Adenosine in the brain: recent progress on detection, function and translation

Short title: Adenosine in the brain

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Conflict of Interest

The authors declare no competing interests.

ABSTRACT

Although adenosine was identified in the brain many decades ago, our understanding of when, where, and how it functions has expanded rapidly in recent years, driven in part by innovative technological advances. Adenosine is now increasingly recognized as a key neuromodulator that dynamically regulates brain circuits important for sleep/wakefulness, movement, cognition and homeostasis. In addition, growing attention has been directed toward the molecular mechanisms governing adenosine production and its downstream signaling pathways, both of which hold great promise as therapeutic targets for neuropsychiatric disorders and neurodegenerative diseases. This review highlights recent progress in detecting adenosine, unraveling its signaling pathways *in vitro* and *in vivo*, and understanding how it regulates brain function under physiological and pathological conditions.

Introduction

Adenosine is a naturally occurring nucleoside distributed ubiquitously throughout the body. It has long been recognized that adenosine levels rise markedly in response to ischemia, hypoxia, excitotoxicity, inflammation and other brain insults (Dunwiddie and Masino, 2001; Latini and Pedata, 2001; Fredholm, 2007). More recently, it became appreciated that adenosine is released moment by moment during normal brain function and animal behavior, functioning as a gliotransmitter and neuromodulator (Badimon et al., 2020; Peng et al., 2020; Ma et al., 2022; Theparambil et al., 2024; Chen et al., 2025; Xin et al., 2025). Extracellular adenosine acts through four adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R, respectively), which are G-protein coupled receptors (GPCRs), to exert a vast variety of physiological effects (Fredholm et al., 2011).

Over the last two decades, extensive progress has been made to elucidate adenosine signaling and function. For example, i) $A_{2A}R$ has become the first Gs-coupled receptor with a resolved crystal structure (Jaakola et al., 2008; Lebon et al., 2011; Xu et al., 2011); ii) genetic knockouts of each of the four adenosine receptor subtypes, as well as enzymes involved in adenosine generation and metabolism, have shed light on adenosine's physiological roles (Chen et al., 2013); iii) the development of agonists and antagonists with high affinity [Kd ~ nM] and selectivity [>100 fold among adenosine receptor subtypes] has enabled selective manipulation of specific adenosine pathways, even in human (Ijzerman et al., 2022); iv) an $A_{2A}R$ antagonist has been approved for treating Parkinson's disease and is being tested in multiple clinical trials for treating tumors (Jacobson et al., 2022); and v) both adenosine and its downstream signaling pathways can be imaged in behaving animals (Ma et al., 2018, 2022; Joya et al., 2020; Peng et al., 2020; Massengill et al., 2022; Wu et al., 2023; Wei et al., 2025). The convergence of

interdisciplinary evidence strongly supports the role of adenosine and adenosine receptors in regulating brain function and animal behavior, and uncovers the potential of adenosine signaling pathways as therapeutic targets for various neuropsychiatric disorders and neurodegenerative diseases (Borea et al., 2018).

This review highlights recent progress in uncovering the critical roles of adenosine signaling in a variety of physiological and pathophysiological processes in the brain, including: 1) The development of novel genetically encoded sensors that enable the monitoring of the spatiotemporal dynamics of extracellular and intracellular adenosine and its downstream intracellular signaling pathways with exquisite sensitivity, even in behaving animals, thus paving the way to revealing the complex mechanisms underlying adenosine release, uptake, and degradation. 2) The use of this novel adenosine detection technology combined with advanced molecular biology and pharmacology to demonstrate that adenosine concentrations may be regulated moment by moment to actively shape neuronal circuits and brain function and ultimately control animal behavior. 3) The increased understanding of the mechanisms underlying adenosine production and release with highlights in the multifaceted roles of equilibrative nucleoside transporters (ENTs) in adenosine release and a previously underappreciated contribution of adenosine production by microglia. 4) The emerging concept of adenosine augmentation therapy, which harnesses both adenosine's neuromodulatory function and its homeostatic function, which coordinates metabolic activity across eukaryotic cells; and 5) the growing potential of adenosine-based interventions in sleep disorders and Alzheimer's disease.

Detecting adenosine

Traditional approaches for monitoring adenosine dynamics *in vivo* often rely on microdialysis coupled with high-performance liquid chromatography (HPLC) or mass spectrometry (Figure 1A) (Bito et al., 1966; Porkka-Heiskanen et al., 1997). This method quantifies time-averaged extracellular adenosine accumulation and enables multiplexed detection of neurotransmitters and metabolites at nanomolar sensitivity. However, the utility of this method is constrained by low spatiotemporal resolution (~minutes) (Porkka-Heiskanen et al., 2000). Other methods, including microelectrode biosensors, which rely on enzymatic cascades to convert adenosine into electrochemically detectable H_2O_2 (Dale et al., 2000; Gourine et al., 2005; Dale, 2021), and fast-scan cyclic voltammetry (FSCV), which detects direct electrochemical oxidation of adenosine (Nguyen et al., 2014), achieve improved temporal resolution (~1 s and ~100 ms, respectively). Nevertheless, critical limitations have persisted: 1) poor spatial resolution: neither biosensors nor FSCV localize adenosine release sites or diffusion gradients; and 2) exclusive extracellular focus: these methods cannot resolve intracellular adenosine dynamics, which govern metabolic flux and nucleoside trafficking (Garcia-Gil et al., 2021; Liu et al., 2022).

In contrast to these traditional methods, recently developed genetically encoded adenosine indicators (Figure 1A) enable cell-type-specific expression and long-term monitoring of either extracellular or intracellular adenosine signals (using different sensors). When combined with advanced imaging techniques, these tools offer superior spatiotemporal resolution. Furthermore, engineered variants now provide tunable affinities and multi-color options, significantly expanding their versatility for diverse experimental applications (Wu and Li, 2020; Wu et al., 2022b).

