 3A and 3B). Overall, in Fig. 3A, there is a main effect of time on dopamine signal (one-way ANOVA, n=7, overall p 0.0004). Dopamine was significantly reduced by more than 50% during OGD condition (one-way ANOVA, n=7, p=0.0004), as seen in Figure 3A. Dopamine release was even lower during the first 30 minutes of reperfusion, 90 % of pre-OGD (compared to pre-OGD, p=0.0003). After 30 mins of reperfusion, dopamine begins to recover and the dopamine release is not significantly different than pre-OGD values, although there is significant variance in the recovery between samples. By 120 min after reperfusion, dopamine is about 90 % of the pre-OGD value.

One reason that dopamine may be depleted during OGD is that it may be spontaneously released during OGD. During OGD, small rapid dopamine events of exocytosis were observed without electric stimulation. In the color plot, which is all the FSCV data, a background increase in dopamine can be seen when there is no stimulation. We did not collect continuous FSCV data, and it is difficult to measure background changes with FSCV, but future experiments could

try to examine basal levels of dopamine, to see if that release leads to the decrease in stimulated release.

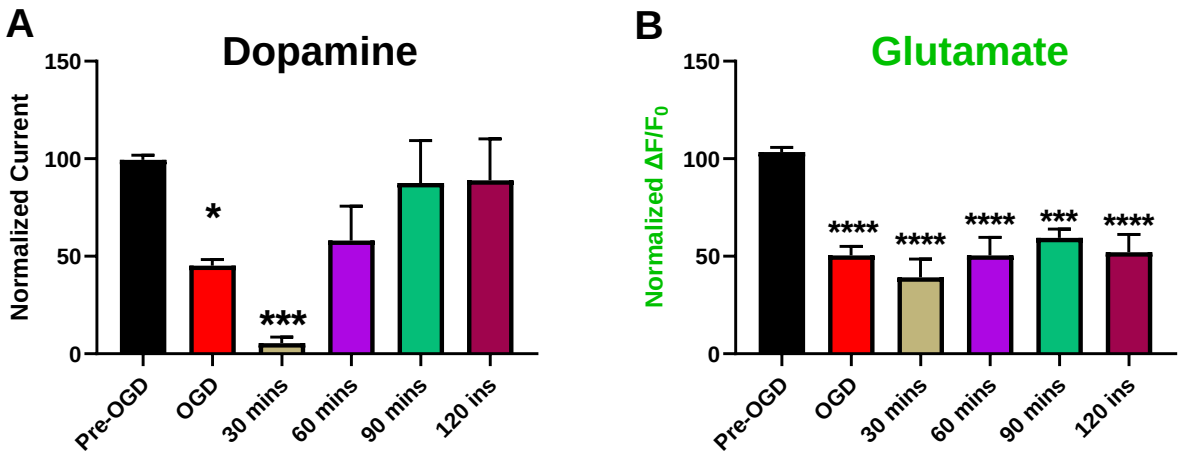


Figure 17: Dopamine and glutamate response to OGD and reperfusion in WT. A. Each bar represents the normalized dopamine current relative to the average of three pre-OGD scans (pre-OGD, OGD, 30 mins, 60 mins, 90 mins, and 120 mins). OGD had a significant effect on dopamine levels (one-way ANOVA, n = 7, overall p = 0.0004; *p = 0.0488, ***p = 0.0003). Dopamine levels progressively recovered to baseline as reperfusion time increased (*p = 0.0488, ***p = 0.0003). B. The effect of OGD and reperfusion on stimulated glutamate fluorescence. Transient OGD significantly suppressed glutamate levels (One-way ANOVA, n = 7, overall p < 0.0001, ***p < 0.0001), and glutamate did not return to the initial baseline during the reperfusion period (***p ≤ 0.0005, ****p ≤ 0.0001).

Figure 3B shows that stimulated glutamate release followed similar trends to dopamine but does not recover as much during reperfusion. There is a main effect of time and OGD condition on glutamate release (During OGD, glutamate goes down by 50% of pre-OGD level, a significant effect release (One-way ANOVA, n=7, p<0.0005). However, the level stays about 50% of pre-OGD for all of the reperfusion time periods and it never recovers, as all the reperfusion time periods are significantly different than pre-OGD (One-way ANOVA, n=7, p<0.0001). There is no way to look at basal release of glutamate with the glutamate sensor because all traces are background subtracted.

4.3.2 NMDA receptor antagonist on dopamine and glutamate during OGD and reperfusion

Prolonged high levels of extracellular glutamate can lead to neuronal death. NMDA receptors, a subtype of ionotropic glutamate receptors, play a dual role of promoting recovery and preventing delayed neuronal loss, while also mediating glutamate-induced excitotoxicity when overstimulated.[12] Overactivation of NMDA receptors increases Ca^{2+} permeability and results in incomplete desensitization, contributing to excitotoxic cell death. NMDA receptor antagonists, on the other hand, exhibit anti-excitotoxic effects and may prevent NMDA receptor-mediated cell death during ischemia. Thus, we evaluated the effect of NDMA antagonist D-AP5 on dopamine and glutamate during OGD.

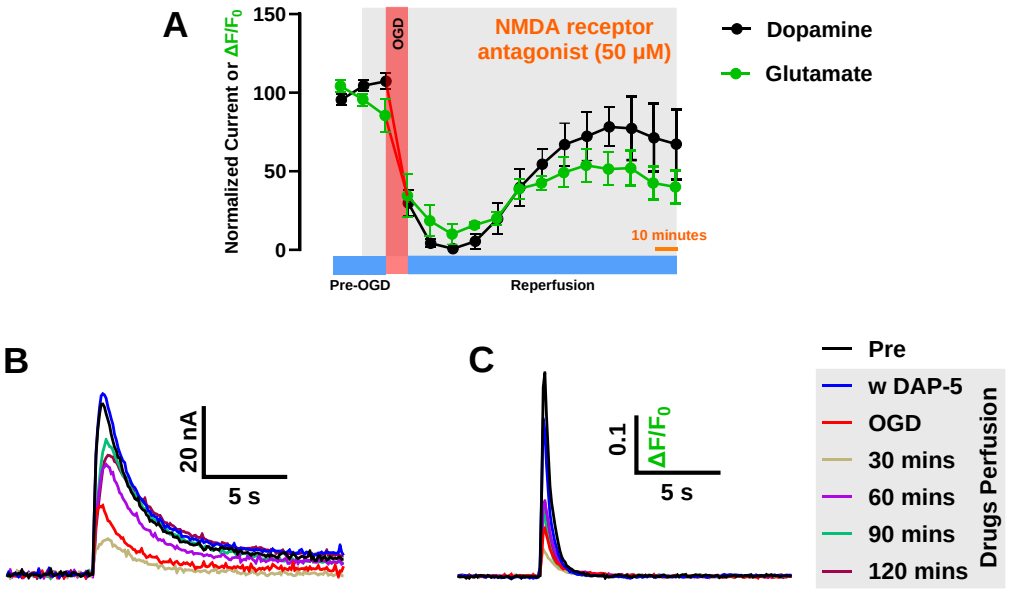


Figure 18: Role of NMDA Receptors Antagonist D-AP5 during OGD. The first two stimulations were performed under normal oxygenated aCSF conditions, while subsequent stims were conducted in the presence of the NMDA receptor antagonist D-AP5 (50 μM), as indicated by the shaded region. Following exposure to OGD, both dopamine and glutamate levels decreased by more than 60% relative to pre-OGD levels. Over time, dopamine recovered to baseline, while glutamate levels remained at OGD-induced levels. (B) Dopamine i v t trace (C) iGluSnFR3 response of stimulated glutamate during pre-OGD, OGD and reperfusion with DAP-5.

