Published in final edited form as:

J Neurochem. 2015 January ; 132(1): 51–60. doi:10.1111/jnc.12946.

Adenosine transiently modulates stimulated dopamine release in the caudate putamen via A₁ receptors

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Abstract

Adenosine modulates dopamine in the brain via A₁ and A_{2A} receptors, but that modulation has only been characterized on a slow time scale. Recent studies have characterized a rapid signaling mode of adenosine that suggests a possible rapid modulatory role. Here, fast-scan cyclic voltammetry was used to characterize the extent to which transient adenosine changes modulate stimulated dopamine release (5 pulses at 60 Hz) in rat caudate putamen brain slices. Exogenous adenosine was applied and dopamine concentration monitored. Adenosine only modulated dopamine when it was applied 2 or 5 s before stimulation. Longer time intervals and bath application of 5 μ M adenosine did not decrease dopamine release. Mechanical stimulation of endogenous adenosine 2s before dopamine stimulation also decreased stimulated dopamine release by $41 \pm 7\%$, similar to the $54 \pm 6\%$ decrease in dopamine after exogenous adenosine application. Dopamine inhibition by transient adenosine was recovered within 10 minutes. The A₁ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) blocked the dopamine modulation, whereas dopamine modulation was unaffected by the A_{2A} receptor antagonist SCH 442416. Thus, transient adenosine changes can transiently modulate phasic dopamine release via A₁ receptors. These data demonstrate that adenosine has a rapid, but transient, modulatory role in the brain.

Keywords

adenosine; dopamine; neuromodulator; A₁ receptor; fast-scan cyclic voltammetry; caudate putamen

Introduction

Adenosine has been traditionally studied as it builds up slowly in the extracellular space (Cunha 2008;Latini and Pedata 2001), however rapid changes in adenosine have been recently characterized. Amperometric biosensors and fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes have been used to demonstrate that adenosine is released and cleared on fast time scales, from a few seconds to a minute (Cechova and Venton 2008;Pajski and Venton 2013;Klyuch *et al.* 2012). Transient adenosine release can be caused by hypercapnia (Dale 2006) or hypoxia (Dale *et al.* 2000) and has also been

characterized during epilepsy (Dale and Frenguelli 2009). *In vivo* and in brain slices, rapid adenosine release can be stimulated using electrical (Pajski and Venton 2013) or mechanical stimulation (Ross *et al.* 2014a). Street *et al.* discovered rapid adenosine transients in spinal cord slices using FSCV, which did not require a stimulus (Street *et al.* 2011) and our lab characterized spontaneous, transient adenosine release in the prefrontal cortex and caudate putamen of anesthetized rats that lasted only 3 seconds (Nguyen *et al.* 2014). However, the function of these transient adenosine changes to modulate neurotransmission on a rapid time scale has not been studied.

On the minute to hour time scale, adenosine modulates two important processes in the brain: cell metabolism (Cunha 2001; Newby *et al.* 1985) and neurotransmission (Ferre *et al.* 1997; Ferre *et al.* 1992; Quarta *et al.* 2004; Okada *et al.* 1996). Adenosine modulates basal levels of many neurotransmitters, including dopamine, serotonin, glutamate, and GABA via A₁ and A_{2a} receptors (Ferre *et al.* 1997; Ferre *et al.* 1992; Quarta *et al.* 2004; Okada *et al.* 1996; Sperlagh and Vizi 2011) and has been shown to modulate stimulated release of acetylcholine (Pedata *et al.* 1983) and glutamate (Corsi *et al.* 1999b) and GABA (Corsi *et al.* 1999a) *in vivo*. A₁ is the most abundant adenosine receptor in the brain and inhibits neurotransmission by blocking adenylyl cyclase activity while A_{2A} is the second most abundant adenosine receptor in the brain and has an excitatory effect, activating adenylyl cyclase activity (Cunha 2008). Specifically, adenosine modulates basal dopamine levels in the caudate putamen (Okada *et al.* 1996) and the nucleus accumbens (Quarta *et al.* 2004); however, the effect was slow and changes in basal levels were not recorded until 20 minutes after adenosine was applied. Quarta *et al.* found both A₁ and A_{2A} receptors regulate dopamine release but their effect is secondary to glutamate modulation which depends on stimulation of NMDA receptors in the nucleus accumbens (Quarta *et al.* 2004). In other studies, the inhibitory effects of the A₁ receptor was shown to overpower the excitatory effects of A_{2A} receptor activation, and consequently the effect of A_{2A} receptor antagonists and agonists were masked in the presence of activated A₁ receptors (Okada *et al.* 1996). These studies all looked at the effect of basal changes in adenosine on basal dopamine levels. However, dopamine neurons have two firing patterns: slow, tonic firing which produces basal levels and rapid, phasic firing which results in transient dopamine release (Grace 1991). The effect of transient adenosine to modulate phasic dopamine release has not been investigated.

