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Mechanical stimulation evokes rapid increases in extracellular adenosine concentration in the prefrontal cortex

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Abstract

Mechanical perturbations can release ATP, which is broken down to adenosine. In this work, we used carbon-fiber microelectrodes and fast-scan cyclic voltammetry to measure mechanically-stimulated adenosine in the brain by lowering the electrode 50 μm . Mechanical stimulation evoked adenosine *in vivo* (average: $3.3 \pm 0.6 \mu\text{M}$) and in brain slices (average: $0.8 \pm 0.1 \mu\text{M}$) in the prefrontal cortex. The release was transient, lasting 18 ± 2 s. Lowering a 15 μm diameter glass pipette near the carbon-fiber microelectrode produced similar results as lowering the actual microelectrode. However, applying a small puff of artificial cerebral spinal fluid was not sufficient to evoke adenosine. Multiple stimulations within a 50 μm region of a slice did not significantly change over time or damage cells. Chelating calcium with EDTA or blocking sodium channels with tetrodotoxin (TTX) significantly decreased mechanically evoked adenosine, signifying that the release is activity-dependent. An alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), did not affect mechanically-stimulated adenosine; however, the nucleoside triphosphate diphosphohydrolase 1,2 and 3 (NTDPase) inhibitor POM-1 significantly reduced adenosine so a portion of adenosine is dependent on extracellular ATP metabolism. Thus, mechanical perturbations from inserting a probe in the brain cause rapid, transient adenosine signaling which might be neuroprotective.

Keywords

mechanical stimulation; sensor; adenosine; prefrontal cortex; fast-scan cyclic voltammetry; ATP

Introduction

The release of neurotransmitters as a result of a mechanical stimulation, termed mechanosensitive release, has been observed throughout the body (Stalmans and Himpens 1997; Woo *et al.* 2008; Ramsingh *et al.* 2011; Olsen *et al.* 2011). Mechanical damage can lead to cell death and be caused by cell stretching (Stalmans and Himpens 1997), swelling (Xia *et al.* 2012), shear stress (Wan *et al.* 2008) or mechanical perturbation (Xia *et al.* 2012). In the brain, mechanosensitive release of neurotransmitters has been observed from both neurons (Xia *et al.* 2012) and astrocytes (Newman 2001). Some mechanosensitive release is calcium dependent (Newman 2001; Stalmans and Himpens 1997) and a result of exocytosis, such as

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mechanosensitive glutamate release detected from astrocytes (Montana *et al.* 2004;Newman 2001). Other release events are not exocytotic, such as the release of ATP through pannexin channels from neurons after cell swelling (Xia *et al.* 2012). Detection of mechanosensitive release on a rapid time scale has not been explored and would be beneficial in understanding the immediate tissue response to mechanical manipulations.

Mechanosensitive release of ATP has been documented in many parts of the body including the heart (Wan *et al.* 2008), bladder (Ferguson *et al.* 1997;Olsen *et al.* 2011) and retinal neuronal cells (Xia *et al.* 2012). Mechanosensitive ATP release is a response to a normal biological function such as inhalation in the lungs (Ramsingh *et al.* 2011), arterial constriction (Wan *et al.* 2008), or bladder distention (Ferguson *et al.* 1997). In the brain, mechanosensitive ATP release occurs in response to damage due to swelling, mechanical perturbation, and shear stress (Xia *et al.* 2012;Newman 2001). Much of this release is not exocytotic and comes from astrocytes, but some may be calcium dependent and come from neurons (Newman 2001;Ramsingh *et al.* 2011). ATP can stimulate P2Y receptors in the extracellular space (Pankratov *et al.* 2006;Xia *et al.* 2012), but most ATP is quickly broken down to adenosine (Pajski and Venton 2010;Latini and Pedata 2001). Thus, breakdown of ATP is assumed to be the source of most extracellular adenosine during conditions such as brain injury; however, little is known about response to minor tissue injuries such as probe implantation.

Adenosine is an important signaling molecule in the brain which regulates neurotransmission (Rudolphi *et al.* 1992;Okada *et al.* 1996) and blood flow (Sciotti and Vanwylen 1993;O'Regan 2005). It is also neuroprotective during ischemia (Fredholm 1997;Rudolphi *et al.* 1992;Parkinson *et al.* 1994), stroke (Von Lubitz *et al.* 2001), and traumatic brain injury (Lin and Phillis 1992;Fredholm 1997). The neuroprotective effects of adenosine are thought to occur primarily through A1 receptors (Cechova *et al.* 2010;Okada *et al.* 1996), which are inhibitory, and increases in adenosine have been detected for minutes to hours after ischemia and brain injury (Quarta *et al.* 2004). However, direct, calcium-dependent release of adenosine was recently discovered which occurs on the seconds to minute time scale (Pajski and Venton 2010;Wall and Dale 2007). There have been reports of rapid adenosine release in response to implantation of electrodes in slices from murine spinal lamina (Street *et al.* 2011;Chang *et al.* 2012) and deep brain stimulation probes in humans (Chang *et al.* 2012), but this release has not been well characterized.