To evaluate the neuroprotective action of NMDA receptors during transient OGD, brain slices were treated with the NMDA receptor antagonist D-AP5 (50 μM) for 10 minutes prior to

OGD exposure and then through OGD and reperfusion. As shown in Figure 4, the NMDA receptor antagonist had no significant effect on stimulated neurotransmitter release under normal conditions. However, in the presence of D-AP5, there is a main effect of OGD condition on dopamine and glutamate levels (one-way ANOVA, n = 4, $p < 0.0001$), with neurotransmitter levels decreasing by more than 60% compared to pre-OGD levels (One-way ANOVA, n=4, ***p <0.0005). This reduced release persisted during the reperfusion phase with oxygenated aCSF. Dopamine levels showed a partial recovery close to pre-OGD levels during prolonged reperfusion, similar to data without the NMDA receptor antagonist. In contrast, glutamate levels during reperfusion closely mimicked the response observed during OGD, with no significant recovery. Example traces of stimulated dopamine and glutamate response with NMDA receptor antagonist can be seen in Figure 4B and 4C.

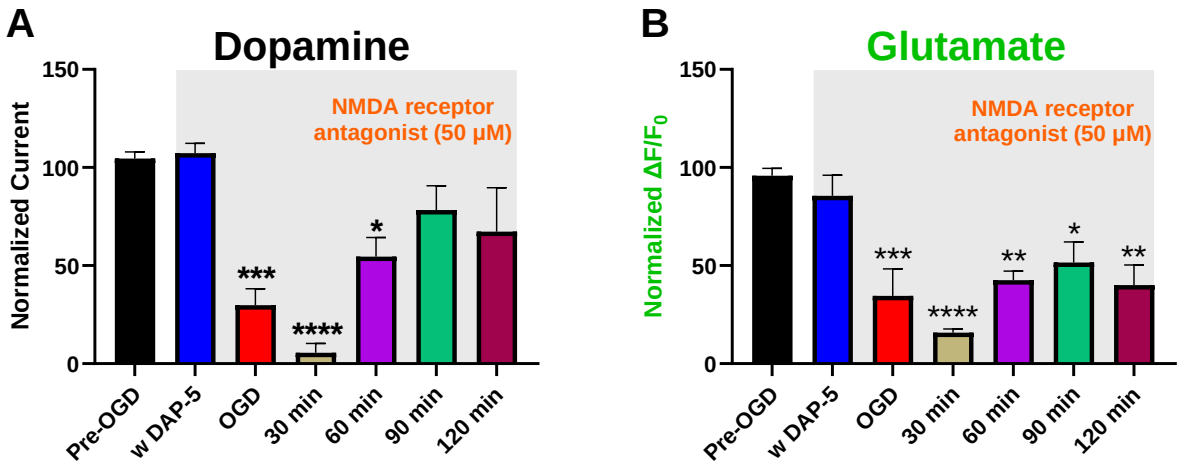


Figure 19: Effect of NMDA antagonist AP5 on stimulated dopamine and glutamate release during OGD. (A) Dopamine levels significantly decreased following OGD exposure and partially recovered to baseline levels during reperfusion (one-way ANOVA, n = 4, overall $p < 0.0001$). (B) Stimulated glutamate levels were reduced by more than 60% compared to pre-OGD levels during OGD and did not return to baseline during reperfusion (One-way ANOVA, n = 4, overall $p < 0.0001$, *p = 0.0105, **p <0.002, ***p <0.0005, ****p <0.0001).

Figures 5A and 5B illustrate the effects of OGD and reperfusion on stimulated dopamine and glutamate levels in the presence of the NMDA receptor antagonist DAP5. Both neurotransmitters were depressed during OGD, even in the presence of a NMDA receptor

antagonist, and then remained significantly decreased during the initial 30 minutes of reperfusion. Dopamine exhibited more recovery with continued reperfusion, while glutamate levels remained unchanged compared to OGD conditions. Thus, there was no real effect of D-AP5 on glutamate and dopamine release during OGD and reperfusion.

4.3.3 Deletion of A_{2A} receptor allows more recovery of glutamate during reperfusion

To evaluate the A2A receptors during OGD insult and reperfusion we used global A2AKO mice.[25] Figure 6A illustrates dopamine (black) and glutamate (green) concentrations during the experiment in the A2AKO slice. Both neurotransmitters remained stable during the initial three pre-OGD runs. Upon switching to N_2-saturated OGD buffer, stimulated dopamine and glutamate release declined to approximately 50% of pre-OGD levels, similar to WT conditions. Both neurotransmitters continued to decrease after the onset of reperfusion with oxygenated aCSF buffer. After 30 minutes of reperfusion, electrically stimulated neurotransmitters showed a slow, gradual recovery. Eventually, both dopamine and glutamate rebounded to their pre-OGD baseline levels. Notably, glutamate recovery in A2AKO slices contrasts with previous WT experiments, where reperfusion glutamate levels remained at OGD levels and exhibited impaired function. Also, for dopamine release, background increases in dopamine were observed in color plots for the OGD condition and the first reperfusion stimulation, indicating that basal increases might inhibit stimulated dopamine, similar to WT conditions.

