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A_1 and A_{2A} receptors modulate spontaneous adenosine but not mechanically-stimulated adenosine in the caudate

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Abstract

Adenosine is a neuromodulator and rapid increases in adenosine in the brain occur spontaneously or after mechanical stimulation. However, the regulation of rapid adenosine by adenosine receptors is unclear, and understanding it would allow better manipulation of neuromodulation. The two main adenosine receptors in the brain are A_1 receptors, which are inhibitory, and A_{2A} receptors, which are excitatory. Here, we investigated the regulation of spontaneous adenosine and mechanically-stimulated adenosine by adenosine receptors, using global A_1 or A_{2A} knockout mice. Results were compared *in vivo* and in brain slices models. A_1 KO mice have increased frequency of spontaneous adenosine events, but no change in the average concentration of an event, while A_{2A} KO mice had no change in frequency but increased average event concentration. Thus, both A_1 and A_{2A} self-regulate spontaneous adenosine release, but A_1 acts on the frequency of events, while A_{2A} receptors regulate concentration. The trends are similar both *in vivo* and slices, so brain slices are a good model system to study spontaneous adenosine release. For mechanically-stimulated adenosine, there was no effect of A_1 or A_{2A} KO *in vivo*, but in brain slices there was a significant increase in concentration evoked in A_1KO mice. Mechanically-stimulated release was largely unregulated by A_1 and A_{2A} receptors, likely because of a different release mechanism than spontaneous adenosine. Thus, A_1 receptors affect the frequency of spontaneous adenosine transients and A_{2A} receptors affect the concentration, so future studies could probe drug treatments targeting A_1 and A_{2A} receptors to increase rapid adenosine neuromodulation.

Keywords

Fast-scan cyclic voltammetry; adenosine receptors; spontaneous adenosine; mechanosensitive adenosine; caudate; neuromodulation

Introduction

Adenosine plays an important role in the brain as a neuromodulator and a neuroprotector.[1–10] There are four known adenosine receptors, A_1, A_{2A}, A_{2B} and A_3 receptors, which are G protein-coupled receptors, but A_1 and A_{2A} receptors are the most prevalent receptors responsible for adenosine modulation in the brain.[11–14] A_1 receptors are the most abundant in the brain and inhibit neurotransmission by blocking adenylyl cyclase activity, while A_{2A}

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receptors, the second most abundant receptor type, are excitatory, activating adenylyl cyclase activity.[15] Adenosine receptors, particularly A_{2A} receptors, are located on blood vessels and modulate blood flow and oxygen consumption.[16,17] Moreover, adenosine receptors are also expressed on many neurons and regulate other neurotransmitters in the brain. For example, A_1 and A_{2A} receptors modulate synaptic release of glutamate, acetylcholine, serotonin, and GABA.[1,2,18–20] A_1 and dopamine D_1 receptors form heteromers as well as A_{2A} and D_2 receptors and they therefore modulate dopamine signaling.[21] However, it is still not clear how adenosine A_1 and A_{2A} receptors self-regulate adenosine release.

Traditionally, concentration changes of extracellular adenosine have been investigated on the minute to hour time scale.[22–24] However, electrophysiology experiments in Dunwiddie's group showed rapid signaling of adenosine, on the timescale of milliseconds to seconds.[25] Spontaneous, transient adenosine signaling was recently measured by fast scan cyclic voltammetry, lasting only few seconds.[9,16,26,27] Transient adenosine is activity dependent, and dependent on CD73, an enzyme that converts AMP to adenosine, suggesting spontaneous adenosine is vesicularly released as ATP and broken down in the extracellular space.[9,22,26,28–33] The frequency of spontaneous release is increased by DPCPX, an A_1 receptor inhibitor[34] and DPCPX also increases electrically-stimulated adenosine release.[35] The A_{2A} receptor antagonist, SCH 442416, eliminated the increase in adenosine and oxygen events during cerebral ischemia/reperfusion (I/R) *in vivo*.[36] Another mode of rapid adenosine release is mechanically-stimulated release, whereby moving a pipette or an electrode in the brain causes rapid adenosine release.[37] Mechanosensitive adenosine release is not dependent on CD73 and thus it may have a different mechanism of formation than spontaneous adenosine.[29,30,37] Mechanosensitive ATP release has been discovered in the brain due to swelling, mechanical perturbation, and shear stress.[38] Thus, while moving the electrode is an easy way to experimentally cause release, mechanically-stimulated adenosine is biologically relevant to physical damage the brain can suffer, such as from shear stress.[37] While adenosine can also be electrically stimulated, mechanically-stimulated adenosine is more reproducible.[35,39] However, little is known about how adenosine receptors modulate mechanically-stimulated adenosine.