In this study, we characterized the rapid rise in adenosine concentration after mechanical stimulation in the prefrontal cortex of brain slices and *in vivo*. A carbon-fiber microelectrode was lowered to cause mechanical perturbation to the tissue and adenosine measured electrochemically using fast-scan cyclic voltammetry. Mechanical stimulation evoked an increase in adenosine concentration in anesthetized rats and in brain slices. Mechanically evoked adenosine was primarily activity-dependent and partially the result of downstream breakdown of ATP. It was not a downstream effect of glutamate signaling at alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors or released from equilibrative nucleoside transporters (ENTs). Thus, adenosine could provide transient neuromodulation during damage caused by electrode implantation.

Methods

Chemicals

All chemicals were from Fisher Scientific (Fair Lawn, NJ, USA) unless otherwise stated. Adenosine, ATP, and inosine 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 calibration concentrations for all brain slice experiments. The aCSF was 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, pH 7.4 was used in slices and for calibration of electrodes used in slices. Phosphate-buffered saline (PBS) containing 131.25 NaCl mM, 3.0 KCl mM, 10.0 NaH₂PO₄ mM, 1.2 MgCl₂ mM, 2.0 Na₂SO₄ mM, and 1.2 CaCl₂ mM with the pH adjusted to 7.4 was used to calibrate electrodes for *in vivo* experiments. Adenosine was tested at 1.0 μ M for brain slice and *in vivo* experiments. For calcium-free experiments, the aCSF solution was made without CaCl₂ and 1 mM ethylenediaminetetraacetic acid (EDTA) was added. Tetrodotoxin (TTX) purchased from Tocris Bioscience (Ellisville, MO), solubilized in 0.2 M citrate buffer (pH 4.8) and frozen as 50 μ M aliquots that were diluted to 0.5 μ M for experiments. A safety protocol for TTX use was approved by the Office of Environmental Health and Safety at the University of Virginia. The AMPA antagonist, 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), nucleoside triphosphate diphosphohydrolase 1,2 and 3 (NTPDase 1,2 and 3) inhibitor (POM-1), and equilibrative nucleoside transporter inhibitor S-(4-Nitrobenzyl)-6-thioinosine (NBTI) were purchased from Tocris Bioscience (Ellisville, MO).

Electrochemistry

Carbon-fiber microelectrodes were fabricated from T-650 carbon-fibers as previously described (gift from Cytec Engineering Materials, West Patterson, NJ) (Swamy and Venton 2007a). Cylinder electrodes, 50 μ m long and 7 μ m in diameter, were used with fast-scan cyclic voltammetry (see supplemental methods for details).

Brain slice preparation/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 *ab libitum*. Rats were anesthetized with isoflurane (1 mL/100 g rat weight) in a desiccator and immediately beheaded. 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 prefrontal cortex were prepared using a vibratome (LeicaVT1000S, Bannockburn, IL), transferred to oxygenated aCSF (95% oxygen, 5% CO₂), and recovered for an hour before the experiment. A pump (Watson-Marlow 205U, Wilmington, MA) was used to flow 35-37°C aCSF over the brain slice at 2 mL/min for all experiments. The electrodes were inserted 75 μ m into the tissue. After equilibration, 60 sec of baseline data was collected and the brain slice was mechanically stimulated by lowering the electrode 50 μ m with a micromanipulator. See supplementary methods for staining procedure, pharmacology experimental details and picospritzing experiment.

In vivo experiments

Male Sprague-Dawley rats (250-350 g, Charles River, Willmington, MA) were anesthetized with 50% wt urethane in saline solution (1.5 g/kg, i.p). Bupivacaine (0.25 mL, s.c., APP Pharmaceuticals, LLC; Schaumburg, IL, USA) was administered for local analgesia. The rat's temperature was maintained at 37°C using a heating pad with a thermistor probe (FHC, Bowdoin, ME, USA). The working electrode was implanted in the prefrontal cortex: coordinates in mm from bregma: anterior-posterior (AP): +2.7, mediolateral (ML): +0.8, and dorsoventral (DV): -3.0 (Paxinos and Watson 2007). A Ag/AgCl reference electrode was inserted on the contralateral side of the brain. The electrode was lowered 100 μ m to stimulate mechanosensitive release every 30 minutes for 2 hours.