Adenosine receptors are GPCRs – the largest family of membrane-spanning proteins – that have evolved as specific detectors for adenosine. Upon ligand binding, GPCRs undergo

significant conformational changes (Weis and Kobilka, 2018), offering opportunity for sensor development (Vilardaga et al., 2003; Wang et al., 2018; Sabatini and Tian, 2020). Hoffmann et al. engineered, to our knowledge, the first genetically encoded indicator for extracellular adenosine by fusing a cyan fluorescent protein (CFP) to the C-terminus and the fluorescein arsenical hairpin (FlAsH) label to the third intracellular loop of the $A_{2A}R$. The conformational change elicited by adenosine binding alters the distance between these moieties and thereby Förster resonance energy transfer (FRET) (Hoffmann et al., 2005). However, the small signal (≤20%) as well as the requirement and associated challenges for FlAsH dye incubation limit its application.

A breakthrough emerged with the development of GPCR-activation-based (GRAB) fluorescence indicators, $GRAB_{Ado}1.0$ (Peng et al., 2020) and $GRAB_{Ado}1.0m$ (Wu et al., 2023), which couple the conformational changes in $A_{2A}R$ upon adenosine binding to a circular-permutated EGFP (cpEGFP) to result in a great increase in its brightness. $GRAB_{Ado}$ enables real-time detection of extracellular adenosine in cultures, acute brain slices, and *in vivo* (Figure 1B and 1C). Additionally, this design framework has been extended to other purinergic transmitters, including extracellular adenosine triphosphate (ATP) and uridine diphosphate (UDP) (Wu et al., 2022a; Umpierre et al., 2024).

An alternative approach to probe adenosine signaling is to examine its downstream intracellular signaling pathways. As detailed further below, all adenosine receptors converge onto the adenylyl cyclase–cyclic AMP (cAMP) pathway, and some receptors also tap into the Gq–phospholipase C–inositol trisphosphate/diacylglycerol (IP3/DAG) pathway. It is now possible to image cAMP and the activity of its major effector protein kinase A (PKA) in behaving animals with cellular resolution (Ma et al., 2018; Massengill et al., 2022; Wang et al.,

2022). *In vivo* imaging of protein kinase C (PKC) activity, which is a major effector of IP3/DAG, has also been established recently (Yahiro et al., 2025). While other neuromodulators also regulate these signaling pathways, the highly selective agonists and antagonists of adenosine receptors have enabled dissection of the unique contribution of adenosine (Ma et al., 2022; Theparambil et al., 2024).

While most of the above methods are designed for detecting extracellular adenosine, a genetically encoded intracellular adenosine indicator, HypnoS (Wei et al., 2025), was recently developed by coupling cpEGFP to the *Plasmodium vivax* adenosine deaminase (PvADA). HypnoS is sufficient for real-time monitoring of intracellular adenosine dynamics in living cells and model organisms (Figure 1D and 1E). Ongoing efforts to expand the spectral range of adenosine and downstream signaling sensors to encompass red, far-red, and near-infrared options should enable the monitoring of multiple signals in living systems, potentially offering insights into intracellular adenosine's role in both physiological and pathological conditions.

Adenosine accumulation in physiological and behavioral contexts

The above developments in sensing technology, combined with other modern genetic and pharmacological approaches, have started to uncover adenosine dynamics during normal physiological and behavioral contexts, such as sleep/wakefulness and movement.

Extracellular adenosine can promote sleep, as well as other hypometabolic states, such as torpor and hibernation (Ma et al., 2023). It has long been known that injection of adenosine into the cerebral ventricle of cats causes a state resembling natural sleep (Feldberg and Sherwood, 1954). Subsequent studies suggest that adenosine, particularly that released in the basal forebrain, is a primary physiological driver of homeostatic sleep need (Porkka-Heiskanen et al.,

1997; Basheer et al., 2004; Saper et al., 2005; Vyazovskiy et al., 2009; Bjorness et al., 2016; Bringmann, 2018; Jagannath et al., 2021). Fiber photometric measurements of $GRAB_{Ado}1.0$ show that adenosine levels in the basal forebrain are dynamically regulated such that its concentration is increased during wakefulness and decreased during non-rapid-eye-movement (non-REM, or NREM) sleep (Peng et al., 2020). This study also found a prominent increase in adenosine concentrations during REM sleep (Figure 1B). In addition, 40-Hz flickering light promotes sleep by raising cortical levels of extracellular adenosine (Zhou et al., 2024) (see later for its translational potential).

While many studies suggest that neurons are the source of extracellular adenosine in the brain, others indicate that glia also contribute. For example, a recent study showed that chemogenetic activation of astrocytes in the nucleus accumbens (NAc) increases extracellular adenosine levels and promotes NREM sleep via A_{2A}Rs (Roy et al., 2024). In contrast, astrocytes in the parafacial zone (PZ) promote wakefulness through adenosine–A_1R signaling (Zhu et al., 2024). It is also important to note that adenosine is not the only mediator by which astrocytes regulate sleep (Peng et al., 2023). Finally, activation of microglial Gi signaling promotes sleep by increasing extracellular adenosine levels (Ma et al., 2024), potentially by modulating the conversion of extracellular ATP to adenosine (Badimon et al., 2020).