In this study, we tested the hypothesis that transient adenosine release modulates phasic dopamine release. Adenosine was exogenously applied to slices of the caudate putamen to mimic adenosine transients and time varied between adenosine application and stimulation of phasic dopamine release. Alternatively, transient adenosine release was mechanically evoked (Ross *et al.* 2014). Adenosine temporarily inhibits dopamine release if it is applied 2–5 s before the stimulation but perfusing the slice with 5 μ M adenosine for 30 minutes did not affect stimulated dopamine release. A₁ receptors mediated the transient effects of adenosine but A_{2a} receptors did not. Thus, transient changes in adenosine causes temporary inhibition of dopamine release in the caudate putamen via A₁ receptors, proving that adenosine can modulate dopamine on a rapid time scale.

Methods

Chemicals

All chemicals were from Fisher Scientific (Fair Lawn, NJ, USA) unless otherwise stated. Adenosine and dopamine were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.1 M HClO₄ for 10 mM stock solutions. Stock solutions were diluted daily in artificial cerebral spinal fluid (aCSF) to 1 μ M for electrode calibrations for brain slice experiments. The aCSF consisted of 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 2.4 mM CaCl₂ dehydrate, 1.2 mM MgCl₂ hexahydrate, 25 mM NaHCO₃, 11 mM glucose, and 15 mM tris (hydroxymethyl) aminomethane and was adjusted to pH 7.4 every day. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma) and SCH 442416 (Tocris Biosciences, Ellisville, MO) stock solutions were made in dimethylsulfoxide (DMSO) and diluted in aCSF on the day of the experiment.

Electrochemistry

Cylinder microelectrodes were made from carbon-fibers as previously described (gift from Cytec Engineering Materials, West Patterson, NJ) (Ross and Venton 2012) and were 50 μ m long and 7 μ m in diameter. Fast-scan cyclic voltammetry (FSCV) was used with carbon-fiber microelectrodes. Cyclic voltammograms (CVs) were collected using a ChemClamp (Dagan, Minneapolis, MN). Data was collected using Tarheel CV software (gift of Mark Wightman, UNC) using a homebuilt data analysis system and two computer interface boards (National Instruments PCI 6052 and PCI 6711, Austin TX). The electrode was scanned from -0.4 V to 1.45 V (vs Ag/AgCl) and back with a scan rate of 400 V/s and a repetition rate of 10 Hz. Electrodes were pre-calibrated for both 1 μ M dopamine and 1 μ M adenosine in aCSF prior to the experiment using flow injection analysis. Electrodes were equilibrated for 30 min in tissue with the waveform being applied before measurements taken. Cyclic voltammograms which exhibited large amount of high frequency noise were filtered to remove the noise using Origin Pro (CVs for Figure 2, 4, and 5C).

Brain slice experiments

All animal experiments were approved by the Animal Care and Use Committee of the University of Virginia. Male Sprague-Dawley rats (250–350 g, Charles River, Wilmington, MA) were housed in a vivarium and given food and water *ad libitum*. Rats were anesthetized with isoflurane (1 mL/100 g rat weight) in a desiccator and beheaded immediately. The brain was removed within 2 min and placed in 0–5°C aCSF for 2 min for recovery. Four hundred micrometer slices of the caudate putamen were prepared using a vibratome (Leica VT1000S, Bannockburn, IL), transferred to oxygenated aCSF (95% oxygen, 5% CO₂), to recover for an hour before the experiment. aCSF (maintained at 35–37°C) flowed over the brain slices using a pump (Watson-Marlow 205U, Wilmington, MA) at a rate of 2 mL/min for all experiments. The medial caudate putamen was tested and the coordinates are approximately (in mm from bregma): +1.2 anterior-posterior, +2.0 mediolateral, and -5.5 dorsoventral. The carbon-fiber electrode was inserted 75 μ m into the tissue of the medial caudate putamen (see Figure S1 for diagram). Dopamine was stimulated using a biphasic stimulating electrode, with wires spaced 800 μ m apart and placed 300 μ m from the working electrode (PlasticsOne, Inc., Roanoke, VA). Two stimulations of