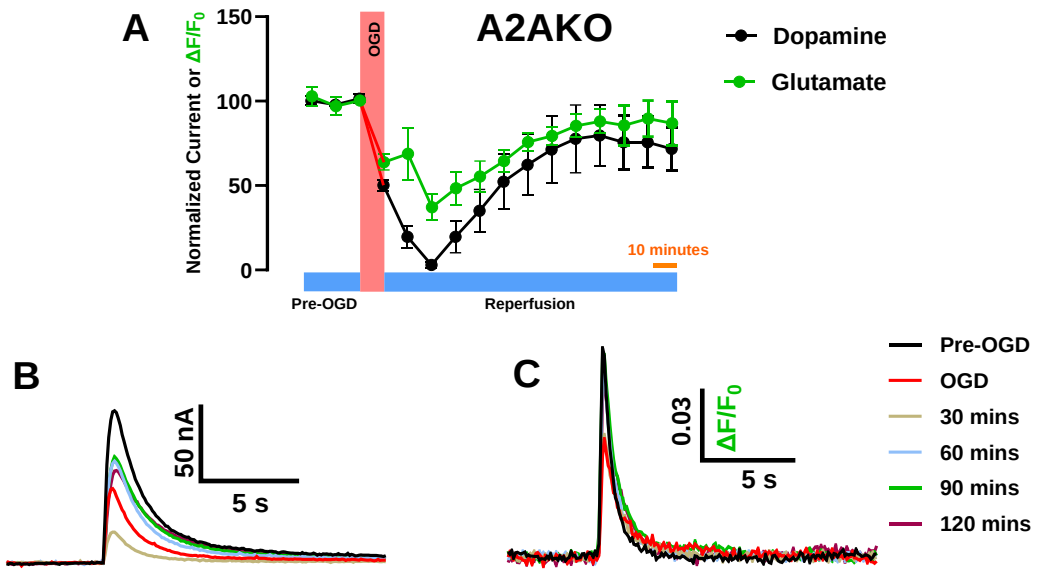


Figure 20: Neuroprotective role of A2A receptors. Data were collected in slices from A2A knockout (A2AKO) mice. Following exposure to OGD, both dopamine and glutamate levels decreased by more than 50% compared to pre-OGD levels. However, with extended perfusion using oxygenated aCSF, both neurotransmitters gradually returned close to baseline levels. (B) Dopamine i v t trace (C) iGluSnFR3 traces after.

Figure 6B shows i v t traces of dopamine release at different time points. Dopamine levels peaked during pre-OGD, halved during the OGD insult, and further declined during the first 30 minutes of reperfusion before gradually recovering over time. Similarly, Figure 6C shows stimulated glutamate following the same trend: a reduction during OGD, further decline during the initial reperfusion period, and eventual recovery nearing pre-OGD levels.

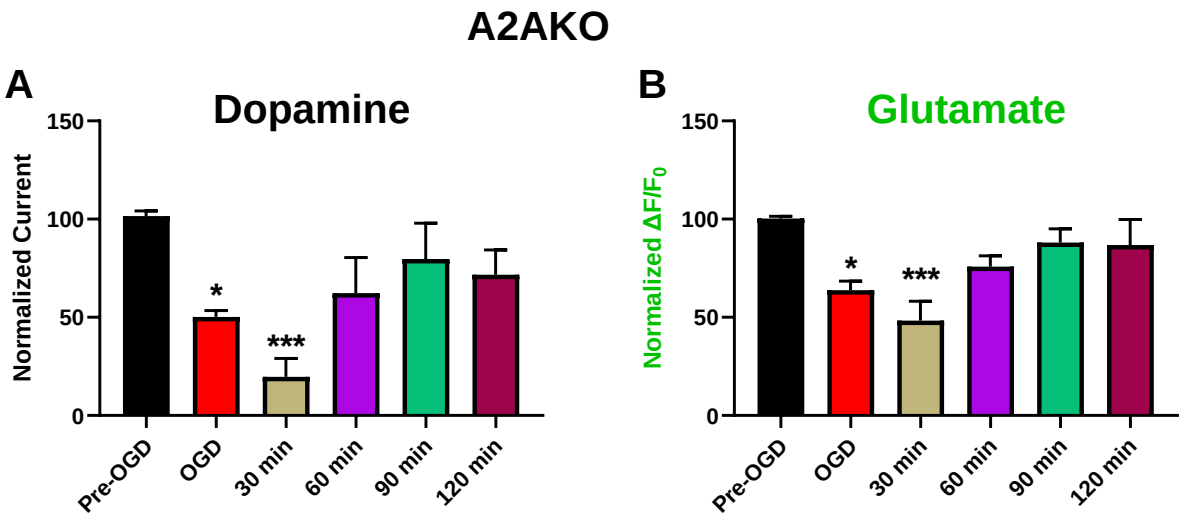


Figure 21: Stimulated dopamine and glutamate level A2AKO mice. (A) Dopamine levels were significantly suppressed following OGD insult (One-way ANOVA, n = 6, overall p = 0.0019; *p = 0.0275; *** = 0.0003). During reperfusion, dopamine was more depressed during the first 30 minutes but returned closer to the baseline. (B) Glutamate mimicked the trend observed in dopamine, experiencing significant suppression due to OGD insult and the first 30 min of reperfusion (One-way ANOVA, n = 6, overall p = 0.0008; 8p = 0.0109; ***p = 0.0003). Glutamate also recovered from 60-120 min after reperfusion and was not significantly different than baseline.

Figures 7A and 7B summarize electrically stimulated dopamine and glutamate levels, in A2AKO mice during OGD and reperfusion. As shown in Figure 7A, there is a significant overall effect of OGD condition/time on dopamine release (one-way ANOVA, n = 6, p = 0.0019). After 10 minutes of OGD, dopamine release was significantly reduced at approximately 50% of pre-OGD levels (one-way ANOVA, n = 6, p = 0.0275). During reperfusion with oxygenated aCSF, there was a further decrease in stimulated dopamine during the first 30 minutes (one-way ANOVA, n = 6, p = 0.0003). However, at longer reperfusion times, dopamine levels began to gradually increase and were not significantly different than pre-OGD levels.

A similar trend was observed for glutamate, as shown in Figure 7B. There is an overall effect of OGD condition on glutamate release in A2AKO mice (One-way ANOVA, n = 6, overall p = 0.0008). After 10 minutes of OGD insult, stimulated glutamate levels were significantly reduced (One-way ANOVA, n = 6, p ≤ 0.0109) and they continued to decline in the first 30

minutes of reperfusion (One-way ANOVA, n=6, p = 0.0003). However, in contrast to WT slices, A2AKO glutamate levels showed better recovery over time, eventually returning to pre-OGD levels, and the 60-120 min time points were not significantly different than pre-OGD. This improved recovery is attributed to the global deletion of A2A receptors, which may reduce the excitotoxic effects of neurotransmitter release during reperfusion.

Additionally, the study demonstrates distinct outcomes in A2A receptor knockout (A2AKO) slices. Unlike WT slices, glutamate levels in A2AKO slices showed recovery after extended reperfusion periods. This phenomenon could be attributed to the neuroprotective role of A2A receptors.[16,18] The genetic elimination of A2A receptors likely reduces excessive adenosine binding and diminishes the potential for additive excitatory glutamate release, contributing to improved recovery.

4.3.4 A_1 antagonist is neuroprotective for both dopamine and glutamate after OGD insult

Adenosine is rapidly elevated during ischemia, which is an inherent neuroprotective mechanism. One of the primary responses involves the activation of widely expressed A1 receptors, which inhibit excitatory synaptic transmission. The neuroprotective role of adenosine during ischemia is largely attributed to A1 receptor activation, which decreases Ca^{2+} influx and reduces presynaptic neurotransmitter release.