Extracellular adenosine signaling also plays a role in other physiological processes besides sleep. For example, Ma et al. (2022) observed an acute increase in PKA activity in D2 dopamine receptor (D2R)-expressing indirect-pathway striatal projection neurons (iSPNs) during animal locomotion, and this increase was eliminated by antagonizing A_{2A}Rs. Furthermore, fiber photometric measurements of $GRAB_{Ado}$ revealed acute accumulation of extracellular adenosine during locomotion (Figure 1C). This acute extracellular adenosine accumulation antagonized the

actions of dopamine by oppositely regulating adenylyl cyclase–cAMP–PKA activities, SPN function, and animal locomotion (Ma et al., 2022). The interaction between dopamine and adenosine signaling is further discussed in later sections. In parallel, other studies found that adenosine and its precursor ATP are released by neuronal stimulation (Roberts et al., 2022; Nasrallah et al., 2024; Theparambil et al., 2024), and may regulate neuronal functions and plasticity (via A_1Rs and A_{2A}Rs) or astrocytic metabolism (via A_{2B}Rs). Finally, several recent studies also suggest a role for acute adenosine regulation of neuronal circuit function and animal biological states, which implies activity-dependent adenosine release (Badimon et al., 2020; Chen et al., 2025; Xin et al., 2025), although additional work will be needed to fully investigate the precise dynamics and release mechanisms of adenosine in these processes.

Adenosine production, release, and regulation

Adenosine is both a building block and a degradation product of ATP. At rest, its intracellular concentration is kept low by being phosphorylated by adenosine kinase (ADK) or broken down to inosine by adenosine deaminase (ADA). However, breakdown of ATP during energy consumption or decreased energy production, such as in hypoxia, can lead to increased intracellular adenosine concentrations (Dunwiddie and Masino, 2001; Latini and Pedata, 2001; Fredholm, 2007). This intracellular adenosine can be released into the extracellular space or slow the clearance of extracellular adenosine by equilibrative nucleoside transporters (ENTs), which transport adenosine across the plasma membrane following the concentration gradient. When ATP is released to the extracellular space, it can be broken down into adenosine via evolutionarily conserved ectonucleotidases CD39 and CD73 (Sandau et al., 2016; Jacobson and Reitman, 2020).

The regulation of ATP release is still not fully understood and probably varies under different conditions. Possible mechanisms include co-release with other neurotransmitters via synaptic vesicles, ATP-permeable ion channels, and leakage after cell damage (Fredholm et al., 2011; Chen et al., 2013). Adenosine has also been suggested to undergo vesicular release by itself (Corti et al., 2013). Importantly, the spatiotemporal resolution of modern adenosine sensors has revealed that activity-dependent release of extracellular adenosine can occur at a local scale, suggesting a potential role of adenosine beyond volumetric transmission (Wu et al., 2023).

ENTs: Source or sink?

ENTs facilitate adenosine movement across the plasma membrane. They can limit the level of extracellular adenosine when intracellular adenosine concentrations are low, but can also reverse to provide a supply of extracellular adenosine when intracellular adenosine concentrations increase.

The role of ENTs has been studied, among other brain regions, in the striatum, where ENT1 is abundantly expressed (Anderson et al., 1999; Jennings et al., 2001), particularly on astrocytes (Peng et al., 2005). Studies measuring extracellular adenosine with either FSCV or the $GRAB_{Ado}$ sensor (Peng et al., 2020) either *in vivo* and *ex vivo* have found that the ENT1 inhibitor nitrobenzylthioinosine (NBTI) limits the clearance of extracellular adenosine, prolongs its extracellular lifetime (Nguyen et al., 2015; Adhikary et al., 2022; Roberts et al., 2022), and increases striatal tonic adenosine levels (Roberts et al., 2022), indicating that ENT1 in the striatum limits tonic extracellular adenosine levels through uptake. Notably, ENT1 uptake of adenosine is impaired by ethanol (Choi et al., 2004), a motor depressant.

On the other hand, ENT1 in the striatum might also contribute to adenosine release. Detection of evoked adenosine transients by imaging $GRAB_{Ado}$ in striatal slices indicated that, while an ENT1 inhibitor prolongs the lifetime of evoked adenosine transients, it also decreases their peak amplitude (Roberts et al., 2022). After application of the gliotoxin fluorocitrate to render astrocytes inactive, clearance of evoked extracellular adenosine levels was prolonged and peak adenosine levels were elevated, indicating that astrocytic ENT1 supports adenosine uptake, but not its release. This implicates non-astrocytic (presumably neuronal) ENT1 in adenosine release (Roberts et al., 2022), as seen elsewhere (Wu et al., 2023).

To date, the exact mechanism of striatal adenosine accumulation remains to be defined. Additional candidates include catabolism of extracellular ATP or cAMP (Wall and Dale, 2013; Adhikary et al., 2022; Ma et al., 2022) and vesicular exocytosis of adenosine or ATP (Klyuch et al., 2012; Corti et al., 2013). Notably, in neurons of the hippocampus and basal forebrain, the adenosine sensor HypnoS detects activity-dependent increases in intracellular adenosine levels, and blocking ENT1/2 elevates intracellular adenosine, both *in vitro* and *in vivo* (Wei et al., 2025). This suggests that ENTs mediate adenosine effluxes in certain brain regions.

Adenosine production by microglia

While neurons and astrocytes have long been recognized as the cellular sources of extracellular adenosine (Latini and Pedata, 2001; Pascual et al., 2005; Lovatt et al., 2012), recent work has identified microglia, the brain's resident immune cells, as a critical and previously underappreciated contributor to adenosine production (Matyash et al., 2017; Badimon et al., 2020). Microglial-produced adenosine plays a critical role in the modulation of neuronal activity and associated behavioral responses in mice, enabling microglia not only to sense and respond to

changes in neuronal activity but also to provide negative feedback control of neuronal activation (Badimon et al., 2020). Indeed, ablation of microglia amplifies and synchronizes the activity of neurons.