dopamine, 10 minutes apart, were performed prior to exogenous or endogenous adenosine application to ensure signal stability. Previously, our lab provided evidence that the medial caudate putamen does not produce as much adenosine as other regions of the caudate (Pajski and Venton 2010); however, if stimulated adenosine was detected, experiments were not performed in that location of the medial caudate to minimize interference from stimulated adenosine release. Pulse trains of 5 biphasic pulses, each 200 μ A and 4 ms long (2 ms per phase), were applied at a frequency of 60 Hz using a stimulus isolator (Dagan). For exogenous application of adenosine experiments, 25 μ M adenosine was pressure ejected onto brain slices either 2, 5, 10, 30, or 60 s prior to dopamine stimulation using a Parker Hannifin picospritzer (Picospritzer III, Cleveland, OH). Each pressure ejection time before dopamine stimulation was completed in a separate set of slices. The picospritzing pipette was placed 20–30 μ m from the working electrode. Low pressures and short puff-times were used to minimize tissue damage from the pressure application. The ejection parameters were 10 psi for 50–100 milliseconds and 100–200 nL of 25 μ M adenosine was delivered (2.5–5.0 pmol). High concentrations of adenosine are needed with exogenous application in order to detect any at the carbon-fiber microelectrode due to diffusion and rapid uptake. For mechanical stimulation of adenosine in slices, an empty pulled glass pipette (approximately 15–20 μ m) was inserted into the slice 20–30 μ m from the working electrode, and lowered 50 μ m with a micromanipulator 2 s prior to dopamine stimulation. Background subtraction was taken 1 s before dopamine which subtracts out much of the adenosine, to clearly see the dopamine response.

Pharmacology experiments

For pharmacology experiments, two pre-drug dopamine stimulations were collected, followed by perfusion of either 200 nM 8-Cyclopentyl-1,3-dipropylxanthine, DPCPX, (Sigma Aldrich, St. Louis MO) or 1 μ M SCH 442416 (Tocris Biosciences, Ellisville MO) for 30 minutes. Dopamine was stimulated to measure if the drugs affected stimulated dopamine release. Next, adenosine was either exogenously applied or mechanically stimulated 2 s prior to the next dopamine stimulation (10 minutes later) in the presence of the drug. As a control, dopamine stimulations were repeated without drug to make sure dopamine is stable over the length of the experiment.

Statistics

All values are reported as the mean $\pm$ standard error of the mean (SEM). Paired t-test were performed to compare the initial dopamine stimulation to the dopamine stimulation immediately after exogenous adenosine application or after mechanical adenosine stimulation. Paired t- test were also performed to compare initial dopamine stimulation versus the last stimulation within each set of control experiments. One-way ANOVA with Bonferroni post-tests was used to assess the overall effect of adenosine on dopamine concentration after either exogenous adenosine application, mechanically evoked, or in the presence of either DPCPX or SCH 442416. All statistics were performed in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) and considered significant at the 95% confidence level ($p < 0.05$).

Results

FSCV allows the electrochemical monitoring of electroactive species on the millisecond timescale (Keithley *et al.* 2011; Venton *et al.* 2003). Both adenosine and dopamine are electroactive and can be co-detected with FSCV (see supplemental Figure S2 for an example color plot and cyclic voltammograms) (Swamy and Venton 2007; Cechova *et al.* 2010). Additionally, the presence of adenosine does not interfere with dopamine detection (see Figure S3). Adenosine undergoes a series of two electron oxidations (Ross and Venton 2012) at carbon-fiber microelectrodes (primary oxidation at 1.40 V and secondary oxidation at 1.0 V) while dopamine has one oxidation peak at 0.6 V and a reduction peak at -0.2 V (Robinson *et al.* 2003). Oxidation of adenosine requires a higher switching potential than dopamine, so the waveform is scanned from -0.4 to 1.45 V and back at 400 V/s and a 10 Hz repetition rate (Cechova *et al.* 2010).

To study dopamine modulation, exogenous adenosine was applied to the slice at different intervals before dopamine release was stimulated. Figure 1A shows a schematic of the electrode, stimulating electrode, and picospritzing pipette placement in the caudate-putamen. The example 3-dimensional color plot shows adenosine and dopamine are both detected (Fig. 1B). The applied waveform voltage is on the y-axis, time is on the x-axis, and current is in false color. The green color represents oxidative current and the cyclic voltammograms also help identify the analytes. Background subtraction was taken 1 s before dopamine to subtract out much of the adenosine response in order to clearly see dopamine. Stimulated dopamine release lasted on average between 1.5–3 s and was 350–600 nM which is of similar duration and concentration as behaviorally-evoked dopamine transients previously studied in the brain (Robinson and Wightman 2007). Here, 5 pmol of adenosine was exogenously applied 30 seconds prior to dopamine stimulation, which resulted in 1.7 μ M of adenosine detected at the electrode that lasted 7.3 s. On average, the concentration of adenosine detected at the electrode was 1.0 ± 0.1 μ M and lasted 4.6 ± 0.3 s ($n = 19$), which is within range of larger spontaneous adenosine release *in vivo* (Nguyen *et al.* 2014).