In this study, we investigated the function of A1 receptors in regulating synaptic release of dopamine and glutamate during OGD insult and reperfusion conditions by administering the A1 antagonist DPCPX. Figure 8A shows the levels of stimulated neurotransmitters throughout the experiment, with shaded regions indicating A1 antagonist drug perfusion across all

conditions. Dopamine levels showed no change during DPCPX perfusion. When slices were subjected to transient OGD conditions in the presence of the A1 antagonist, stimulated dopamine decreased by 30%. Glutamate levels remained unaffected however, which is very different than WT.

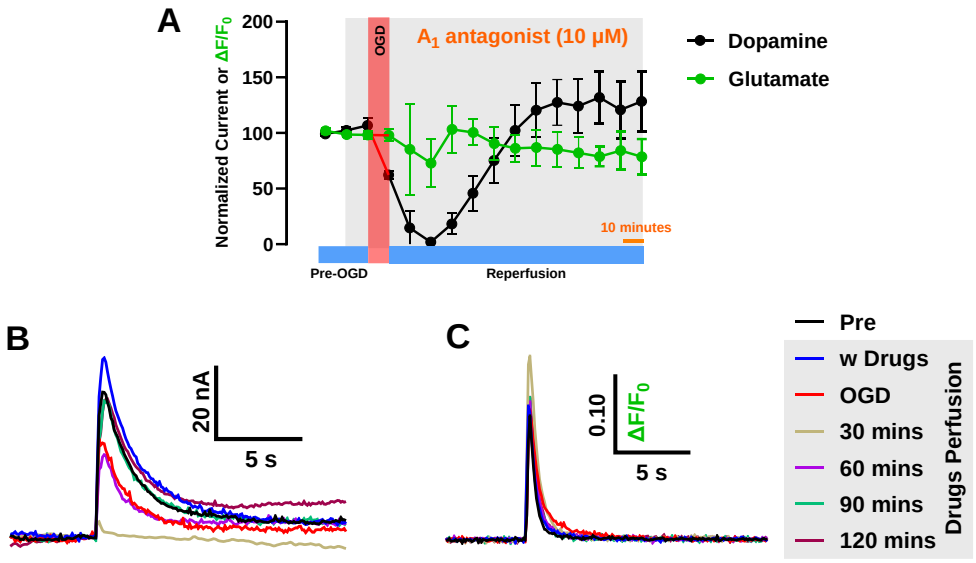
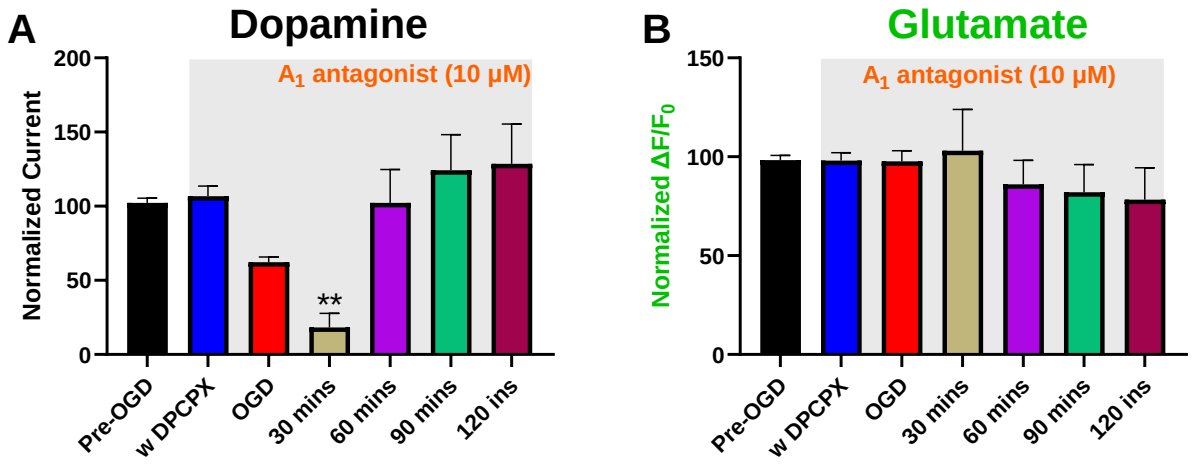


Figure 22: Neuroprotective effect of A1 antagonist (DPCPX, 10 uM) on stimulated dopamine and glutamate in WT mice. (A) The shaded region represents the DPCPX perfusion period. Exposure to OGD significantly reduced dopamine release, with suppression persisting for the subsequent 30 minutes of reperfusion. In contrast, glutamate levels remained stable during and after the OGD insult. (B) Dopamine i v t trace (C) iGluSnFR3 trace after electrical stimulation.

During reperfusion with the A1 receptor antagonist, dopamine levels continued to decline over the first 20 minutes but rapidly recovered to pre-OGD levels within 60 minutes of reperfusion—more efficiently than in experiments without A1 antagonist drugs. Stimulated dopamine eventually exceeded pre-OGD levels. Glutamate levels never significantly decreased during OGD or reperfusion in the presence of the A1 antagonist. Thus, DPCPX had a major effect on glutamate release during OGD and reperfusion.

Figure 8B illustrates example time traces of dopamine release across different time points. With the A1 antagonist, dopamine release was slightly increased pre-OGD, but there is

still a modest decrease in dopamine during OGD. Dopamine levels declined during the initial 30 minutes of reperfusion but rebounded rapidly to pre-OGD levels. Figure 8C shows simultaneous measurements of stimulated glutamate responses, which are similar during pre-OGD, OGD,



and reperfusion. Although there was a slight increase in glutamate during the first 30 minutes of reperfusion with the drug, glutamate levels remained stable for the remainder of the experiment.

Figure 9. A1 antagonist DPCPX is neuroprotective during OGD insult. (A) Dopamine levels remained unchanged during the perfusion of the A1 antagonist. Dopamine decreased during OGD and was significantly lower than pre-OGD during reperfusion at the 30-minute mark (One-way ANOVA, n = 3, p = 0.0016; **p = 0.0094). Dopamine returned to baseline by 60 min and even went slightly above baseline with reperfusion time. (B) Stimulated glutamate release in the presence of A1 antagonist DPCPX. There were no significant differences in glutamate during OGD or reperfusion.