Suppression of neuronal activation by microglia occurs in a highly region-specific fashion and depends on the ability of microglia to sense and catabolize extracellular ATP/ADP, which is released upon neuronal activation by both neurons and astrocytes (Corkrum et al., 2020; Dale et al., 2023; Hatashita et al., 2023; Lalo and Pankratov, 2023). ATP detection by the high-affinity P2Y purinoceptor 12 (P2Y12), which is uniquely expressed in microglia, triggers rapid recruitment of microglial processes to active synapses (Haynes et al., 2006; Eyo et al., 2014; Sipe et al., 2016; Mo et al., 2019; Badimon et al., 2020; Cserép et al., 2022), and initiates a catabolic cascade wherein ATP is converted to AMP by the microglial ectonucleotidase CD39 (Badimon et al., 2020), and subsequently to adenosine by the more broadly expressed CD73 (Matyash et al., 2017; Badimon et al., 2020; Yang et al., 2020).

This microglia-specific ATP–adenosine pathway, coupled with the subsequent A_1R-mediated inhibition of neurons ((Badimon et al., 2020) and unpublished data), is essential for regulating local neuronal activity. Disruption of this pathway, via microglia ablation, genetic or pharmacological impairment of microglial ATP sensing and its downstream Gi signaling, AMP/adenosine production, or neuronal A_1R activation, leads to pathological neuronal hyperactivity and behavioral abnormalities (Badimon et al., 2020; Liu et al., 2021; Merlini et al., 2021). In addition, dysregulation of this pathway alters neurovascular coupling, affecting blood flow responses to neuronal activation (Fu et al., 2025). Collectively, these data reveal a fundamental role for microglia in maintaining the excitatory–inhibitory balance. This microglial activity-dependent feedback mechanism resembles inhibitory neuron functions, albeit with

markedly different spatiotemporal dynamics (Badimon et al., 2020; Pfeiffer and Attwell, 2020) that need to be fully elucidated.

The broader functional scope of the microglial ATP–adenosine pathway remains incompletely understood. Both adenosine and microglia have been suggested to contribute to brain state transitions, such as sleep–wakefulness and arousal (Porkka-Heiskanen et al., 1997; Bjorness et al., 2009). Another possibility is that the microglial ATP–adenosine pathway participates in the dynamic tuning of sensory gain under normal conditions (Giménez-Llort et al., 2005; Bayazitov et al., 2024). Overall, in a newly emerging framework, microglia, alongside neurons and macroglia, dynamically shape neuronal excitability thresholds through activity-dependent adenosine signaling. It is tempting to speculate that, by integrating systemic cues such as metabolic or inflammatory states with local circuit modulation, microglia may serve as a functional link between immune signals and brain function that has potential implications for decoding brain state transitions and for developing new therapeutic approaches targeting neuropsychiatric and neurodegenerative disorders.

Adenosine receptors and their downstream signaling

As mentioned earlier, adenosine functions through four G protein-coupled receptors: A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R. Each receptor exhibits distinct expression patterns, G-protein coupling profiles, and intracellular signaling pathways, thereby differentially shaping neuronal, glial, and vascular responses under physiological and pathological contexts.

A_1Rs

A_1Rs are the most evolutionarily conserved subtype of ARs and they exhibit the highest affinity for adenosine (Kd ~70 nM) (Daly and Padgett, 1992; Fredholm et al., 2000; Dunwiddie and

Masino, 2001; Stockwell et al., 2017). They are widely expressed across various tissues, with particularly high levels in the central nervous system, especially in the cortex, thalamus, hippocampus, cerebellum, and dorsal horn of the spinal cord. A_1Rs are also present in the olfactory bulb, striatum, mesencephalon, and retina (Mahan et al., 1991; Dixon et al., 1996; Fredholm et al., 2000, 2005; Schwarzschild et al., 2006). At the cellular level, A_1Rs are localized to both presynaptic and postsynaptic sites, where they play a pivotal role in modulating neuronal excitability (Rebola et al., 2003).

Upon activation, A_1Rs couple to inhibitory Gi/o proteins, leading to suppression of adenylyl cyclase activity and thus reduced cAMP production and PKA signaling (Calker et al., 1979). A_1Rs can also activate phospholipase C, leading to increased intracellular calcium and IP_3 signaling in certain cellular contexts (Chen et al., 2013). These downstream pathways activate pertussis toxin-sensitive K^+ channels, as well as K_{ATP} channels, and inhibit Q-, P-, and N-type Ca^{2+} channels, resulting in neuronal hyperpolarization and suppression of neurotransmitter release (Fredholm et al., 2000; Jacobson and Gao, 2006).

In several regions of the central nervous system (CNS), including the striatum and the spinal cord, A_1R can form heteromers with dopamine D1 receptors (D1Rs) (Shen et al., 2013; Cortés et al., 2019; Rivera-Oliver et al., 2019). In these heteromers, A_1R activation antagonizes dopaminergic transmission by desensitizing D1Rs, reducing dopamine binding to D1R, and thus decreasing activation of adenylyl cyclases without the involvement of G-protein activation (Shen et al., 2013; Cortés et al., 2019; Ferré et al., 2019; Rivera-Oliver et al., 2019). A_1R–D1R heteromers may be therapeutic targets that provide unique advantages in several diseases, such as Parkinson's disease, attention-deficit hyperactivity disorder (ADHD), Restless Legs Syndrome, and spinal cord injury (Cortés et al., 2019).

A_{2A}Rs

A_{2A}Rs also exhibit high affinity (Kd~150 nM) for adenosine, although slightly lower than that of A_1Rs (Dixon et al., 1996; Dunwiddie and Masino, 2001). Within the brain, they are highly expressed in the striatum, particularly on iSPNs, and they are also enriched in the olfactory tubercle. Lower levels of A_{2A}R are present in the cortex, thalamus and hippocampus (Dixon et al., 1996; Rosin et al., 1998). A_{2A}Rs activate the adenylyl cyclase–cAMP–PKA pathway. While this was originally attributed to the coupling with Gs proteins, studies have shown that A_{2A}Rs primarily couple to Golf proteins in the striatum, and may also involve the βγ7 subunit of G proteins in certain contexts (Schwindinger et al., 2010). Beyond the canonical cAMP–PKA signaling pathway, A_{2A}Rs can engage in PKC-dependent pathways, particularly in the hippocampus (Cunha and Ribeiro, 2000; Pinto-Duarte et al., 2005). A_{2A}R can also form functional heteromers with D2Rs in the SPNs of the basal ganglia that are pharmacologically distinct from monomeric receptors (Ferré et al., 1997; Hillion et al., 2002; Fuxe et al., 2007; Bonaventura et al., 2015). Similar to A_1R–D1R heteromers, A_{2A}R–D2R heteromers engage in antagonistic allosteric interactions and modulation of downstream signaling.