Exogenously-applied, transient adenosine modulates dopamine on a rapid time scale

To test the effect of transient adenosine release on stimulated dopamine levels, the time interval was varied between exogenous application of adenosine and dopamine stimulation. In all experiments, dopamine stimulations were performed twice, 10 minutes apart, before adenosine application to ensure the dopamine signal was stable. Phasic dopamine release was stimulated by 5 pulses at 60 Hz and stimulated dopamine was normalized to the initial stimulations in each animal to account for variation between animals. Electrical stimulation causes an immediate release of dopamine that is detected within 0.5–1 s of stimulation. Figure 2A compares stimulated dopamine release 10 min before adenosine, 2 s after adenosine was applied, and 10 min after adenosine was applied. Two seconds after adenosine was applied, stimulated dopamine release was half of the initial or the recovery peak. Overall, dopamine inhibition was significantly dependent on the time interval between adenosine administration and dopamine stimulation (Fig. 2B, one-way ANOVA, $p = 0.0060$). Dopamine release decreased by 54 ± 6 % when adenosine was applied 2 s before (paired t-test, $p = 0.006$, $n = 14$) and 35 ± 6 % when adenosine was applied 5 s before

(paired t-test, $p = 0.0129$, $n = 5$). Supplemental figure 4 shows the current vs time traces for stimulated dopamine with no adenosine or after adenosine was applied 2 s before stimulation. The peak height decreases after adenosine application, but the normalized curves show no apparent difference in uptake. When adenosine was applied 10 s, 30 s, or 60 s before the stimulation, dopamine was not significantly reduced (paired t-test compared to initial stimulation in each animal, $p > 0.05$ in all cases, $n = 4$).

Two controls were performed to ensure that the adenosine was causing the inhibitory response. First, dopamine release was stimulated every 10 minutes for 40 minutes with no adenosine applied. There was no significant decrease in dopamine for the last stimulation compared to the first (Fig. 2B, paired t-test, $p = 0.7760$, $n = 6$). Second, aCSF was puffed on 2 s prior to dopamine stimulation instead of adenosine. There was no significant decrease in dopamine release from the aCSF puff, indicating it was not simply due to tissue disturbance due to puffing that caused release (Fig. 2B, paired t-test, $p = 0.0691$, $n = 4$).

Figure 2C shows the average stimulated dopamine concentrations when adenosine was transiently applied 2 s before dopamine release. There was an overall effect (repeated measures one-way ANOVA, $p = 0.0003$, $n = 14$) and the stimulated dopamine concentration 2 s after adenosine was significantly different than both the pre-adenosine stimulations and the recovery (repeated measures one-way ANOVA Bonferroni post-test, $p = 0.0014$ and $p = 0.0022$ respectively, $n = 14$).

Bath application of adenosine does not modulate stimulated dopamine release

To test the extent to which an increase in the basal levels of adenosine would modulate stimulated dopamine release, slices were perfused with 5 μM adenosine for 30 minutes, with dopamine being stimulated every 10 minutes. Fig. 3A shows CVs of stimulated dopamine release before, 30 min after adenosine bath, and after adenosine washout are all about the same. Bathing the slice in adenosine did not significantly reduce the amount of dopamine at 10, 20, or 30 minutes of perfusion and dopamine concentration was not significantly different than the pre-adenosine or post-adenosine (washout) stimulation (Figure 3B, repeated measures one-way ANOVA, $p = 0.5175$, $n = 5$). The adenosine bath experiments were performed in a separate set of slices than the transient adenosine application experiments.

Mechanically stimulated, endogenous adenosine modulates stimulated dopamine release

Recently, mechanically evoked transient increases in extracellular adenosine levels were discovered and adenosine could be evoked by lowering either the microelectrode or a glass pipette in brain slices (Ross *et al.* 2014a). Here, we tested the extent to which mechanically evoked adenosine modulates stimulated dopamine release (Figure 4). A glass pipette was implanted near the electrode and was lowered 50 μm into the tissue 2 s prior to the dopamine stimulation. Lowering the pipette caused an immediate increase in extracellular adenosine which was $1.4 \pm 0.4 \mu\text{M}$ and lasted $6.3 \pm 1.3 \text{ s}$ (for CV, see Figure S5). Dopamine release was inhibited by the endogenous transients (Figure 4A, red trace), but recovered after 10 minutes (dashed line). There was an overall effect (repeated measures one-way ANOVA, $p = 0.0092$, $n = 6$) and on average, mechanically evoked adenosine significantly decreased

stimulated dopamine (repeated measures one-way ANOVA Bonferroni post-tests, $p = 0.0350$, $n = 6$) and this decrease was on average $41 \pm 7\%$. Thus, both exogenously-applied and endogenously-evoked adenosine transients modulate stimulated dopamine release.