Figure 9A illustrates the effects of OGD insult and reperfusion on stimulated dopamine release, in the presence of an A1 antagonist. Pre-OGD dopamine levels were consistent and unaffected by 10 minutes of A1 antagonist perfusion. Dopamine release had significant overall change due to OGD condition/time (One-way ANOVA, n = 3, overall p = 0.0016). Upon exposure to OGD with A1 antagonist, a reduction in dopamine levels was observed, although this change was not significantly different from pre-OGD levels, likely due to a low n number. Dopamine continued to decline during the first 30 minutes of reperfusion with the A1 antagonist, resulting in a significant reduction in stimulated dopamine release at 30 minutes (One-way ANOVA, n = 3, p = 0.0016; **p = 0.0094). However, dopamine levels recovered rapidly within 60 minutes of reperfusion, returning to pre-OGD levels faster than in experiments conducted

without A1 antagonists. Dopamine subsequently maintained pre-OGD levels or slightly higher for the remainder of the experiment.

Figure 9B shows the effects of A1 antagonist on glutamate response during OGD insult and reperfusion. Notably, stimulated glutamate release was not significantly decreased by OGD or reperfusion with an A1 receptor antagonist. There was no main effect of OGD condition/time on glutamate release (One-way ANOVA, n=3, overall p 0.7339). The maintenance of stimulated glutamate release is a significant contrast to WT slices without drug, which show a marked, prolonged decrease during OGD and reperfusion.

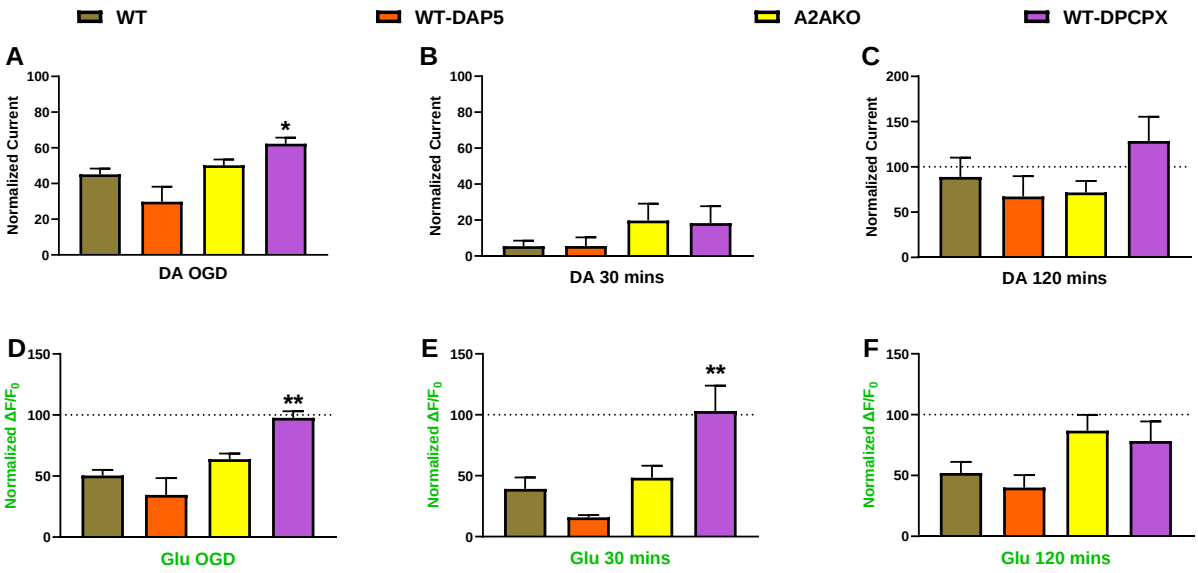


Figure 10: Overall effect of OGD and reperfusion on dopamine and glutamate in WT, WT-DAP5, A2AKO and WT-DPCPX. (A) OGD. Stimulated dopamine levels decreased significantly during OGD across all conditions. A1 antagonist compared to WT (One-way ANOVA, F(3,17), overall p 0.0026, *p 0.0421). (B) During the first 30 minutes of reperfusion, dopamine levels were further suppressed with no significant differences observed across treatments. (C) By 120 minutes of reperfusion, dopamine levels recovered to and there are no differences among groups. (D) Stimulated glutamate levels were significantly decreased during OGD in all conditions except those treated with the A1 antagonist. (One-way ANOVA, F(3,16), overall p 0.0006, **p=0.0014) (E) Glutamate levels continued to decline in all conditions, except for the A1 antagonist treatment, during the first 30 minutes of reperfusion. (One-way ANOVA, F(3,16), overall p 0.0019, **p 0.0042) (F) Overall there was significant differences in glutamate recovery during 120 minutes of perfusion (One-way ANOVA, F(3,16), overall p 0.0475). Glutamate recovery was absent in WT and WT-NMDA antagonist treatments; however, both A2AKO and A1 antagonist treatments showed an 80% recovery, but it was no significantly different than WT.

Figure 10 summarizes the impact of oxygen-glucose deprivation (OGD) and reperfusion on stimulated dopamine and glutamate levels in the striatum with the different conditions. In Figure 10A, for dopamine, there is a main effect of treatment (one-way ANOVA, F(3,17), overall p= 0.0026) and the A1 antagonist is significantly higher than the WT condition (one-way ANOVA, F(3,17), p=0.0421). For 30 min of reperfusion (Fig. 10B), dopamine decreased more and there are no significant differences among groups (One-way ANOVA, F(3,17), overall p 0.3231). For 120 min of reperfusion, there is not a significant main effect of treatment on dopamine release (One-way ANOVA, F(3,17), p=0.2620).

Figure 10D shows glutamate results during OGD. There is significant main effect of treatment (One-way ANOVA, F(3,16), overall p=0.0006) and the A1 antagonist is significantly higher than the WT condition (One-way ANOVA, F(3.16), **p 0.0014). In Figure 10E, during 30 mins of reperfusion, there is a significant main effect of reperfusion among the groups (One-way ANOVA, F(3,16) , overall p=0.0019) and A1 antagonist showed significantly higher effect with respect to the WT treatment (One-way ANOVA, F(3,16) , p=0.0042). Figure 10F shows significant main effect during 120 minutes of reperfusion (One-way ANOVA, F(3,16) , overall p 0.0475), however no significant effects were observed for A2AKO and A1 antagonist treatment.

Thus, there are differences in neurotransmitters after OGD and reperfusion. While perfusion with an A1 receptor antagonist did not eliminate the effect of OGD on stimulated dopamine, it successfully abolished glutamate inhibition throughout the experimental duration. This observation suggests that the excessive release of adenosine during OGD activates A1 receptors, thereby inhibiting stimulated glutamate release from presynaptic neurons.[1,2] These findings highlight the neuroprotective role of A1 receptors during ischemia and reperfusion.