Statistics

All values are reported as the mean $\pm$ standard error of the mean (SEM). Paired t-test were performed to compare data before and after drugs in the same slices. All statistics were performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and considered significant at the 95% confidence level ($p < 0.05$). A one-way ANOVA with Bonferroni post tests was performed to compare consecutive mechanical stimulations *in vivo* and across stimulations for both CFME and glass pipettes. A two-way ANOVA was used to compare the two stimulation techniques.

Results

Mechanically-stimulated adenosine in brain slices and in vivo

Fast-scan cyclic voltammetry was used to electrochemically detect adenosine in brain slices. A large background current is obtained because of the fast scan rate, but it is removed with background subtraction (Millar *et al.* 1985). However, basal levels cannot be measured and the technique instead measures fast changes in concentration. The mechanical stimulation in this study was lowering the carbon-fiber microelectrode 50 μ m in the prefrontal cortex of a brain slice. Electrically-stimulated adenosine release has been found to be ATP dependent in the prefrontal cortex (Pajski and Venton 2013), and thus this region has the capability for evoked adenosine release. Adenosine was observed immediately following carbon-fiber microelectrode movement 100% of the time ($n=25$ slices, 39 mechanical stimulations).

Fig. 1A shows a color plot of adenosine release after mechanical stimulation (red arrow) in a brain slice. Applied voltage is on the y-axis, time is on the x-axis, and the green and purple colors represent oxidative current, which is proportional to concentration. Before the electrode was lowered, a background signal was obtained, denoted by the tan color representing no current change. The primary oxidation peak for adenosine occurs immediately after the mechanical stimulation at approximately 1.4 V and the secondary peak occurs at 1.1 V (denoted by the black arrows in Fig. 1A and B). The peak positions and shape of the cyclic voltammogram (CV) are characteristic of two, two-electron oxidations for adenosine (Swamy and Venton 2007b; Dryhurst 1977). The third peak observed at 0.1 V could be the third oxidation peak for adenosine, but that peak is not often observed at our carbon-fiber microelectrodes (Swamy and Venton 2007b). That peak and the blue (negative) currents on the bottom of the color plot are likely due to other chemical and ionic changes

that occur with mechanical stimulation. The current after adenosine is released often goes below baseline due to ionic changes (Pajski and Venton 2013). A current vs. time trace at the main oxidation potential for adenosine shows a rapid increase in current immediately following the mechanical stimulation of the electrode. In Fig. 1A, the signal lasts about 10 seconds and the peak current corresponds to 0.77 μM adenosine.

Mechanosensitive release has most often been attributed to ATP, which can then break down to form adenosine (Olsen *et al.* 2011; Ramsingh *et al.* 2011). ATP and adenosine have similar cyclic voltammograms because the adenine group is electroactive in both; however, sensitivity for ATP at carbon-fiber microelectrodes is 3-6 fold less than for adenosine (Ross and Venton 2012). To confirm our sensors were measuring adenosine, mechanically-evoked release was measured with selective biosensors for adenosine, ATP, and inosine biosensors in brain slices (Llaudet *et al.* 2003). Inosine sensors lack adenosine deaminase, the first enzyme in the cascade that breaks adenosine down to inosine (Llaudet *et al.* 2003), and are used as a null sensor to ensure other compounds are not causing a false signal at the adenosine biosensor. While the sensitivity for the biosensors (adenosine sensitivity: 0.8 nA/ μM , ATP: 0.25 nA/ μM , inosine: 0.7 nA/ μM) was not as good as with the carbon-fiber microelectrodes on our system, adenosine release was observed 50% of the time with mechanical stimulation ($n=5$ slices, 10 mechanical stimulations, Figure S1 and Table 1). However, no changes were observed at ATP or inosine biosensors. Thus, the signal detected at carbon-fiber microelectrodes is likely to be adenosine. These data did not rule out ATP metabolism as a source of adenosine because ATP can breakdown to adenosine within 200 ms (Dunwiddie *et al.* 1997) and the response time of the ATP biosensors are on the magnitude of seconds (Llaudet *et al.* 2005). Thus, if ATP is rapidly metabolized to adenosine, then the biosensor is unlikely to detect much ATP.

Mechanically-evoked adenosine was also observed *in vivo* in the prefrontal cortex of anesthetized rats. Fig. 1B shows an example of mechanically-evoked adenosine *in vivo* after the electrode is lowered 100 μm . The peak current corresponds to 2.4 μM adenosine and the duration is approximately 80 s. Average results for carbon-fiber microelectrodes in slices and *in vivo* are summarized in Table 1. The concentration and duration of adenosine release is on average much larger *in vivo* compared to brain slices.