Previous studies used pharmacology to understand the regulation of adenosine by blocking different receptors, but drugs cannot block or excite all receptors, and drugs must cross the blood-brain barrier to be utilized *in vivo*. Thus, genetically-altered mice provide an alternative to understand the global effects of receptors on adenosine regulation. Studies using A_1 knockout (A_1KO) and A_{2A} knockout (A_{2A}KO) mice have revealed the importance of these receptors for adenosine regulation.[40–42] For example, mice lacking adenosine A_1 receptors showed decreased hypoxic neuroprotection and an increased renal injury following ischemia and perfusion.[40,42–44] A_{2A}KO mice have less exploratory activity, more anxiety, and are less sensitive to depressant challenges than wild-type mice; moreover, A_{2A} KO mice have an attenuated response to focal ischemia, suggesting that removing A_{2A} receptors is neuroprotective.[45–47] Adenosine receptor knockout mice were also used to investigate adenosine-dopamine interactions, and A_{2A}-mediated neural functions are partially independent of D_2 receptors.[21,48] However, spontaneous, transient adenosine or mechanically-evoked adenosine events have not been measured in A_1 or A_{2A} KO mice.

In this study, we investigated whether adenosine receptors regulate spontaneous transient adenosine and mechanically-stimulated adenosine release *in vivo* and in brain slices using global knockout mice. Brain slice experiments are a useful biological model that bypasses the blood-brain barrier, however, spontaneous and mechanically-evoked adenosine events have not been directly compared between brain slice experiment and *in vivo* measurements. Data was collected in the caudate-putamen region in wild-type (C57BL/6), A_1KO, and A_{2A}KO mice. Both *in vivo* and brain slice results show A_1KO mice had an increased frequency of spontaneous adenosine events, without changing the mean concentration of each adenosine transient event. A_{2A}KO mice had no change in spontaneous adenosine event frequency but an increase in concentration. For mechanically-evoked adenosine, there was no significant difference in concentration *in vivo*, but in brain slices, A_1KO mice had a significantly higher concentration compared to wild-type mice. Overall, A_1 and A_{2A} receptors self-regulate spontaneous adenosine but self-regulation is less evident for mechanically-evoked release. Differential regulation of different modes of adenosine release may be useful to develop strategies that specifically target spontaneous adenosine to harness its neuromodulatory effects.

Results and Discussion

In this study, spontaneous and mechanically-stimulated adenosine release were compared in wild type mice (C57BL/6), A_1KO, and A_{2A}KO mice. All experiments were performed in the caudate-putamen, which has abundant A_1 and A_{2A} receptors,[49] and two model systems were compared: brain slices and anesthetized mice. The hypothesis is that the frequency or concentration of adenosine will change in the knockout mice if A_1 or A_{2A} receptors self-regulate adenosine.