4.4 Discussion:

The brain consumes significant amounts of glucose and oxygen to sustain proper synaptic signaling and physiological functions.[27] Pathological conditions, such as cerebral ischemia, disrupt transient blood flow to the brain, impairing the continuous supply of oxygen and glucose.[23,24,28] This study examined the effects of transient ischemia and subsequent reperfusion on neurotransmitters, particularly dopamine and glutamate, in the striatum using brain slice models. The striatum is highly susceptible to ischemia, second only to the hippocampal CA1 region.[16] Ischemia profoundly affects synaptic signaling by disrupting neurotransmitter dynamics and transmission due to energy and oxygen depletion, which compromises normal cellular processes.[10,16,24]

Upon the early onset of ischemia, extracellular adenosine levels surge due to ATP breakdown, as cytosolic ATP production declines.[9,10,16] This triggers several downstream events, including ion gradient disruption across the cell membrane, excessive excitatory neurotransmitter release into the extracellular space, increased intracellular calcium (Ca^{2+}) concentrations, and neuronal excitotoxicity.[14,18,29,30] Using a well-established oxygen-glucose deprivation (OGD) model, we simulated transient ischemic conditions and measured neurotransmitter release every 10 minutes over a 10-min OGD and 120-minute reperfusion period.[6] In this model, glucose was substituted with sucrose, and the medium was saturated with nitrogen gas to replace oxygen.[31] The deprivation of oxygen and glucose during OGD disrupts cellular homeostasis, causing energy production failure, oxidative stress, and activation of excitatory neurotransmitters, potentially leading to neuronal damage or death. In our studies, stimulated dopamine and glutamate decreased about 50% after 10 min of OGD. Thus, a transient OGD resulted in less pools of dopamine and glutamate to be available for stimulated release. It is possible that basal levels increase during OGD due to spontaneous release, but both FSCV and iGluSnFR are background subtracted techniques, and so slow basal changes

are difficult to measure. We did see some evidence of spontaneous dopamine release, but the experiment was not optimized to detect us. Thus, the main conclusion is that 10 min of OGD reduces the amount of dopamine and glutamate available for stimulated release during phasic type firing.

Reperfusion is crucial for preserving and restoring tissue, preventing irreversible damage following a transient blockage of glucose and oxygen supply. However, studies have shown that reperfusion can exacerbate ischemia-induced tissue damage.[24,32] This is often driven by the release of reactive oxygen species (ROS) and a cascade of inflammatory responses, leading to localized damage and systemic insults. During reperfusion, the excess release of glutamate into the extracellular space contributes to excitotoxicity, which can further harm neuronal tissue. In contrast, the excessive release of dopamine during reperfusion may serve a protective role, mitigating glutamate-induced toxicity.[19,31,33] In this study, dopamine release decreased further than OGD during the initial 30 minutes of reperfusion and was at barely detectable levels in some slices. Thus, the effects of OGD on dopamine last longer than just during OGD. Dopamine gradually recovered to about 80-90% pre-OGD levels after 120 minutes of reperfusion, which was not significantly different than pre-OGD. Glutamate followed a similar trend initially, decreasing to about 50% during OGD, but the release stayed at a similar level during the whole 120 min of reperfusion, and it did not return to baseline levels. One advantage of the simultaneous measurements of dopamine and glutamate is that the time courses were measured in the same slice, which showed that recovery from reperfusion was different for the two neurotransmitters. While both had similar decreases during OGD, dopamine went down more than glutamate but then recovered much more than glutamate. Thus, the simultaneous measurements with FSCV and iGluSnFR facilitated an understanding of the different time courses during reperfusion.

To further understand receptor involvement, pharmacological treatment with and NMDA receptor antagonist D-AP5 was applied in WT slices. Glutamate levels rise drastically during OGD due to increased intracellular Ca^{2+} and compromised membrane integrity, leading to excessive ionotropic receptor activation (e.g., NMDA) and potential excitotoxicity.[12,22,34] Thus, we hypothesized that NMDA antagonist might be neuroprotective for at least glutamate, However, preconditioning slices with an NMDA antagonist (50 μM D-AP5) prior to OGD failed to prevent the suppression of neurotransmitter release during OGD and the first 30 minutes of reperfusion, suggesting limited effectiveness during transient ischemia.

The striatum has the highest concentration of A2A receptors in the brain and is particularly vulnerable to ischemia.[25,35,36] During oxygen and glucose deprivation, extracellular adenosine levels rise and activate A2A receptors, which leads to glutamate release.[37–39] In A2AKO slices, both dopamine and glutamate levels were significantly reduced during OGD and the first 30 minutes of reperfusion but eventually recovered to pre-OGD levels. Importantly, suppression of glutamate release due to OGD was less pronounced in A2AKO slices compared to WT and the slices recovered faster during reperfusion. This faster recovery may stem from the absence of A2A receptors, which reduces glutamate overactivation in response to increased adenosine during OGD. These findings suggest that while A2A receptors do not directly regulate dopamine release, they play a crucial role in restoring synaptic glutamate release in the striatum during reperfusion.

Adenosine typically exerts inhibitory effects via A1 receptors, and the inhibition of glutamate release by A1 receptors is thought to be neuroprotective.[1,40–42] To examine the role of A1 receptors, slices were perfused with A1 antagonist (DPCPX, 10 μM). Previous studies from our lab demonstrated that A1 receptor activation suppresses dopamine and glutamate release by reducing Ca^{2+} influx essential for synaptic vesicle docking. During OGD, dopamine levels decreased, but in the presence of DPCPX, they recovered rapidly during reperfusion, indicating

improved resilience compared to experiments without the antagonist. Furthermore, the A1 receptor antagonist prevented glutamate suppression throughout OGD and reperfusion, emphasizing its protective effects on glutamate signaling. By inhibiting A1 receptor functions with DPCPX, synaptic transmission inhibition was eliminated during OGD, preserving glutamate dynamics. The application of an A1 antagonist blocked this inhibitory effect by preventing Ca^{2+} influx into presynaptic neurons, resulting in disrupted regulation of glutamate release.[12,26] Thus, A1 receptors mediated the rapid decrease in stimulated release during OGD and immediate reperfusion. These findings suggest that A1 antagonist minimizes the reduction in stimulated dopamine levels caused by OGD and promotes quicker recovery during reperfusion. Meanwhile, glutamate release remains largely unaffected, further supporting the role of A1 receptors in maintaining synaptic neurotransmitter release by counteracting the inhibitory effects of adenosine via A1 receptor activation.

Overall, these findings demonstrate that both A1 and A2A receptors are integral to playing neuroprotective roles decreasing dopamine and glutamate signaling during transient ischemia and reperfusion. A2A receptors are more important for regulating longer time periods after reperfusion, while A1 receptors regulated glutamate release more during OGD and immediate reperfusion. Adenosine receptors had more effects on synaptic glutamate release than extrasynaptic dopamine release, which indicate that while adenosine does regulate both neurotransmitters, that there are more profound effects on glutamate. The ability to monitor more than one neurotransmitter at time gives a more complete picture of the complex changes in neurochemistry during ischemia and reperfusion. Overall, these results highlight that adenosine receptors could be potential therapeutic targets for mitigating excitotoxic damage and preserving neuronal function under ischemic stress.