Fig. 2A shows an example current versus time trace of consecutive mechanical stimulations every 30 minutes *in vivo*. The traces are similar, although there is variation that could be due to different areas of tissue being stimulated. The normalized current versus time trace in the inset shows that the temporal profile of adenosine is relatively stable. Fig. 2B shows the average concentration for each successive mechanical stimulation as well as the average for all stimulations (blue bar). There was no significant effect of stimulation number on the concentration of adenosine evoked ($n=5$ rats, $p=0.6781$, repeated measures one way ANOVA) or duration (Fig. 2C, $n=5$ rats, $p=0.6224$, repeated measures one way ANOVA). Thus, the concentration and duration of adenosine signaling is constant for multiple mechanical stimulations.

Evaluation of other methods for mechanical stimulation

Next, we examined the extent to which other mechanical stimulations evoked adenosine. An empty, pulled glass pipette approximately 15 μm in diameter was lowered 50 μm into the slice near the electrode. A similar increase in adenosine was detected at the carbon-fiber microelectrode when either the carbon-fiber microelectrode itself or a pipette near it was moved (Fig. 3A and 3B). Repeated mechanical stimulations were also tested. The electrode or glass pipette was lowered 50 μm , moved back up 50 μm , and finally lowered again 50 μm , with 10 min intervals between stimulations for recovery. Figure 3A and 3B show cyclic voltammograms for both the carbon-fiber microelectrode and glass pipette stimulations, respectively. The cyclic voltammograms for adenosine were similar for all stimulations, which were performed in the same area of tissue. The magnitude of adenosine measured by repeated stimulations within each technique was not statistically different (repeated measures one-way ANOVA, $F=0.3522$, $p=0.7882$, $n=5$). Carbon-fiber microelectrodes and glass pipettes were also compared and there was no main effect of stimulation technique (repeated measures two-way ANOVA, $F(1,7)=0.2229$, $p=0.6512$) or stimulation number ($F(1,7)=5.046$, $p=0.0595$), and no interaction ($F(1,7)=0.1402$, $p=0.7192$). Thus, mechanically-evoked adenosine can occur by both physically lowering the working electrode or by moving something comparable in size nearby.

Local tissue damage associated with multiple electrode stimulations was characterized by staining for dead cells and was analyzed using multiphoton microscopy. A LIVE/DEAD stain was used to analyze cells with compromised cell membranes (necrosis) and a counter-stain, 4',6-diamidino-2-phenylindole (DAPI), stained for all nuclei. The first 50 μm of the slice tissue was found to be highly damaged, which is expected in slice experiments. A multiphoton Z-stack through the initial dead segment and into the underlying healthy tissue at the point of electrode insertion did not reveal a clear path for electrode insertion. There was no noticeable hole in the tissue and no track of cell death (Fig. 3D and E), indicating that the electrode did not disrupt the cell membranes of the cells as it moved up and down through the tissue. Figure 3D shows all nuclei stained with DAPI colored blue and dead cells colored red. The percentage of dead cells was counted and compared in the region of electrode implantation and a control region (Fig. 3E) and there was no significant difference between these two regions (paired t-test, $p=0.9096$, $n=4$). This is consistent with previous work indicating that FSCV probe insertion does not significantly damage the tissue (Peters *et al.* 2004; Jaquins-Gerstl and Michael 2009).

Mechanical stimulation of adenosine was not observed by pressure ejecting aCSF into the slice close to the electrode. A glass pipette was implanted, the tissue allowed to recover, and then a picospritzer was used to deliver 100-200 nL volumes of aCSF to the brain slice tissue 30 μm away from the carbon-fiber microelectrode. Low pressures were used to avoid significant tissue damage and the parameters chosen are similar to those used for pressure ejection studies of transport in the literature (Zahniser *et al.* 1999). Figure 4A shows the concentration over time profile for a mechanical stimulation by lowering the electrode and by pressure ejecting aCSF in the same slice. An increase in adenosine concentration occurred immediately following the electrode lowering; however, no adenosine changes were observed with aCSF injection. A slight decrease in current was observed followed by a

slow return to baseline after aCSF puffing, likely due to a disturbance of the background current by the fluid movement. The cyclic voltammograms (Fig. 4B) confirm no adenosine was detected for aCSF application and adenosine was not observed after aCSF application in any slice (n=3).