Adenosine modulation of dopamine is regulated by A₁ receptors but not A_{2A} receptors

The A₁ receptor antagonist DPCPX was perfused through the slice to test if the modulation of dopamine release occurred via A₁ receptors for both exogenous application (Figure 5A and B) and mechanically evoked adenosine (Figure 5C). Separate set of slices were used for both exogenous adenosine and mechanically evoked adenosine. Initial dopamine stimulations were collected (Figure 5A, B, and C), then, the slice was perfused with 200 nM DPCPX for 30 minutes and another stimulation performed to ensure the drug did not affect dopamine release (Figure 5A, B, and C). Next, adenosine was puffed onto the slice 2 s prior to dopamine stimulation in the presence of 200 nM DPCPX (Figure 5A blue trace and 5B). Overall, there was no significant effect of DPCPX or adenosine on stimulated dopamine release (repeated measures one-way ANOVA, $p = 0.4146$, $n = 6$). Similarly, there was no significant effect of DPCPX or adenosine on stimulated dopamine release when adenosine was mechanically stimulated in the presence of the drug instead of puffed on (repeated measures one-way ANOVA, $p = 0.5690$, $n = 6$, Figure 5C). In addition, a lower dose of DPCPX, 100 nM, was tested to test for non-specific pharmacological effects and it has the same effects as the higher dose. DPCPX alone or puffing on adenosine in the presence of 100 nM had no effect on stimulated dopamine release (repeated measures one-way ANOVA, $p = 0.7183$, $n = 5$, Supplemental Figure S6). DPCPX blocked the inhibition of dopamine release by adenosine; thus, the inhibition of dopamine release is regulated by A₁ receptors.

The effect of A_{2A} receptors was tested with 1 μ M SCH 442416, an A_{2A} antagonist. The same procedure for DPCPX was repeated for SCH 442416 in a separate set of slices. On average, there was an overall effect in the presence of SCH 442416 (Figure 5E, repeated measures one-way ANOVA, $p = 0.0001$, $n = 6$; Figure 5F, $p = 0.0247$, $n = 4$). SCH 442416 alone did not significantly change dopamine release (Figure 5E, repeated-measures one-way ANOVA Bonferroni post-test $p > 0.9999$, $n = 6$; Figure 5F, $p > 0.9999$, $n = 4$), however, puffing on adenosine 2 s prior to dopamine stimulation in the presence of the drug still resulted in a significant decrease in dopamine concentration (Figure 5E, one-way ANOVA Bonferroni post-test, $p = 0.0010$, $n = 6$; Figure 5F, $p = 0.0247$, $n = 4$). Overall, dopamine was decreased by $54 \pm 6\%$. Therefore, A_{2A} receptors do not affect the rapid modulation of adenosine by dopamine.

Discussion

In this paper, we show that adenosine transiently modulates phasic dopamine release in the caudate putamen. Transient adenosine release that occurred 2 to 5 s before stimulation modulated dopamine release but adenosine release that occurred over 10 s before stimulation did not. In addition, bath application of adenosine had no effect on phasic dopamine release. Dopamine was modulated by both adenosine that was exogenously applied, where a picospritzer was used to mimic spontaneous adenosine release that last a

few seconds (Nguyen *et al.* 2014), or adenosine that was endogenously evoked by mechanical stimulation. The modulation of phasic dopamine release was temporary and fully recovered by the next stimulation 10 min later. The inhibition of dopamine release by adenosine was regulated by the A₁ receptor. This work demonstrates the first neuromodulatory function for transient adenosine release: modulation of phasic dopamine release on a rapid time scale.

Transient adenosine modulates phasic dopamine release

Neurotransmitter and neuromodulator signaling occurs on different time scales. Basal levels are maintained by low-frequency, tonic firing and typically change on a slow, minute to hour time scale (Grace 1991). Larger responses to salient events occur after higher frequency, phasic firing but these responses are transient, often lasting for only seconds (Robinson *et al.* 2003). For dopamine, regulation of tonic signaling can be very different than that of phasic signaling (Floresco *et al.* 2003; Cragg and Rice 2004; Zhang and Sulzer 2004). Previous studies of adenosine modulation of dopamine release characterized that changes in basal adenosine modulated tonic dopamine levels on a slow time scale. For example, basal dopamine levels decrease after a 60 minute perfusion with the stable adenosine analogue 2-chloroadenosine (2-CADO) (Zetterstrom and Fillenz 1990) or 40 min after perfusion with 50 μ M adenosine (Okada *et al.* 1996). These studies showed that the modulation of basal dopamine levels by adenosine was slow, on the 40 minute to hour time frame, and required large amounts of adenosine. However, no studies have explored the effects of adenosine to modulate phasic dopamine.

In our study, we compared the effects of bath application of 5 μ M adenosine and transient adenosine release to modulate phasic dopamine release. Bath application of adenosine, which mimics increases in basal levels, did not change phasic dopamine release within 30 min. The adenosine concentration used here was not as high as in the studies examining adenosine neuromodulation of basal levels of dopamine (which were >50 μ M, (Okada *et al.* 1996)), but was of similar order of magnitude to the adenosine transients previously observed (Nguyen *et al.* 2014). While future studies could examine longer time periods or higher concentrations, the main conclusion was that increasing basal adenosine levels did not modulate phasic dopamine release. This demonstrates that tonic activation of the adenosine receptors does not result in modulation of phasic dopamine release.