Spontaneous Adenosine Release

Brain slice measurements—Brain slice experiments are easier than *in vivo* studies, especially for pharmacology studies, where there is no blood-brain barrier.[22,26] Figure 1 shows example color plots for spontaneous adenosine release in brain slices of control, A_1KO and A_{2A}KO mice. The color plots display the results in 3 dimensions, with potential on the y-axis, time on the x-axis, and current in false color. The green/purple circles in the center represent the primary adenosine oxidation peak (at 1.4 V), and the green/purple circles slightly below (at 1.0 V) is the secondary oxidation peak of adenosine. The concentration vs time graphs on top plot show the change of primary adenosine peak, which is converted to concentration using a calibration factor. Starred peaks were identified as spontaneous adenosine release via our automated algorithm.[50] Wild type (Fig. 1A) has fewer events than either A_1KO (Fig. 1B) or A_{2A}KO (Fig. 1C), and A_1KO has the highest number of spontaneous adenosine events.

Figure 2 compares average data for spontaneous adenosine events in brain slices among different genotype mice. Figure 2A shows the number of spontaneous events for one-hour measurement and there is a significant main effect of genotype (One-way ANOVA, $p = 0.023$, n=8–9 brain slices); A_1KO mice have significantly more spontaneous events than wild type mice (Tukey's multiple comparisons, $p<0.05$). However, the number of

spontaneous adenosine events is not significantly different between wild type and A_{2A}KO mice (Tukey's multiple comparisons, p>0.9999). Inter-event time is the time between two consecutive transients and is a measure of the frequency of adenosine events. Figure 2B shows the distribution of inter-event times in a histogram, with relative frequency of each bin on the y-axis and inter-event time on the x-axis (times were binned in 50 s bins). There is a main effect of genotype (Kruskal-Wallis test, p<0.0001, n=8–9 brain slices), with a higher frequency of events in A_1KO mice than wild type mice or A_{2A}KO mice (Dunn's multiple comparisons test, A_1KO vs WT: p=0.0019, A_1KO vs A_{2A}KO: p<0.0001). There is no significant difference in inter-event time distribution between WT and A_{2A}KO mice (p>0.99). Figure 2C shows the mean concentration of the first 10 spontaneous adenosine events from every slice (the first 10 are used in order to avoid the overrepresentation of some animals which have more transients). There is a significant main effect of genotype (One-way ANOVA, p=0.0004, n=80 transients/genotype) with A_{2A}KO mice having a significantly higher concentration than wild type or A_1KO mice (Tukey's multiple comparisons, WT vs A_{2A}KO: p=0.0016, A_{2A}KO vs A_1KO: p=0.0019) but there is no significant difference between wild type and A_1KO mice (p=0.99). Fig. 2D shows duration, which is defined as peak width at half height, and there is a main effect of genotype (One-way ANOVA, p=0.026, n=8–9 brain slices). A_{2A}KO mice have a shorter average duration compared to the other two genotypes (Tukey's multiple comparison, A_{2A}KO vs WT p=0.0463, A_{2A}KO vs A_1KO p=0.043) and there is no significant difference between WT and A_1KO (p=0.99). The concentration and duration distributions also proved that A_{2A}KO mice have a higher concentration and shorter duration (Figure S1).

***In vivo* measurements—**In order to confirm that the brain slice model is reliable for spontaneous adenosine measurements, we also measured spontaneous adenosine release *in vivo* in anesthetized mice. Since experiments can last longer *in vivo*, spontaneous adenosine was measured for four hours in each mouse.

Figure 3 shows examples of spontaneous adenosine measurements *in vivo*. In the example data, the number of transients is higher in A_1KO mice than WT or A_{2A}KO mice, and the concentration of adenosine transients in A_{2A}KO mice is higher than the other two genotype mice. The trend for number of transients in WT mice is the same for *in vivo* measurements as in brain slice measurements, with fewer transients in WT mice and more transients in A_1KO. However, *in vivo* data has higher concentration adenosine events than the brain slice experiment. Moreover, the number of spontaneous adenosine is more stable *in vivo* during the entire duration of the experiment, but most spontaneous adenosine in brain slice experiment happens during the first 30–45 minutes because of the difficulty of synthesis in brain slices.[22] In slices, adenosine synthesis is not as well maintained without the addition of adenine and ribose, but adenine can't be added in FSCV experiments because it is electroactive, so experiments are kept to one hour.[51]