Mechanism of mechanically-evoked adenosine

Electrically-stimulated adenosine release is activity-dependent in brain slices (Klyuch *et al.* 2012; Pajski and Venton 2010). Thus, we tested whether mechanically-evoked adenosine is activity-dependent by bathing the brain slices in calcium-free aCSF with 1 mM EDTA for 30 minutes. To prevent action potentials, 0.5 μ M TTX was applied for 30 minutes in separate experiments. In each of these experiments, a baseline of mechanically evoked adenosine was collected, then drug was applied and 30 min later, the electrode was lowered a second time (see Fig. 2S for all concentration versus time plots). Control data show that mechanical stimulations 30 minutes apart have the same signal when drugs are not applied (Fig. 5A and 5E, n = 5, p = 0.1507, paired t-test). Fig. 5B and 5C show adenosine cyclic voltammograms from mechanical stimulation before and after perfusion with EDTA and TTX, respectively. EDTA significantly reduced the amount of adenosine released by 93% (Fig. 5E, n = 6, p = 0.0482, paired t-test) whereas TTX significantly reduced the signal by 61% (Fig. 5E, n = 3, p = 0.0337, paired t-test). These reductions in adenosine release demonstrate mechanically evoked adenosine is mostly calcium dependent and activity dependent.

Figure 5D shows that blocking equilibrative nucleoside transporters with 10 μ M NBTI did not significantly change the amount of adenosine released compared to the predrug signal (Fig. 5E, n=9, p=0.1675, paired t-test). However, perfusion with NBTI did increase duration of signal (see Figure S3) so transport into the cell was blocked. Activity dependent adenosine release was not facilitated through equilibrative nucleoside transporters.

Next, the dependence of mechanically-evoked adenosine on glutamate or ATP was tested. Activity dependent adenosine release has been proposed to be AMPA receptor dependent in the cerebellum (Klyuch *et al.* 2011) and glutamate has been shown to be mechanically released from astrocytes (Montana *et al.* 2004). To test for AMPA receptor dependence, the antagonist CNQX was used. Fig. 6A shows a cyclic voltammogram of mechanically evoked adenosine before and after perfusion with 10 μ M CNQX. Mechanically evoked adenosine after CNQX increased by 20%, but it was not significantly different from the predrug stimulation (Fig. 6C, n = 5, p=0.2935). To test for dependence on ATP release, a recombinant NTPDase 1, 2 and 3 inhibitor, POM-1, was used (Wall *et al.* 2008). Fig. 6B shows mechanically evoked adenosine decreased after 100 μ M of POM-1 was perfused and on average significantly reduced mechanically evoked adenosine by 62% (Fig. 6C, n = 6, p=0.0098). POM-1 did not completely block all of the adenosine detected so other mechanisms of adenosine accumulation could also contribute to the signal. Less sensitive ATP metabolism inhibitors, ARL 67156 and AOPCP (Wall *et al.* 2008), were also used but they did not significantly reduce the amount of adenosine detected (data not shown). Overall, our data show mechanically evoked adenosine is not caused by glutamate acting at AMPA receptors but a portion is due to extracellular ATP breakdown.

Discussion

Mechanosensitive release of purines due to physical perturbation of cells has been mostly attributed to ATP release, which slowly builds up over time (Woo *et al.* 2008;Olsen *et al.* 2011;Ramsingh *et al.* 2011). In this study, we demonstrate that in the prefrontal cortex, mechanical stimulation elicits a rapid, transient release of ATP which is immediately metabolized to adenosine. In brain slices, peak adenosine release was on average 0.8 ± 0.1 μ M and lasted 18 ± 2 s. Mechanically-evoked adenosine was activity-dependent but was not dependent on equilibrative nucleoside transporters or a downstream effect of AMPA receptors. Pharmacologically blocking ATP metabolism demonstrated that mechanically evoked adenosine was largely a downstream effect of ATP release. This implies a large releasable pool of ATP that breaks down quickly to adenosine. These studies provide new insight that ATP is released and rapidly metabolized to adenosine in the extracellular space after electrode implantation and that adenosine could act as a signaling molecule during tissue damage.

Mechanically evoked adenosine occurs via different methods

In this experiment, we primarily mechanically stimulated the tissue by lowering the working electrode 50-100 μ m in tissue. This method of stimulation would be relevant to any type of probe implantation, for example electrochemical, electrophysiological, or deep brain stimulation (DBS) probes. Because lowering the carbon-fiber microelectrode could change the background current, the response was compared to lowering a glass pipette of similar size near the stationary microelectrode. The evoked adenosine was similar for both techniques and thus the signal is not an artifact of moving the electrode. However, simply puffing on small amounts of aCSF, close to the electrode did not evoke any adenosine response. The aCSF puff might slightly move cells, but did not cause sufficient damage to elicit adenosine release.