In contrast, adenosine transients modulate phasic dopamine when administered directly prior to the electrical stimulation. Stimulated dopamine release was only inhibited when adenosine was administered 2 or 5 s prior to dopamine stimulation. Because the exogenous adenosine transients typically last on average 5 s, modulation by adenosine was limited to when adenosine was present. Once the transient adenosine release was cleared, no modulation of dopamine was observed. In agreement with that observation, adenosine modulation of dopamine release was fully recovered by the next stimulation, when adenosine would have been cleared for minutes. Thus, transient adenosine modulates phasic dopamine, but it is a temporary modulation that occurs only on a short time scale when adenosine is present.

Both exogenously applied adenosine and mechanically-stimulated adenosine modulated dopamine release, proving physiological amounts of adenosine can modulate dopamine. Exogenously-applied adenosine caused a larger inhibition, which might be due to a more global adenosine elevation that leads to greater adenosine receptor activation. Mechanically-evoked adenosine is more localized than the exogenously applied adenosine (Ross *et al.* 2014a), and may only activate adenosine receptors within the vicinity of the stimulation. The exogenous and mechanically-stimulated adenosine transients were large and future studies could examine the extent to which the concentration of adenosine transients effects modulation.

Adenosine modulates phasic dopamine release via A₁ receptors

A₁ receptors are expressed throughout the brain, particularly in the caudate putamen (Rivkees *et al.* 1995; Cunha 2008), and are responsible for most inhibitory actions of adenosine (Gomes *et al.* 2011). Here, we found that the inhibition of phasic dopamine release by transient adenosine is regulated by the inhibitory A₁ receptor. DPCPX alone did not affect phasic dopamine release, in contrast to studies of tonic dopamine levels, where basal dopamine increased within 20 minutes after DPCPX perfusion (Okada *et al.* 1996; O'Neill *et al.* 2007). Basal levels are not measured in our study, but the presence of DPCPX did attenuate the modulation of phasic dopamine release by transient adenosine.

A₁ receptors have nanomolar affinity for adenosine and the concentration of adenosine in both the transient activation and the bath application experiments was sufficient to completely activate A₁ receptors (Ballarin *et al.* 1991). Therefore, there must be a compensatory mechanism by which continuous receptor activation does not produce the same effect as transient receptor activation. In the brain, repetitive adenosine receptor stimulation changes the adrenergic signaling cascade (Roman *et al.* 2008) and prolonged exposure to adenosine analogs causes A₁ receptor desensitization in 15–30 minutes (Abbracchio *et al.* 1992; Klaasse *et al.* 2008; Mundell and Kelly 2011; Dunwiddie *et al.* 1997). A₁ receptors may desensitize after bath application of adenosine, which explains why modulation of phasic dopamine does not occur after exposure to adenosine for 30 minutes.

A_{2A} receptors are the primary excitatory receptor in the brain and thus were not hypothesized to cause dopamine inhibition. A_{2A} receptors are highly expressed in the caudate putamen, and they can be colocalized with D₂ dopamine receptors (Fuxe *et al.* 2005). Transient adenosine modulation of dopamine release was not affected by blocking A_{2A} receptors with SCH 442416, similar to studies of basal levels that found no change in dopamine levels after perfusion with an A₂ antagonist (Okada *et al.* 1996). Past studies have suggested that A₂ receptor action can be masked by A₁ receptors (Okada *et al.* 1996) and more detailed studies could address this with combinations of drugs in the future. Overall, A_{2A} receptors do not appear to be important for the observed transient modulation of dopamine.

Function of transient adenosine release

Rapid release of adenosine has been recently characterized after electrical stimulation (Pajski and Venton 2010; Pajski and Venton 2013) and mechanical stimulation (Ross *et al.*

2014a). In addition, spontaneous adenosine release has been characterized *in vivo* (Nguyen *et al.* 2014). Transient adenosine release also occurs during pathological states such as hypercapnia (Dale 2006), ischemia (Dale and Frenguelli 2009), and epilepsy (Dale and Frenguelli 2009). This study demonstrates the first transient, neuromodulatory function of rapid adenosine release: modulating phasic dopamine release. The modulation of dopamine release is local and fast; it only happens where and when adenosine is present. Adenosine modulation provides temporary inhibition in response to salient events and is easily reversed. However, the mechanism of spontaneous adenosine release in the brain is not clear and we do not know how transient adenosine release is correlated with the cues that release transient dopamine release during behaviors such as drug administration. Adenosine release can be stimulated in some subregions of the caudate putamen (Pajski and Venton 2010), so stimulated adenosine release may be able to regulate stimulated dopamine release. Recently, adenosine was discovered in synaptic vesicles of the rat brain (Corti *et al.* 2013), so vesicular release of adenosine, possibly co-released with another neurotransmitter, after a behavioral or other physiological stimulation could also result in a transient adenosine increase that would regulate phasic dopamine. Future studies are needed to correlate adenosine and dopamine transient *in vivo*, especially in behaving animals.