Figure 4 shows averaged results of spontaneous adenosine measurements. For the average number of spontaneous adenosine events (Fig. 4A), there is a significant main effect of genotype on the number of transients (One-way ANOVA, main effect p=0.0026, n=8 animals). A_1KO mice have a significantly higher number of events compared to wild type mice (Tukey's multiple comparisons test, p=0.0019) but were not statistically different than

A_{2A}KO (p=0.074). The average number of spontaneous adenosine events in A_{2A}KO mice is not significantly different than wild type mice (p=0.24). Figure 4B shows the distribution of inter-event times, with data binned in 50 s intervals and there is a main effect of genotype on inter-event time (Kruskal-Wallis test, p<0.0001, n=8 animals). A_1KO mice have a significantly different distribution compared to WT and A_{2A}KO mice and A_{2A}KO are also different than WT (Dunn's multiple comparisons test, p<0.0001, n=8 animals). The inter-event time in A_1KO is much shorter than the other two types of mice, indicating the frequency of events is higher. Figure 4C shows the mean concentration of the first 60 spontaneous adenosine events from each animal (to use the same number from each to avoid overrepresentation), and there is a significant main effect of genotype (One-way ANOVA, p<0.0001, n=8 animals). A_{2A}KO mice have a significantly higher concentration than wild type or A_1KO mice (Tukey's multiple comparisons test, both p<0.0001), but there is no difference between wild type and A_1KO mice (p>0.99). Thus, A_{2A} receptors influence the concentration of individual transient adenosine events. Furthermore, there was a main effect of genotype on duration (Fig. 4D, One-way ANOVA, p=0.017, n=8 animals), and the average duration of each event in A_{2A}KO mice is shorter (Tukey's multiple comparisons test, p=0.015). There was no significant difference between wild type and A_1KO mice (p=0.10) or A_1KO and A_{2A}KO mice (p=0.62). Differences in duration are slight because the temporal resolution of FSCV is only 0.1 s. Figure S2 presents the concentration and duration histograms of spontaneous adenosine in all genotypes, and the distributions also proves that A_{2A}KO have a higher concentration and shorter duration.

Mechanically-Stimulated Adenosine Release

Brain slice measurements.—Mechanically-stimulated adenosine was measured in brain slices by lowering the electrode 50 μm three times per slice. Because slices are only 400 μm thick and measurements are generally performed in the middle to avoid dead layers on the edges, moving the electrode ~150 um is about the maximum possible. Separate slices were used, not the same slices where spontaneous events were measured, because slices have a limited time for viability.

Figure 5A shows example results of mechanically-stimulated adenosine. The CV shape and the current do not change for three mechanical stimulations in a one-hour period. Figure 5B is the current vs time trace for the stimulations in Figure 5A. Both the rise time and the duration of these 3 stimulated adenosine traces were similar and relatively stable. Figure 5C compares the average concentration by genotype and there is a main effect of genotype (One-way ANOVA, Tukey's multiple comparisons, p<0.0001, n=8 slices). The concentration in A_1KO mice is significantly higher than the other two genotypes (p<0.0001), but wild type mice and A_{2A}KO mice are not significantly different in concentration (p=0.85). Figure 5D compares duration using $t_{1/2}$ of the primary oxidation peak and there is a significant effect of genotype on duration (One-way ANOVA, p=0.025, n=8 slices). Wild-type mice have a significantly longer duration compared to A_1KO (Tukey's multiple comparisons, p=0.019). A_1KO mice had the fastest clearance among these three types of mice, but duration was not significantly different from A_{2A}KO mice (p=0.64).