Traditional mechanical perturbation techniques, including cell swelling (Xia *et al.* 2012), cell stretching (Ramsingh *et al.* 2011), and shear stress (Olsen *et al.* 2011;Woo *et al.* 2008), are modeled after normal biological functions. Our study does not directly mimic one of these processes, but an interesting phenomenon was observed in the experiments where the electrode or glass pipette was lowered and raised multiple times. The adenosine increase after pulling the pipette up was slightly larger than for lowering it down. While this phenomenon would need to be characterized much further, it suggests some cell stretching may be a cause of adenosine increase when raising the electrode. These experiments also show that the tissue is not dying, as the same response can be measured repeatedly, and staining reveals no significant increase in local cell death after multiple electrode stimulations. Thus, the electrode insertion is not causing a significant amount of permanent tissue damage, in line with previous reports of minimal tissue damage associated with carbon-fiber microelectrode insertion *in vivo* (Jaquins-Gerstl and Michael 2009;Peters *et al.* 2004).

An adenosine response to probe implantation has been suggested in the past but never fully characterized. Tawfik et al. discovered that adenosine was elevated immediately after electrode implantation, preceding the microthalamotomy effect in the ferret thalamus

(Tawfik *et al.* 2010). The microthalamotomy effect is observed in essential tremor and Parkinson's disease patients and refers to the immediate relief from tremors following electrode implantation during DBS procedures (Chang *et al.* 2012). Chang et al implanted a DBS probe into 7 human patients and also saw the microthalamotomy effect along with release of adenosine in all patients (Chang *et al.* 2012). Future studies could probe whether adenosine is a cause or effect of the microthalamotomy effect during implantation in DBS. Elevated levels of adenosine were also discovered during and after acupuncture in the tibialis anterior muscle close to the knee (Goldman *et al.* 2010). While this study was outside the central nervous system and used bigger needles, adenosine was detected and quantified on the 10-30 min. time scale using microdialysis coupled to HPLC. Thus, other forms of probe implantation cause increases in adenosine, which may be similar or complementary to the transient adenosine release observed in this study.

Mechanical stimulation evokes large, transient adenosine changes

Mechanically-stimulated adenosine varied widely in both slices and *in vivo*. The concentration of adenosine detected in slices ranged from 0.1 μM to 3.3 μM and *in vivo* from 0.3 to 10 μM . Larger amounts of adenosine were released *in vivo* but the stimulation was 100 μm compared to 50 μm in slices, which would result in more cells being stimulated. The activity dependent nature of release suggests that intact circuits *in vivo* may also facilitate larger amounts of release. The large range is not surprising because the density of adenosine releasing cells around the electrode could vary based on electrode location. Also, speed of insertion was not tightly controlled which could cause varying tissue responses. The average amount of adenosine released by mechanical stimulation was higher than previously detected with electrical stimulation (Pajski and Venton 2010); thus, tissue perturbation causes higher amounts of release than electrical stimulation.

The duration of adenosine release varied from 2 s to 110 s in brain slices and *in vivo*. The duration was correlated with the concentration, as larger amounts of adenosine lasted longer because more time was needed to clear the adenosine from the extracellular space. Mechanically-evoked adenosine signaling is fast compared to other studies that measure mechanically-evoked ATP release (Ramsingh *et al.* 2011). Mechanosensitive ATP release is usually detected using luciferin-luciferase assay techniques (Woo *et al.* 2008), which allow measurements to be taken only every 30 s. The duration of the adenosine response is similar to electrically evoked adenosine signaling that has been shown in multiple brain regions (Pajski and Venton 2010;Pajski and Venton 2013;Klyuch *et al.* 2011). Mechanical evoked release is easier than electrical stimulation and could be used to study the function of transient adenosine signaling, including any neuroprotective effects.

Mechanism and function of mechanically evoked adenosine release

Mechanically evoked adenosine in the prefrontal cortex is primarily activity dependent. Chelating calcium with EDTA or preventing action potentials with TTX blocked most of the adenosine detected. Activity-dependent adenosine release was completely blocked by TTX in other regions (Wall and Dale 2013;Wall and Dale 2007) while mechanically-stimulated release in the prefrontal cortex was reduced by 65%; therefore, there may be a portion that is not activity dependent. Calcium chelators were more effective in blocking release, reducing

it by over 90%, indicating most of the release is calcium dependent. Adenosine can also be released via equilibrative nucleoside transporters (ENTs) (Wall and Dale 2013; Lovatt *et al.* 2012); however, inhibiting equilibrative nucleoside transporters with NBTI did not significantly change mechanically-evoked adenosine, ruling this mechanism out. Activity-dependent release of ATP, which breaks down to adenosine, was also explored. Blocking ATP metabolism with POM-1 significantly reduced the amount of adenosine detected. However, as with TTX, POM-1 did not completely block all adenosine; so while ATP metabolism contributes to the signal, some adenosine may be formed by another mechanism. Stimulated adenosine release can also be a downstream action of glutamate acting at ionotropic receptors (Pajski and Venton 2010; Wall and Dale 2008; Klyuch *et al.* 2011). Blocking AMPA receptors with CNQX had no effect on mechanically-stimulated adenosine release, so this release was not AMPA receptor dependent. Future studies could examine the effect of other glutamate receptors such as NMDA receptors, which could also regulate adenosine release (Wall and Dale 2013), as well as other neurotransmitters to examine if adenosine is a downstream effect of other receptor activation. Our studies show that mechanically-evoked adenosine release in the prefrontal cortex is primarily activity-dependent and mostly due to downstream breakdown ATP. This is in contrast to measurements of slower adenosine build up during seizures that suggest that release from ENTs is the primary mechanism and not breakdown of ATP (Lovatt *et al.* 2012). Thus, the mechanism of transient release may be different than other forms of adenosine signaling.