The extent to which this transient adenosine modulation is a general mechanism for modulating other neurotransmitters is not known. Adenosine can also modulate serotonin (Okada *et al.* 2001), glutamate (Quarta *et al.* 2004), and GABA (Sperlagh and Vizi 2011) via A₁ receptors (Quarta *et al.* 2004; Cunha 2008). However, previous studies have focused on basal increases in adenosine regulating neurotransmission on a slow time scale. More studies are needed to address the effects of transient adenosine on phasic release of other neurotransmitters.

The transient modulation of dopamine could be important in diseases. Phasic dopamine release is required for drug seeking behavior (Phillips *et al.* 2003) and modulation by transient adenosine could be a novel way to regulate drug seeking and addiction. Parkinson's disease patients have fewer dopamine releasing neurons, therefore an overall lower tonic level of dopamine. A₁ receptor agonists in combination with high frequency stimulation treatment (Lee *et al.* 2006) may be useful for preventing temporary inhibition of phasic dopamine by adenosine in Parkinson's disease patients. Our study, along with the previous studies on basal dopamine modulation, points to two different mechanisms of adenosine neuromodulation of dopamine: a slow mechanism that modulates basal dopamine levels after long perfusion times and a rapid modulation of phasic dopamine which occurs during a temporary increase in adenosine levels. Understanding and manipulating adenosine on fast and slow time scales could lead to differential modulation of tonic and phasic dopamine release.

Conclusions

In conclusion, this paper demonstrates rapid modulation of phasic dopamine release in the caudate putamen. Both exogenous and endogenous adenosine transients inhibited stimulated dopamine release by 50 % if adenosine release occurred 2 s before stimulation. However, bath application of adenosine or applying adenosine more than 10 s before the stimulation

did not inhibit dopamine release. The inhibition was reversible, and stimulated dopamine was fully recovered within 10 minutes. Inhibition of dopamine release was regulated by the A₁ receptor, and not by the A_{2A} receptor. Future work could focus on the extent to which transient adenosine transiently modulates other neurotransmitters in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the National Institute of Health for funding this work (R01NS076875).

Abbreviations used

DPCPX	8-cyclopentyl-1,3-dipropylxanthine
FSCV	fast-scan cyclic voltammetry
aCSF	artificial cerebral spinal fluid
DMSO	dimethylsulfoxide
DA	dopamine
AD	adenosine

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A. Experimental schematic

B. Sample data

Figure 1.

Experiment set-up and example data. A) Schematic for exogenous application of adenosine in brain slices. A bipolar stimulating electrode, carbon-fiber microelectrode (CFME), and picospritzer pipette are placed in a slice of the caudate putamen. The CFME and stimulating electrode form a shallow triangle and the picospritzer pipette is placed 20–30 μm away from the CFME. B) A color plot shows voltage on y-axis, time on x-axis, and current in false color. A blue arrow indicates when adenosine was applied and the red arrow indicates the stimulation. Here, 25 μM adenosine was exogenously applied with a picospritzer (10 psi, 50 ms, 200 nL) 30 s prior to dopamine stimulation (60 Hz, 5 pulses, 4 ms pulse width). Adenosine has two oxidative peaks (primary 1.4 V and secondary at 1.0 V) and dopamine oxidizes at 0.6 V. The adenosine CV is shown above (blue trace) and the dopamine CV is

shown below (red trace). The adenosine transient lasted only 7.3 seconds and the local concentration maximum at the electrode was 1.7 μM . The maximal dopamine concentration was 0.3 μM .

A. Adenosine modulates stimulated dopamine

B. Effect of time between puff and stimulation

C. Average data for exogenous adenosine 2s prior to DA

Figure 2.