In vivo measurements.—Mechanical stimulation was also performed *in vivo* by lowering the electrode 4 times, every 15 minutes. These experiments were performed in the same animals as spontaneous adenosine, after the 4 hours of spontaneous data collection. Fig. S3 shows the data by stimulation number and the concentration and duration of every mechanical stimulation is stable *in vivo* for multiple stimulations (Figure S3A–3F). Figure 6A shows an example of mechanically-evoked adenosine release in A_1KO mice *in vivo*, with a large adenosine event that begins with the mechanical stimulation at 30 s. The top cyclic voltammogram proves adenosine is detected and the concentration vs time curve shows the response is rapid and cleared within 60 s. *In vivo*, mechanically-evoked adenosine release is larger and longer in duration than spontaneous adenosine release. Figure 6B shows that the average concentration of mechanically-stimulated release is not significantly different by genotype (One-way ANOVA, p=0.31, n=8 animals per genotype). For duration, there are no significant differences among the three genotypes of mice (Fig 6C, One-way ANOVA, p=0.74, n=8 animals per genotype). Overall, knocking out A_1 or A_{2A} receptors does not change the concentration or duration of mechanically-stimulated adenosine *in vivo*.

Discussion

In this study, we investigated the role of A_1 and A_{2A} receptors to regulate spontaneous and mechanically-stimulated adenosine release both *in vivo* and in brain slices. Deletion of A_1 receptors increased the frequency of spontaneous adenosine events but did not change the concentration of the events. Knockout of A_{2A} receptors did not influence the frequency of spontaneous adenosine events but increased the concentration of each event. Brain slice and *in vivo* data had the same trends for spontaneous adenosine release, showing that brain slices are a good model system. For mechanically-stimulated adenosine, the concentration of adenosine was higher in A_1 KO mice in the brain slice model, but not *in vivo*. There was no effect of A_{2A} receptors on mechanically-stimulated adenosine. Thus, A_1 and A_{2A} receptors have greater effects on spontaneous adenosine release, and serve to differentially regulate frequency and concentration. This knowledge of adenosine receptor regulation of adenosine signaling is important for future development of drug treatments targeting A_1 and A_{2A} receptors to regulate rapid adenosine release and harness its rapid mode of neuromodulation.

A_1 receptors regulate spontaneous adenosine frequency—A_1 receptors are the most abundant adenosine receptors in the brain and inhibit adenylyl cyclase activity.[49] A_1 receptors are located presynaptically[1,21,52] and can inhibit vesicular release, including that of glutamate and ATP.[10] The mechanism of spontaneous adenosine formation is through the rapid breakdown of extracellular ATP,[53] and thus regulation of ATP release will also regulate adenosine formation. In addition, previous studies demonstrated DPCPX, an A_1 receptor antagonist, regulated the frequency of spontaneous adenosine events, but not the concentration of adenosine release.[32,34,54]

Here, we studied global deletion knockout mice, where the receptor is fully deleted, to better understand the effects of A_1 receptors on spontaneous adenosine. A_1 receptor KO mice have previously been used to determine the effects of A_1 receptors in regulating sleep[55] and seizures after traumatic brain injury.[56] The hypothesis is that deleting inhibitory A_1 receptors would remove presynaptic inhibition of adenosine release and increase the

frequency of adenosine transients. Indeed, in both brain slices and *in vivo*, there was a significantly higher number of spontaneous adenosine events in A_1KO mice compared to wild type. Deletion of A_1 receptors only increased the frequency of spontaneous adenosine, but did not affect the average concentration or the duration. A_1 receptors regulate the frequency of exocytotic events, and global deletion therefore allows more exocytosis, causing more adenosine events. However, the loading of the vesicles remains the same so the average concentration is not affected. Previous studies have shown that spontaneous adenosine is regulated by the frequency of release, particularly when pharmacological agents are given to block A_1, $GABA_B$, or NMDA receptors.[32,34] Mice with global deletions of CD39 or CD73, the enzymes that breakdown extracellular ATP, also had lower frequency of release, but little to no effect on concentration.[34,53,54] Therefore, A_1 receptors, acting presynaptically, regulate the frequency of spontaneous adenosine release, and this mechanism could be explored in the future as a method of harnessing the rapid neuromodulatory properties of adenosine.