This work demonstrates that a local mechanical stimulation causes an elevation of adenosine concentration for less than a minute; thus transiently activating adenosine receptors. The function of rapid adenosine signaling after probe implantation remains unknown; however, adenosine signaling resulting from various forms of damage has been well documented in the literature (Frenguelli *et al.* 2007; Shen *et al.* 2011; Haselkorn *et al.* 2008). In particular, activation of A₁ receptors is inhibitory, which could dampen neurotransmission that causes excitotoxicity in the brain (Sperlagh *et al.* 2007). Adenosine could also stimulate blood flow after probe implantation, which would deliver nutrients for tissue repair. The immediate nature of the response allows for rapid activation of the pathway, but the fast duration would not allow long-term depression of neuronal activity. Further investigations of downstream effects of rapid adenosine signaling after mechanical stimulation as well as interactions of adenosine with other signaling molecules that are released would be interesting to better understand the function of adenosine as a rapid modulator in the brain.

Conclusion

Mechanically evoked adenosine was observed immediately following mechanical stimulations in the prefrontal cortex in both brain slices and *in vivo*. Adenosine could be evoked by moving the microelectrode or a glass pipette implanted near the microelectrode and stimulations could be repeated, indicating the tissue was not dying. Pharmacological studies confirm most of the adenosine detected is an extracellular breakdown product of ATP. Mechanically evoked adenosine was calcium dependent and activity-dependent but was not dependent on AMPA receptors. The study shows that implantation of a probe into brain tissue causes a transient adenosine response which could be neuroprotective.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CV	cyclic voltammograms
FSCV	fast-scan cyclic voltammetry
AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate
TTX	tetrodotoxin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
aCSF	nucleoside triphosphate diphosphohydrolase 1,2, and 3 (NTPDase 1,2, and 3), artificial cerebral spinal fluid
NBTI	S-(4-Nitrobenzyl)-6-thioinosine
POM-1	sodium polyoxotungstate
DAPI	4',6-diamidino-2-phenylindole

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A. Prefrontal Cortex, brain slices B. Prefrontal Cortex, anesthetized rat

Figure 1.

Adenosine after mechanical stimulation. The stimulation was quickly lowering a carbon-fiber microelectrode 50 μm in a prefrontal cortex slice (Panel A) or 100 μm in the prefrontal cortex of an anesthetized rat (Panel B) (red arrow denotes when electrode was lowered). A color plot with voltage on the y-axis, time on the x-axis and current in false color is shown. Adenosine occurs immediately after mechanical stimulation and is the green/purple region in the middle of the plot. Plots of the change in concentration for adenosine over time, located above the color plots, show that the adenosine signal lasted approximately 10 seconds with a peak concentration of 0.77 μM for slices (A) and lasted 80 s with a maximal concentration of 2.4 μM *in vivo* (B). The cyclic voltammograms have the typical primary and secondary adenosine oxidation peaks which confirm adenosine was detected.

Figure 2.

Four consecutive stimulations *in vivo*. In the prefrontal cortex of an anesthetized rat, 4 consecutive mechanical stimulations were performed every 30 minutes for 2 hours. A) Example current versus time traces are shown for four consecutive mechanical stimulations. There is no pattern of release decreasing over time. A) normalized current versus time plot in the inset demonstrates that the temporal profile of the release is not changing. B) Average concentration of adenosine for each stimulation was not significantly different ($n = 5$ rats, $p = 0.6781$, repeated measures one way ANOVA). The last (solid) bar shows the average for all 4 stimulations. C) The duration of adenosine signaling was also not significantly affected by the number of the stimulation ($n = 5$ rats, $p = 0.6224$, repeated measures one way ANOVA). The last (solid) bar is the average for all 4 stimulations.

Figure 3.