A_{2A}Rs interact with and regulate diverse signaling cascades through direct and/or signaling interactions with other receptors, including A_1Rs (O'Kane and Stone, 1998; Ribeiro, 1999; Ciruela et al., 2006), D1Rs and D2Rs (Ferré et al., 1997; Hillion et al., 2002), metabotropic glutamate receptor 5 (mGlu5Rs) (Ferré et al., 2002; Tebano et al., 2005), and N-methyl-D-aspartate receptors (NMDARs) (Nörenberg et al., 1998; Ribeiro, 1999). A_{2A}R activation also influences neurotrophic factor signaling in the striatum (Flajolet et al., 2008; Fontinha et al., 2008; Tebano et al., 2008). An example of such signaling interaction occurs in the striatum, where A_{2A}R activation during mouse locomotion dominates the effect of dopamine

on D2R-expressing iSPNs, resulting in increased downstream PKA activity in these neurons (Ma et al., 2022).

Through these various signaling pathways, A_{2A}Rs play pivotal roles in regulating motor control, motivation, psychiatric behaviors, the sleep-wake cycle, synaptic plasticity, neuronal survival, inflammation, myocardial oxygen consumption, coronary blood flow, angiogenesis, and cancer pathogenesis (Fuxe et al., 2007; Wei et al., 2011; Bonaventura et al., 2015).

A_{2B}Rs

A_{2B}Rs are characterized by a low affinity for adenosine (Kd~5100 nM) (Peakman and Hill, 1994; Dunwiddie and Masino, 2001). They are therefore generally thought to require pathophysiological concentrations of adenosine, activated primarily during conditions of cellular stress, inflammation, and ischemia (although Theparambil et al., 2024 described its role under physiological conditions). A_{2B}R is broadly, but sparsely, expressed in diverse types of cells in the CNS, including astrocytes and neurons (Dixon et al., 1996; Sun and Huang, 2016). A_{2B}R signaling involves dual coupling to Gs and Gq proteins, simultaneously increasing cAMP and activating Phospholipase C (PLC) (Peakman and Hill, 1994; Gao et al., 1999; Linden et al., 1999). A_{2B}R-mediated signaling plays a significant role in gliovascular coupling mechanisms, astrocyte function, cerebral blood flow regulation, and neuronal survival after ischemic events. Therapeutically, targeting A_{2B}R signaling pathways has potential for managing neuroinflammatory diseases, ischemic stroke and chronic pain due to its central role in tissue adaptation to stress and inflammation.

A_3Rs

A_3Rs also exhibit low affinity for adenosine (Kd~6500 nM) (Dunwiddie and Masino, 2001), as well as low expression in the CNS, except within microglia and astrocytes (Hammarberg et al., 2003; Björklund et al., 2008). A_3Rs activate Gi/o to inhibit adenylyl cyclases, thereby reducing cAMP production and PKA activity. In some contexts, they are coupled to Gq to activate PLC, resulting in activation of PKC and mobilization of intracellular calcium (Palmer et al., 1995; Merighi et al., 2003; Haskó and Cronstein, 2004). Activation of A_3R has also been suggested to engage multiple kinases other than PKA and PKC to influence diverse cellular processes, including cardioprotection, neuroprotection, anti-inflammatory responses, and tumor growth regulation, depending on different cell types and pathophysiological states (Merighi et al., 2003; Haskó and Cronstein, 2004; Borea et al., 2015). While the expression level of A_3R is thought to be low in the brain, its global knockout surprisingly resulted in notable brain phenotypes, such as increased neuronal death and decreased cognitive function during hypoxia (Fedorova et al., 2003; Yang et al., 2010).

Physiological functions of adenosine at A_1 and A_{2A} receptors

It is increasingly appreciated that the concentration of adenosine is dynamically regulated to control neuronal excitability and neurotransmitter release. As mentioned earlier, the high-affinity A_1R and A_{2A}R are the most abundant adenosine receptor types in the brain, where they are located on diverse neurons (Benarroch, 2008) as well as in astrocytes, oligodendrocytes and microglia (Haskó et al., 2005). A_1Rs and A_{2A}Rs inhibit and activate the cAMP–PKA pathway, respectively, to oppositely affect target cells (Benarroch, 2008; Ferré et al., 2023). One of the brain regions where this has been extensively studied is the striatum, where both A_1Rs and A_{2A}Rs are expressed and play critical roles in modulating basal ganglia function.

A_1R action on neurons in the striatum

A_1Rs are expressed by striatal cells, including GABAergic, D1R-expressing direct-pathway SPNs (dSPNs) and cholinergic interneurons (ChIs), as well as in the axonal terminals of glutamatergic corticostriatal and thalamostriatal inputs and dopaminergic mesostriatal axons (Ferré et al., 1996; Song et al., 2000; Borycz et al., 2007; Fritz et al., 2021). In the NAc of the ventral striatum, adenosine has been shown to provide a tonic level of A_1R-mediated inhibition of glutamatergic, GABAergic, and dopaminergic transmission (Solinas et al., 2002; Quarta et al., 2004a, 2004b; Borycz et al., 2007; Roberts et al., 2022). Furthermore, A_1R activation hyperpolarizes ChIs and subsequently reduces acetylcholine (ACh) release (Brown et al., 1990; Preston et al., 2000; Song et al., 2000).