A_{2A} receptors regulate the concentration but not the frequency of spontaneous adenosine—A_{2A} receptors are the second most abundant adenosine receptors in the brain, and are expressed at high levels in the caudate-putamen region.[1,49,57,58] A_{2A} receptors are excitatory receptors that stimulate adenylyl cyclase activity to increase cAMP. A_{2A}KO mice have been widely used for behavioral or pharmacology research; for example, A_{2A}KO attenuates brain injury in mice, A_{2A}KO mice are less sensitive in depression tests, and deletion of A_{2A} receptors influences anxiety in mice.[44,47,59,60] A_{2A} receptors are most densely located post-synaptically in the striatum.[61] However, A_{2A} receptors are also located presynaptically, where they control the release of glutamate[62,63] by tightly interacting with A_1 and other receptors.[64] Moreover, A_{2A} receptors are engaged in neuromodulation in the caudate.[65–69] Here, A_{2A} KO mice had no change in frequency of spontaneous events, suggesting that presynaptic A_{2A} receptors do not control the frequency of spontaneous adenosine events. A_{2A} receptors do not regulate baseline neurotransmitter release but do regulate faster events related to long-term potentiation (LTP)[65] however the lack of A_{2A} receptors does not change adenosine frequency and so spontaneous adenosine release does not appear linked to LTP processes.

The main effect observed in A_{2A} KO mice is that the average concentration of each adenosine event was larger. There are a few possible explanations for this increase in concentration. A_{2A}KO may enhance the breakdown of ATP to extracellular adenosine by enzymes such as CD73, because CD73 is colocalized with A_{2A} receptors in the caudate putamen and activation of A_{2A} receptors requires CD73.[70] Therefore, knocking out A_{2A} receptors may change the expression of CD73, which could result in a higher extracellular adenosine concentration. Another possible mechanism is compensation by adenosine A_{2B} receptors, which are also excitatory. A_{2B} receptor expression is 4.5 fold higher in A_{2A}KO mice than wild type mice.[71] The regulation of spontaneous adenosine concentration by A_{2B} receptors is not known, but could be investigated as a compensatory mechanism.

The duration of spontaneous adenosine in A_{2A}KO mice is also significantly shorter than wild type mice both *in vivo* and in brain slice (Fig. 2D, 4D). A_{2A} receptors may regulate adenosine deaminase or adenosine kinase, the main metabolic enzymes which are

responsible for fast extracellular breakdown of adenosine.[26] For example, A_{2A} receptors are affected by adenosine deaminase binding and A_{2A}KO could increase adenosine deaminase activity, which speeds up spontaneous adenosine clearance. Spontaneous adenosine is also cleared by equilibrative nucleoside transporters (ENTs),[26,72] and these ENTs are modulated by A_{2A} receptors to control the extracellular adenosine level in rat hippocampus.[26,73] Future studies could study adenosine clearance in A_{2A}KO mice with pharmacology to determine the mechanism of clearance.

Mechanically stimulated adenosine is not dependent on A_1 or A_{2A} receptors— Moving an electrode in a brain slice causes mechanically-stimulated adenosine without causing significant tissue damage.[37] Mechanical stimulation is therefore a way of causing shear stress in the brain, without killing the cells. The mechanism of formation of mechanically-stimulated release is different than spontaneous adenosine release, as it is not dependent on the adenosine breakdown enzymes CD39 or CD73, implying that it is not due to extracellular breakdown of ATP.[30] Thus, we hypothesized that regulation of mechanically-stimulated release by A_1 and A_{2A} receptors would be different than regulation of spontaneous adenosine release. In A_1 and A_{2A}KO mice, there were no changes in mechanically-stimulated concentration or duration *in vivo*. However, in brain slices, the concentration was significantly higher in A_1KO mice and the duration was significantly lower (Fig. 5C, 5D). These data are the only data from brain slices and *in vivo* which do not agree, as all other data showed the same trends. The reason might be that smaller concentrations are elicited in brain slices in WT, and thus larger concentrations in A_1KO are easier to observe. Larger concentrations are expected if you remove inhibition. However, the *in vivo* data do not show that trend, and stimulations are large for all genotypes, likely due to robust pools of adenosine maintained by synthesis. The source of mechanically-stimulated release is less understood. There is some evidence for exocytosis, as tetrodotoxin or EDTA decrease the concentration of mechanically-stimulated adenosine, suggesting it is activity dependent.[25,37,39] However, other studies suggest it could be regulated by hemichannels such as pannexins or connexins, which are mechanosensitive.[74] Thus, regulation may not be through presynaptic mechanisms and the overall results here suggest mechanically-stimulated release is not as strongly regulated by A_1 and A_{2A} receptors. The data also support that A_1 and A_{2A} drugs would preferentially regulate spontaneous and not mechanically-stimulated adenosine release, allowing a way to tap into the rapid neuromodulatory properties of spontaneous release.