Adenosine is mechanically evoked either by both lowering the working electrode or a glass pipette close to the working electrode. Multiple electrode stimulations did not cause significant cell death. Adenosine can be mechanically stimulated by A) moving the carbon-fiber microelectrode and B) moving a 15 μ m-diameter glass pipette. Repeated stimulations were tested by moving the carbon-fiber microelectrode (A) or the glass pipette (B) 50 μ m down into the tissue, 50 μ m back up, and 50 μ m back down. Ten minutes of recovery time was given between each move. C) The bar graph compares the second (up) and third (down) stimulation for both techniques. Data is graphed as a percentage of first stimulation and there are no significant differences between the two techniques (repeated measures two-way ANOVA, $F(1,7)=0.2229$, $p=0.6512$) or stimulation number ($F(1,7)=5.046$, $p=0.0595$) and no interaction ($F(1,7)=0.1402$, $p=0.7192$). Repeated adenosine measurements within each technique are not statistically different (repeated measures one-way ANOVA, $F=0.3522$, $p=0.7882$, $n=5$). D) Cell imaging of the insertion site where there was multiple electrode stimulations reveals no obvious significant cell death. Blue represents DAPI staining, showing all nuclei. Red represents dead cells stained with a LIVE/DEAD cell assay and scale bar represents 50 μ m. E) There was no significant difference in dead cell percentage between a control region (no electrode insertion) and the electrode insertion site (paired t-test, $p=0.9061$, $n=4$).

Figure 4.

Adenosine is not mechanically evoked by small amounts of pressure ejected aCSF. Mechanically evoked adenosine by carbon-fiber microelectrode insertion was compared to pressure ejecting 100-200 nL volumes of aCSF 30 μm from the electrode in the same slice. A) Concentration versus time profile shows an increase in current when the carbon-fiber microelectrode was lowered and only a shift in current was detected after puffing on aCSF. B) Cyclic voltammograms show an adenosine CV for mechanical stimulation with the electrode by no identifiable adenosine with pressure ejection of aCSF.

Figure 5.

Mechanically evoked adenosine is activity dependent but is not released by equilibrative nucleoside transporters. A predrug mechanical stimulation was performed using a carbon-fiber microelectrode and then slices were perfused with drug and a second mechanical stimulation was performed 30 min later. Release was compared pre and post drug in the same animals using paired t tests. A) Control data. Cyclic voltammograms for mechanically-evoked adenosine are similar for two consecutive mechanical stimulations when the slice was perfused with aCSF. B) Cyclic voltammograms of adenosine show a large decrease in current after perfusion with 1 mM EDTA. C) Cyclic voltammograms of adenosine show a large reduction in mechanically-stimulated adenosine after perfusion with 0.5 μ M TTX. D) There was no change in the adenosine cyclic voltammograms after 10 μ M NBTI, an nucleoside transport inhibitor. The data were corrected for a 68% decrease in electrode sensitivity after NBTI. E.) The bar graph shows average mechanically-evoked release after drug as a % of the initial stimulation. Thus, no change would 100 %. Statistics were performed using paired t-tests comparing the release for pre- and post-drug stimulation. EDTA (n = 6, p = 0.0482) and TTX (n = 3, p = 0.0337) significantly decreased mechanically-stimulated adenosine release while there was no effect of NBTI (n = 5, p = 0.1507).

Figure 6.

Mechanically evoked adenosine is not dependent on AMPA receptors but is a downstream effect of ATP metabolism. A predrug mechanical stimulation was performed using a carbon-fiber microelectrode and then slices were perfused with drug and a second mechanical stimulation was performed 30 min later. Release was compared pre and post drug in the same animals using paired t tests. A) Cyclic voltammograms of adenosine show no change in current after 10 μ M CNQX, an AMPA antagonist. The data were correct for a 40 % decrease in electrode sensitivity after CNQX. B) Cyclic voltammograms (corrected for 30 % decrease in sensitivity) show that perfusion with 100 μ M POM-1 does change mechanically-stimulated adenosine. C.) Bar graph shows the average data as a % of the initial, pre-drug stimulation. The statistics are paired t-tests that compare the pre-drug and post-drug mechanically evoked adenosine in the same slice. There is a significant effect of POM-1 ($n=6$, $p = 0.0098$) but no significant effect of CNQX ($n = 5$, $p = 0.2935$).

Table 1

Mechanically evoked adenosine

	Concentration (μM)	Concentration Range (μM)	Duration	Duration Range	Percentage of slices detected
CFME in vivo n=5	3.3 ± 0.6	0.3-10	42 ± 5 s	11-110 s	100 %
CFME in slices n=25	0.8 ± 0.1	0.1-3.3	18 ± 2 s	2-46 s	100 %
Adenosine Sensor in slices n=5	0.6 ± 0.03	0.4-0.8	10 ± 2 s	1.8-20 s	50 %

*All values are $\pm$ SEM