A notable function of A_1R in the striatum is to control local dopamine release. *In vivo* microdialysis studies find that systemic administration of A_1R antagonists increased extracellular dopamine levels in the striatum (Okada et al., 1996; Solinas et al., 2002; Quarta et al., 2004a, 2004b). A local effect of A_1Rs has been observed in the dorsolateral striatum in brain slices, where they inhibit electrically-evoked dopamine release detected with FSCV (Ross and Venton, 2015). Similarly, in the NAc, a specific A_1R agonist and an antagonist respectively reduce and enhance dopamine release, evoked electrically or optogenetically (Roberts et al., 2022). These effects are independent of GABA, ACh and glutamate transmission (Borycz et al., 2007; Roberts et al., 2022), suggesting that adenosine acts at A_1Rs located directly on dopamine axons. Notably, the effect of the A_1R antagonist suggests a basal or tonic level of A_1R activity in the NAc. Furthermore, A_1R-mediated inhibition of dopamine release is stronger at lower stimulation frequencies (Roberts et al., 2022), which aligns with the hypothesis that A_1Rs preferentially

influence dopamine functions that are mediated by low/tonic activity, such as ongoing monitoring of reward value (Wang et al., 2021).

A_{2A}R action in the striatum

In contrast to A1Rs, A_{2A}Rs in the striatum are largely restricted to glutamatergic corticostriatal inputs and D2R-expressing iSPNs (Fink et al., 1992; Schiffmann et al., 1993; Quiroz et al., 2009). On glutamatergic inputs, A_{2A}Rs are colocalized with A_1Rs, but they mediate opposite effects (Ferré et al., 2023). *In vivo* microdialysis studies show that local administration of an A_{2A}R agonist or antagonist, respectively, increases or decreases extracellular glutamate levels in the NAc, and the agonist's ability to increase dopamine release can be blocked by co-perfusion with an NMDA receptor antagonist (Quarta et al., 2004a, 2004b), indicating that A_{2A}R effects on dopamine are indirectly mediated via upstream actions on glutamate transmission. Additionally, A_{2A}Rs antagonistically interact structurally and functionally with D2Rs in iSPNs (Schiffmann et al., 2007). A similar antagonistic interaction also occurs between A_1Rs and D1Rs in dSPNs.

Collectively, the interplay between A_1/A_{2A}Rs and D1/D2Rs supports a close modulation of basal ganglia output and motor behavior by adenosine and dopamine. These interactions may contribute to motor effects of adenosine receptor ligands, which span from depressant effects of agonists to stimulant effects of antagonists (e.g., caffeine) and their utility in therapeutics (Jacobson et al., 2022).

Adenosine's translational potential

As mentioned earlier, adenosine and its signaling pathway have long been recognized for their potential for drug development. Caffeine, the most widely used psychostimulant, largely functions as an adenosine receptor antagonist (Jacobson et al., 2022). The A_{2A}R antagonist

istradefylline has recently been approved for treating Parkinson's disease, and there are multiple clinical trials for treating tumors (Chen et al., 2013; Jacobson et al., 2022). Below, we highlight recent progress regarding the potential of targeting adenosine signaling in treating Alzheimer's disease and in enhancing sleep and glymphatic activity.

Adenosine production and synaptic actions in aging and Alzheimer's disease

Synaptic dysfunction is a hallmark of early-stage Alzheimer's disease; in fact, aberrant glutamatergic transmission and impaired synaptic plasticity precede neuronal loss (Palop and Mucke, 2010). Evidence is accumulating that imbalanced adenosine receptor signaling may contribute to the synaptic dysfunction observed in the hippocampus and other brain regions (reviewed in (Temido-Ferreira et al., 2019)).

Adenosine plays a central role in modulating glutamate release and synaptic function in the hippocampus. It is co-released with neurotransmitters during neuronal activity (Temido-Ferreira et al., 2019), and subsequently activates both A_1Rs and A_{2A}Rs. A_1Rs generally act to suppress neuronal excitability and inhibit glutamate release. In contrast, A_{2A}Rs promote synaptic plasticity and facilitate neurotransmitter release (Ribeiro et al., 2003; Fredholm et al., 2005; Temido-Ferreira et al., 2019; Shigetomi et al., 2023), indicating a bidirectional modulatory system.

Disruption of the balance between A_1R and A_{2A}R signaling, particularly that resulting from increased expression and overactivation of A_{2A}Rs in cortical regions, is linked to cognitive deficits in aging and Alzheimer's disease (Temido-Ferreira et al., 2020). In aging, cortical levels of the ectonucleotidase CD73 increase (Sacramento et al., 2019), which is correlated with elevated extracellular adenosine levels (Sebastião et al., 2000; Mackiewicz et al., 2006) and may

differentially affect the two receptors. Furthermore, a particular single nucleotide polymorphism (SNP) of the gene encoding $A_{2A}R$ in the human population is associated with hippocampal atrophy and Alzheimer's disease (Horgusluoglu-Moloch et al., 2017). Additionally, functional imaging in aged humans shows increased hippocampal activity (Yassa et al., 2011), and $A_{2A}R$ transcription and translation can be enhanced by elevated neuronal activity (Canas et al., 2018). Transgenic mice lacking ADK, which increases extracellular adenosine levels, display memory impairments, increased $A_{2A}R$ activity, and reduced A_1R expression (Sandau et al., 2016). While the regulation of the $A_{2A}R$ gene is not fully understood, particularly during aging (Le et al., 1996; Lee et al., 1999), *in vitro* studies indicate that its expression is influenced by transcription factors (Buira et al., 2010), cytokines (Morello et al., 2006), microRNAs (Villar-Menéndez et al., 2014), and DNA methylation (Villar-Menéndez et al., 2013).