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Chapter 5: Conclusions and Future Direction

5.1 Contribution to the field

In this dissertation, I investigated the neuromodulatory function of transient adenosine on other neurotransmitters such as serotonin, dopamine and glutamate. Particularly, I multiplexed fast scan cyclic voltammetry with fluorescence to study neurotransmitter release interactions simultaneously in real time using brain slice model. Furthermore, this multiplexed technique was used to study the role of transient oxygen glucose deprivation insult on dopamine and glutamate. This chapter will briefly cover my projects and future directions.

5.2 Neuromodulatory action of adenosine in real-time using brain slice model

Chapter 2 discusses the modulatory actions of adenosine on serotonergic neurons, which are densely populated in the midbrain. Exogenous adenosine was observed to transiently inhibit serotonergic activity for the initial 20 seconds, with activity recovering as adenosine cleared from the slice. Pharmacological studies revealed that adenosine receptors A1, A2A, and A3 did not directly mediate this inhibitory effect. Instead, adenosine indirectly modulated serotonergic neurons through 5-HT1A autoreceptors. This surprising indirect neuromodulation by adenosine on DRN serotonergic neurons offers a new perspective on its inhibitory action via A1 receptors. Further investigations are needed to examine the expression levels of A1 receptors alongside 5-HT1A receptors in DRN to determine spatial relationship during adenosine application. Utilizing transgenic mice (5HT-A1KO) could help elucidate whether these receptors play primary or secondary roles in the observed serotonin inhibition. Additionally, coupling GCaMP sensors, which respond to intracellular calcium binding, with FSCV could offer valuable insights into the adenosine A1 receptors dynamics over serotonergic activity and receptors interactions. This unique finding also suggests that A1 may not always directly inhibit neurotransmitter release but may instead be coupled with other inhibitory loops or communication pathways via co-expression or receptor dimerization with other inhibitory receptors such as GABAergic to exert their modulatory effects.

Chapter 3 highlights the integration of FSCV and fluorescence (iGluSnFR3) to simultaneously measure adenosine, dopamine, and glutamate. A key finding was the inverse correlation between dopamine release and glutamate response during electric stimulation. Adenosine was observed to inhibit both dopamine and glutamate within a 250 μm range, with A1 receptors playing a pivotal role in mediating this inhibitory effect. This combination provides valuable insights into the spatial and temporal interactions of neurotransmitters. The recent development of multi-color genetically encoded sensors, such as green, yellow and red variants, expands the potential for monitoring multiple neurotransmitters with enhanced spatial-temporal resolution.[1–4] For example, dopamine can be monitored using GRABDA sensors, while glutamate can be monitored with iGluSNFR3 sensors. Integrating these florescence tools with FSCV, limited to detect extra-synaptic electroactive neurotransmitters due to electrode size, provides a deeper understanding of neurotransmitter spatial and extra-synaptic dynamics, especially during adenosine modulation.[5,6] This approach could significantly advance studies of synaptic interactions and neural circuit functionality.

Chapter 4 investigates the effects of transient ischemic conditions on dopamine and glutamate in the striatum using a 10-minute OGD model followed by reperfusion to mimic ischemic/reperfusion injury. This is achieved by substituting glucose with an equimolar concentration of sucrose and replacing oxygen with nitrogen to simulate the deprivation of critical cellular energy substrates. OGD significantly reduced dopamine and glutamate release, with both continuing to decline during the first 30 minutes of reperfusion. Dopamine eventually recovered within 120 minutes, whereas glutamate remained at levels consistent with the OGD condition. Studies with transgenic mice (A2AKO) and pharmacological treatments (NMDA receptor antagonist and DPCPX) revealed that A1 and A2A receptors play critical neuroprotective roles during OGD. Specifically, A1 receptor activation prevents glutamate accumulation in extracellular spaces, reducing excitotoxicity. Further studies are needed to

elucidate the mechanisms by which adenosine receptors influence dopamine and glutamate release during OGD/reperfusion.

It would be particularly insightful to examine the effects of perfusing A1 antagonists or agonists exclusively during the OGD phase only and monitoring their impact during reperfusion. Additionally, investigating GABAergic activity and calcium ion influx by expressing iGABASnFR2 and GCaMP in the striatum could provide valuable insights into synaptic release and modulation under conditions of high extracellular adenosine and glutamate during transient ischemia. A time-dependent OGD/reperfusion study could also be conducted to assess the dynamics changes in neurotransmitter release over extended periods.

The integration of FSCV and fluorescence imaging opens a new scope for studying neurotransmitter interactions under both physiological and pathological conditions. This dissertation demonstrates that adenosine exerts its inhibitory and protective functions directly via A1 receptors, while also indirectly inhibiting serotonergic neurons in the DRN. The combination of FSCV and fluorescence sensors broadens the scope for analyzing complex neurotransmitter interactions in real-time. FSCV currently enables simultaneous detection of 2-3 electroactive neurotransmitters, while fluorescence techniques compliment this by allowing the monitoring of non-electroactive neurotransmitter release, such as GABA (iGABASnFRs), glutamate (iGluSnFRs), acetylcholine (iAChSnFR) and norepinephrine.[2,3,7,8] Advances in fluorescence imaging now offer multi-color probes for neurotransmitters, enhancing multiplexing capabilities. For example, dopamine sensors (dLight) are available in red, yellow and green variants, while glutamate (iGluSnFR) sensors are available in yellow and green.[1,2,7]

Additionally, our lab has been employing machine learning algorithms to analyze and resolve signals from neurotransmitters with closely overlapping oxidation potentials.[9,10] When combined with imaging technique, this approach will further enhance the ability to simultaneously monitor multiple neurotransmitters in FSCV, facilitating a deeper understanding

of intricate synaptic interactions. Multiplexing techniques can also be applied in vivo to explore adenosine's modulation in glutamate dynamics. Similarly, these multiplexing methods can be employed to investigate adenosine's involvement in neuromodulating serotonergic neurons, using serotonin sensors (iSeroSnFR), to unravel how A1 receptors indirectly contribute to inhibitory action. This integrated approach holds significant promise for unraveling the intricate neurotransmitter dynamics implicated in neurological disorders such as epilepsy and Parkinson's disease, circuits and drug development.