Effect of time interval between exogenous application and dopamine stimulation. A) Example of the optimized procedure, the black trace indicates the initial dopamine stimulation, red trace is the dopamine 2 s after adenosine application, and the dotted trace shows dopamine on the subsequent stimulation (10 minutes later). Dopamine decreases by about 50% directly after adenosine application, but the inhibition is gone after 10 min. B) Effect of time between adenosine application and stimulation on dopamine inhibition. Overall, dopamine inhibition was significantly dependent on the time delay of adenosine

administration (one-way ANOVA, $p = 0.0060$). The y-axis is the percent of initial stimulation and the x-axis shows the time at which adenosine was administered prior to stimulating dopamine. Exogenous application of adenosine at 2 s (red bar) and 5 s (blue bar) significantly decreased dopamine to $46 \pm 6\%$ (paired t test, $p = 0.006$, $n = 14$) and $65 \pm 6\%$ (paired t test, $p = 0.0129$, $n = 5$) of initial stimulation respectively. The black bar represents a stimulation control without adenosine and the gray bar represents puffing on aCSF instead of adenosine. Both controls do not significantly change dopamine. C) The average pre-adenosine stimulations, the dopamine stimulation 2 s after adenosine application, and recovery is shown. There was an overall main effect (repeated measures one-way ANOVA, $p = 0.0003$, $n = 14$). After adenosine application, stimulated dopamine release was significantly different than both the pre-adenosine stimulations 1 and 2 and the recovery; however the pre-adenosine stimulations 1 and 2 were not significantly different from one another or from the recovery (one-way ANOVA, Bonferroni post-tests, $n = 14$).

A. 5 μ M adenosine bath does not affect stimulated DA

B. Average data for adenosine bath

Figure 3.

Bath perfusion of 5 μ M adenosine does not modulate stimulated dopamine release. A) Dopamine is not modulated when 5 μ M adenosine in the aCSF is perfused through the slice for 30 minutes (black trace: initial dopamine, orange trace: stimulated dopamine after 30 minute adenosine perfusion, green trace: adenosine washout). B) Comparisons of the average pre-adenosine stimulation, dopamine stimulation at different perfusion time points, and stimulation after adenosine washout are shown. Bathing the slice in adenosine did not significantly reduce the amount of dopamine released at 10, 20, or 30 minute during

perfusion and dopamine concentration was not significantly different than the pre-adenosine or washout (repeated measures one-way ANOVA with Bonferroni post-tests, $p = 0.5175$, $n = 5$).

A. Stimulated DA Release

B. Mechanically stimulated ADO modulates DA release

Figure 4.

Mechanically evoked adenosine transiently modulates dopamine. A) Example data shows dopamine is inhibited by around 50 % after mechanical stimulation by lowering an empty pulled glass pipette (black trace: initial dopamine, red trace: after mechanical stimulation, dotted trace: 10 minute recovery). B) There was a significant main effect (repeated measures one-way ANOVA, $p = 0.0092$, $n = 6$). Mechanically evoked adenosine causes a significant decrease in dopamine concentration (repeated-measures one-way ANOVA Bonferroni post tests, $p = 0.0350$, $n = 6$) and dopamine fully recovers within 10 minutes.

Figure 5.

Dopamine modulation is regulated by the A₁ receptor and not the A_{2A} receptor in the caudate putamen. A) Dopamine modulation was blocked in the presence of 200 nM of the A₁ receptor antagonist, DPCPX. Example data show no change in stimulated dopamine release after both drug perfusion and adenosine application (black trace: initial stimulation, red trace: dopamine after DPCPX perfusion for 30 minutes, blue trace: dopamine after exogenous application 2 s prior in the presence of DPCPX). B) Overall, there was no significant overall effect of DPCPX or exogenous adenosine on stimulated dopamine release (repeated measures one-way ANOVA, $p = 0.4146$, $n = 6$). C) For mechanically-evoked adenosine release, there was no significant overall effect, meaning DPCPX blocked the modulatory effect of stimulated adenosine on dopamine release (repeated measures one-way ANOVA, $p = 0.5690$, $n = 6$). D) Dopamine modulation was unchanged in the presence of 1 μM of the A_{2A} antagonist, SCH 442416. Example data show no change in stimulated dopamine release after 30 minutes of drug perfusion, but dopamine is decreased when adenosine was applied 2 s prior in the presence of the drug (black trace: initial dopamine, orange trace: dopamine after SCH 442416 perfusion for 30 minutes, green trace: dopamine after exogenous application 2 s prior in the presence of SCH 442416). E) There was an overall main effect in the presence of SCH 442416 (repeated measures one-way ANOVA, $p = 0.0001$, $n = 6$) for exogenous adenosine application. Dopamine remained unchanged after 30 minute SCH 442416 perfusion (one-way ANOVA Bonferroni post-test, $p > 0.9999$, $n = 6$) but decreased significantly after exogenous application of adenosine (one-way ANOVA Bonferroni post-test, $p = 0.0010$, $n = 6$). F) For mechanically-evoked release, there was an overall main effect in the presence of the A_{2A} antagonist (repeated measures one-way ANOVA, $p = 0.0247$, $n = 4$). Dopamine remained unchanged after 30 minutes of SCH

442416 perfusion (Bonferroni post-test, $p > 0.9999$, $n = 4$) but decreased significantly after mechanically evoked adenosine (Bonferroni post-test, $p = 0.0022$, $n = 4$).