Other forms of early aging, such as models of sustained stress, also lead to long-lasting upregulation of hippocampal $A_{2A}Rs$, along with impaired glucocorticoid receptor (GR) signaling in adulthood (Batalha et al., 2013; Kaster et al., 2015). Remarkably, pharmacological blockade of $A_{2A}Rs$ in animal models of maternal separation reversed long-term potentiation (LTP) deficits and normalized corticosterone rhythms and GR levels, indicating that early-life stress primes the hippocampus for aging-like changes through persistent $A_{2A}R$ overactivation (Batalha et al., 2016). Notably, overexpression of $A_{2A}R$ alone was sufficient to shift synaptic plasticity from long-term depression (LTD) to LTP in young mice, and this effect was mediated by enhanced glutamatergic activation of NMDA receptors and calcium influx (Temido-Ferreira et al., 2020). Complementarily, a genetic model of $A_{2A}R$ overexpression restricted to forebrain neurons in young-adult rats recapitulated key features of brain aging, including flattened circadian corticosterone oscillations, reduced hippocampal GR expression, altered synaptic excitability,

and memory impairments (Batalha et al., 2013). These findings underscore that excess $A_{2A}R$ activity is not merely a consequence of aging, but can act as a causal factor, driving synaptic and hormonal changes that mirror those observed in aged brains.

The effects of adenosine on aging and Alzheimer's disease may be region-specific. In the striatum of a mouse model of Alzheimer's disease, microglial ATP-to-adenosine processing enzymes are down-regulated (Badimon et al., 2020), along with concurrent neuronal hyperexcitability and sleep disturbances (Brzecka et al., 2018). Regardless of the specific change, dysregulated adenosine production or receptors are observed in aging and Alzheimer's disease, which may perturb the finely tuned balance between A_1R-mediated inhibition and $A_{2A}R$-mediated facilitation critical for normal function.

Adenosine augmentation therapy for sleep and glymphatic activity

As discussed earlier, adenosine signaling has long been recognized for its anticonvulsant, neuroprotective and somnogenic effects (Chen et al., 2013; Cunha, 2016; Borea et al., 2018). Extracellular adenosine levels increase in the basal forebrain during the wake-sleep transition (Peng et al., 2020) and possibly underlies prolonged wakefulness-induced rebound sleep (Porkka-Heiskanen et al., 1997). Furthermore, artificially increasing extracellular adenosine levels by pharmacological inhibition of enzymes involved in adenosine metabolisms (King et al., 2006; Oishi et al., 2008; Boison, 2011) or by optogenetic stimulation of glutamatergic neurons in the basal forebrain (Peng et al., 2020) produces a somnogenic effect. In addition, a positive allosteric modulator (PAM) of $A_{2A}Rs$ has been found to promote sleep in mice (Korkutata et al., 2019; Lin et al., 2023). Furthermore, the arousing effect of caffeine is largely attributed to its inhibition of $A_{2A}Rs$ (Huang et al., 2005; Lazarus et al., 2011). These studies suggest the potential

of enhancing adenosine signaling for treating insomnia. However, the development of systemic adenosine-enhancing drugs, such as ADK inhibitors, has largely been aborted due to risks of toxicity and intolerable side effects, such as hypotension (Boison, 2013). A key challenge is to selectively increase adenosine levels in the brain without affecting the widespread distribution of adenosine receptors in peripheral tissues and vasculatures.

A recent study discovered that stimulation with a 40-Hz light flickering light produced an immediate (within minutes), robust and sustained (lasting for 2.5 hours after cessation of light exposure) elevation of extracellular adenosine levels in the mouse visual cortex (Zhou et al., 2024). This effect was frequency- and light intensity-dependent and was brain region-specific. Cortical glutamatergic and GABAergic neurons, but not astrocytes, were found to be the cellular source of this adenosine, with the trans-membrane transport by ENT2 likely being a critical release mechanism (Zhou et al., 2024). 40-Hz flicker stimulation for 30 min increased REM and NREM sleep amounts by approximately 2-fold, which lasted for 3 hours. This somnogenic effect was abolished by genetic deletion of ENT2, but was dose-dependently recaptured by focal injection of adenosine into the visual cortex. Lastly, visual flicker stimulation (30 min prior to sleep) improved sleep in young insomnia patients by reducing sleep latency and increasing total sleep time and sleep efficiency (Zhou et al., 2024). Thus, our study has established a novel adenosine augmentation therapy for insomnia, in which 40-Hz flicker light-induced rapid and sustained increase of the extracellular adenosine level in the visual cortex via ENT2-mediated adenosine efflux.

It was also discovered that 40-Hz flickering light stimulation enhances glymphatic activity via adenosine signaling (Sun et al., 2024). The glymphatic system is increasingly recognized for its critical role in clearing pathogenic proteins in neurodegenerative diseases.

Impaired glymphatic circulation during disease pathogenesis may lead to deterioration of brain function (Da Mesquita et al., 2018; Harrison et al., 2020; Si et al., 2024). 40-Hz flickering light stimulation for 30 min robustly enhanced glymphatic influx- and efflux- circulations in a frequency-dependent manner in wild-type mice. This was confirmed by both fluorescence tracing in brain slices *in vitro* and dynamic contrast-enhanced magnetic resonance imaging (MRI) *in vivo* (Sun et al., 2024). Importantly, adenosine serves as an essential molecular messenger in linking the 40-Hz stimulation with enhanced glymphatic activity: the ENT1/2 inhibitor dipyridamole or knockout of ENT2 or aquaporin-4 each abolished the enhancement of glymphatic activity (Sun et al., 2024). Taken together, adenosine augmentation therapy by 40 Hz light flicker may also serve as a non-invasive strategy for enhancing glymphatic clearance of brain metabolites, with translational potential to relieve brain disorders.