5.3 Future Directions:

Parkinson's disease (PD) is a chronic and progressive neurodegenerative condition, which predominantly causes motor symptoms such as impaired balance, movement and muscle co-ordination.[11,12] It is second most common age-related neurodegenerative conditions affecting individuals over 65 years old.[13] The aggregation of alpha-synuclein, also known as Lewy-bodies, within neurons is characteristic pathological features of PD and associated with dopaminergic neurodegeneration in the substantia nigra projected to the striatum, resulting dopamine depletion.[14,15]

PD leads to deteriorated motor functions as well as non-motor symptoms, including sleep disorders, cognitive impairment, and psychiatric issues.[16] Traditional PD models primarily emphasize dopaminergic neuron dysfunction and motor impairment that usually appears on later stages of the disease after 50-60% of dopaminergic neurons have degenerated.[17–19] This results to 70-80% dopamine depletion in the striatum.[20] Since the 1960s, L-DOPA and numerous dopamine agonist drugs, such as apomorphine and ropinrole, have been used to treat PD.[12] However, along with dopaminergic neuron degeneration, the complex pathology of PD affects a broader range of neuronal systems, such as serotonergic, glutamatergic, cholinergic and GABAergic pathways in the basal ganglia signaling.[12,21–23] For example, the

gradual degeneration of dopaminergic neurons in PD results in reduced dopamine levels, increased excitatory glutamatergic activity and decreased GABAergic activity in the striatum, further amplifying uncontrolled motor activity.[24,25] This observations suggest that non-dopaminergic approach are also needed to alleviate these motor symptoms.

Adenosine is ubiquitously found neurotransmitter in the central nervous system (CNS) and plays a significant role in the neuromodulation, maintaining normal physiological homeostasis and influencing pathological condition.[26,27] Adenosine can exhibit both excitatory and inhibitory neuromodulatory function via its four GPCR receptors i.e., A1, A2A, A2B and A3 adenosine receptors. For example, our lab demonstrated significant inhibitory action of adenosine on dopamine release via A1 receptors in caudate region.[28] Studies also show that A1 and A2A adenosine receptors dimerizes with other GPCR receptors, such as dopamine, glutamate and GABAergic receptors at the synapse. This dimerization is essential characteristics for crosstalk between different pathways in motor control brain regions.[29,30] A2A receptor are highly expressed in the caudate putamen, nucleus accumben and globus pallidus in rodent as well as human brains.[31,32] In the striatum, a crucial region for behavior control, A2A-D2 direct receptor- receptor interactions coupled with A2A-D1 indirect receptor interactions balances possible outcomes of adenosine receptor activation on PD and mediate motor behavior.[29] Similarly, A1-A2A adenosine receptors were also located in the striatal glutamatergic neuron terminals and are regulated through local adenosine concentration.[33] The use of A2A antagonist drug, istradefylline, in the PD treatment further indicates that PD is not only related with dopamine depletion but also involves other neurotransmitters, such as adenosine.

While most research has focused on adenosine and dopamine interaction, there are relatively few studies investigating adenosine, dopamine and glutamate interaction in real-time at different stages of PD. The striatum contains the highest densities of glutamate receptors that tightly control excitatory neurotransmitter glutamate level along with A2A receptors.[34,35] The

multiplexing techniques I developed can be implemented to investigate the role of A2A receptors, and their influence on dopamine and glutamate dynamics in PD models. Our lab combined FSCV and fluorescence techniques and demonstrated simultaneous measurement of both electroactive and non-electroactive neurotransmitters in real-time. The 6-hydroxydopamine (6-OHDA) is commonly used neurotoxin to induce PD in mice by lesioning the nigrostriatal system.[17–19] However, these models typically assess extensive dopaminergic lesions, which reflect late-stage PD. Consequently, there are relatively few studies addressing the initial stages of PD, where early interventions would be most effective.

The purpose of this study would be to use different doses of 6-OHDA into the substantia nigra (SN) of C57BL/6J mice to mimic the early stages of PD and investigate the neuroprotective role of adenosine receptors on dopamine and glutamate neurotransmission.[17,36] Partial lesions of SN will likely mimic the early stages of PD.[36] First, iGluSnFR3 would be injected into the striatum using 1000 nL of AAV virus. Two weeks later, iGluSnFR3-injected mice would receive different doses (0.5 µg, 1 µg and 2 µg dissolved in 0.02% ascorbic acid) of 6-OHDA, alongside a control injection (0.02% ascorbic acid) in the SN using stereotaxic technique.[19,36] 6-OHDA exerts degenerative activity through reactive oxygen species (ROS) and activates astrocytes and microglia.[18,35]

Injected neurotoxins rapidly induce dopaminergic neurons degeneration as early as 24 hours post-surgery, leading to an 80% loss of dopaminergic neurons within 3–4 days and a complete degeneration within three weeks. This study would also evaluate nigral tyrosine hydrolase (TH)+ cell loss and striatal TH+ fiber loss corresponding to different 6-OHDA doses.[17,19,36] Additionally, transgenic mice (A1KO/A2AKO) and adenosine receptor drugs, such as A2A agonist/antagonist will provide valuable insight into adenosine receptor’s role on dopamine and glutamate signaling during PD.

One week after the 6-OHDA injection, brain slices would be prepared from these PD model mice and investigate electrically stimulated dopamine and glutamate interaction in the striatum using multiplexing tools. CFMEs inserted into the tissue would record electrically stimulated dopamine while fluorescence microscopy collects fluorescence change from iGluSnFR3 simultaneously. Using A2AKO mice, this study would explore how A2A receptors influence dopamine and glutamate neurotransmission. Pharmacological intervention drugs, such as A2A agonist/antagonist as well as adenosine (10 μM) perfusion, would give us an insight on adenosine receptors role in neuromodulation during PD condition.

We hypothesize that adenosine A2A receptors directly regulate dopamine and glutamate neurotransmission in the striatum during PD. This knowledge will deepen our understanding of the early phases of PD and could contribute to the development of adenosine-based drug treatment aimed at alleviating PD associated symptomatic conditions.

5.4 Final Conclusions

In conclusion, this dissertation explores the innovative combination of fast scan cyclic voltammetry (FSCV) and fluorescence imaging techniques to simultaneously monitor both electroactive and non-electroactive analytes in real time. Exogenous adenosine was found to transiently inhibit serotonergic neurons via 5HT1A receptors, and not through A1 receptors. The inhibitory effects of adenosine on other neurotransmitters, such as dopamine and glutamate, were also investigated. Notably, adenosine inhibited glutamate release within a 250 μm range, and this inhibition was globally blocked through perfusion with A1 antagonist drugs. Thus, a major finding is that adenosine often has inhibitory actions through A1 receptors, as it does for dopamine and glutamate, but not always, as in the case of serotonin.

Furthermore, multiplexing techniques were employed to study the effects of oxygen-glucose deprivation (OGD) and reperfusion on dopamine and glutamate release. Various pharmacological tests provided insights into the neuroprotective roles of adenosine. Activation

of A1 receptors demonstrated a neuroprotective effect against OGD and reperfusion by inhibiting glutamate release, thus mitigating excitotoxicity.

Looking ahead, these multiplexing techniques hold promise for studying the roles of neurotransmitters and receptors in pathological conditions such as Parkinson's disease and epilepsy. By facilitating the understanding of complex neurotransmitter interactions in disease states, this approach may pave the way for the development of novel therapeutic strategies.

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