Summary and Outlook

Recent work has greatly advanced our understanding of adenosine biology and pathology, in part thanks to advances in sensing technology and modern pharmacological and genetic tools. These novel technologies combined with advanced molecular biology, electrophysiology and behavioral pharmacology studies have led to i) molecular dissection of the complex mechanisms underlying adenosine release, uptake and degradation; ii) discovery of moment-by-moment regulation of adenosine concentrations by neuronal activity and animal behavior; iii) the development of a new framework involving interplay among microglia, astrocytes and neurons; and iv) novel avenues for translational applications.

It is increasingly clear that adenosine accumulation is more prevalent and dynamic than previously appreciated. Our understanding of its role and potential in translation is only at the

beginning. It is likely that the production, release, and functional role of adenosine vary spatially and temporally within the CNS. For example, neurons and astrocytes have both been suggested to be the source of adenosine in different studies. Furthermore, altered intracellular and extracellular adenosine concentrations may affect adenosine flow and possibly its function. It is likely that different brain regions have different levels of adenosine and engage with different release and clearance mechanisms. Development of methods that can quantify adenosine dynamics with higher signal-to-noise ratios and sensors that can quantify the absolute adenosine concentration may help address some of these issues and further reveal adenosine signaling heterogeneity. Other modern technologies, such as brain-wide cell-specific gene expression profiling, may also provide insights into the cell-specific regulation and function of proteins involved in adenosine signaling and thereby provide new avenues for targeted therapeutic applications.

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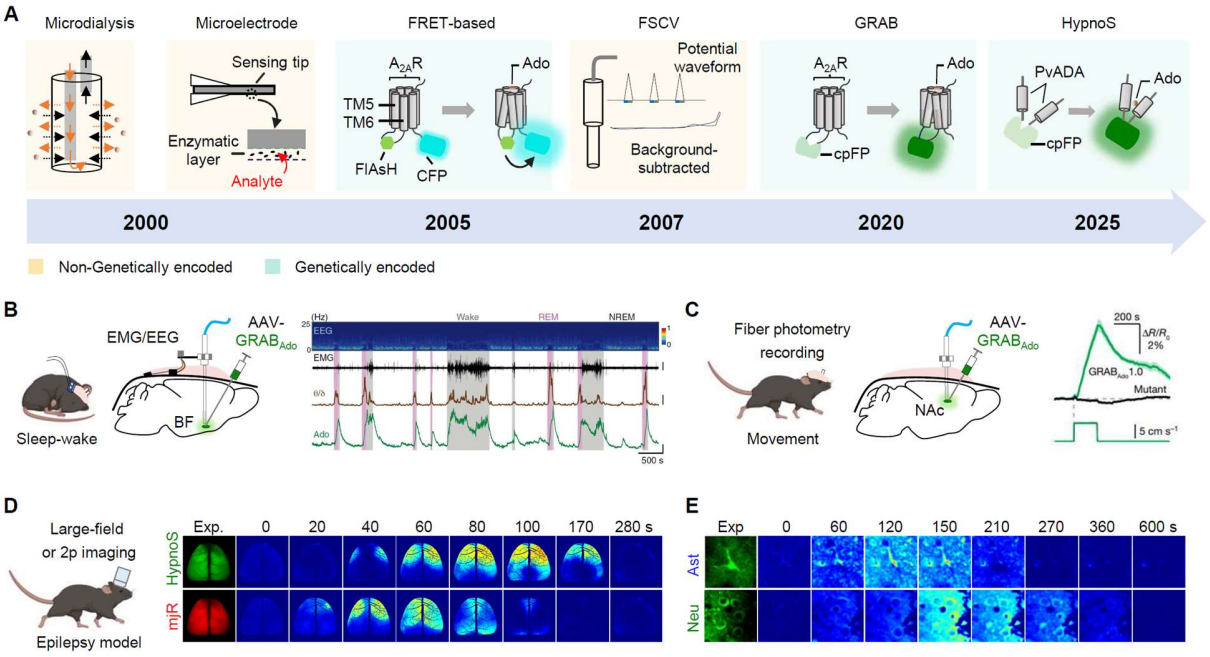

Figure 1. Principles and application examples of methods for detecting adenosine. *A,* History of tool development for detecting adenosine. FRET, fluorescence resonance energy transfer; FSCV, fast-scan cyclic voltammetry; GRAB, G protein-coupled receptor-activation based; PvADA, *Plasmodium vivax* adenosine deaminase; HypnoS, Hypersensitive intracellular adenosine Sensor. *B,* Left: $GRAB_{Ado1.0}$ expressed in the basal forebrain (BF) of fiber-attached mice, driven by the synapsin promoter via an AAV, to monitor eADO dynamics in sleep/wake cycles. Right: (from top to bottom) EEG power spectrogram; electromyogram (EMG); ratio of EEG theta power (θ) to delta power (δ); $GRAB_{Ado1.0}$ fluorescence (Peng et al., 2020). *C*, Left: $GRAB_{Ado1.0}$ expressed in the nucleus accumbens (NAc) of fiber-attached mice, driven by the synapsin promoter via an AAV, to monitor eADO dynamics during running. Right: (from top to bottom) $GRAB_{Ado1.0}$ fluorescence and running speed (Ma et al., 2022). *D*, HypnoS and mjRGECO1a (mjR) expressed in the whole brain of head-fixed mice, driven by the synapsin promoter via an AAV; large-field imaging of dorsal cortical iADO and calcium dynamics during kainic acid (KA)-induced epileptic states (Wei et al., 2025). Exp., expression. *E*, HypnoS expressed in the motor cortex of head-fixed mice, driven by the synapsin or GfaABC1D promoters via an AAV; 2-photon imaging of iADO dynamics during KA-induced epileptic states.