Conclusions

In this paper, we investigated the role of adenosine receptors to regulate spontaneous adenosine and mechanically-stimulated adenosine by using knockout mice. A_1KO mice have an increased frequency of spontaneous adenosine, but no change in concentration, both *in vivo* and in brain slices. A_1 receptors act as presynaptic inhibitors, inhibiting exocytotic events that cause spontaneous adenosine. Deletion of A_{2A} receptors resulted in higher concentrations of spontaneous adenosine, which may be related to interplay of A_{2A} receptors with adenosine breakdown enzymes or compensation by A_{2B} receptors. *In vivo*, mechanically-stimulated adenosine concentration is not dependent on A_1 or A_{2A} receptors, suggesting it is regulated differently than spontaneous release. This differential regulation of

release is important because it could lead to specific pharmacological treatments of A_1 or A_{2A} receptors that target manipulation of spontaneous adenosine release but not mechanically-stimulated release.

Methods

Chemicals

Artificial cerebral spinal fluid (aCSF) consisted of 126mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$ dihydrate, 1.2 mM $MgCl_2$ hexahydrate, 25 mM $NaHCO_3$, 11 mM glucose, and 15 mM tris(hydroxymethyl) aminomethane and was adjusted to pH 7.4 before the experiment. Adenosine was purchased from Acros organics (Morris Plains, NJ, USA) and dissolved in 0.1 M $HClO_4$ for 10 mM stock solution. Stock solutions was diluted to 1 μM in aCSF for the electrode post calibration after brain slice or *in vivo* experiments.

Electrochemistry

Cylinder carbon-fiber microelectrodes were fabricated as described previously.[75] Briefly, electrodes were made by vacuum-aspirating a T-650 carbon fiber (7 μm diameter, Cytec Engineering Materials, West Patterson, NJ) into a glass capillary and pulling into two electrodes by an electrode puller (model PE-21, Narishige, Tokyo, Japan). The pulled electrode tip was cut to 50–100 μm long for brain slice experiment and 150–200 μm long for *in vivo* experiment. Cyclic voltammograms were collected using a ChemClamp (Dagan, Minneapolis, MN, USA) with HDCV (UNC Chemistry, Chapel Hill, NC, USA). The electrode was scanned by a triangular waveform from −0.4 V to 1.45 V with a frequency of 10 Hz at 400 V/s. Electrodes were calibrated after the experiment by testing their response to 1 μM adenosine in a flow cell and a calibration value obtained (in nA/ μM) that was used to convert currents to concentrations.

Brain Slice experiments

All animal experiments were approved by the Animal Care and Use Committee of the University of Virginia. C57BL/6 mice (6–8 weeks old, Jackson Lab), A_1 receptor knockout mice and A_{2A} receptor knockout mice (6–8 weeks old, obtained from Dr. S. Jamal Mustafa, West Virginia University) were housed in a vivarium and given food and water *ad libitum*. Mice were anesthetized with isoflurane and beheaded immediately. The mouse brain was removed within 2 min and placed in 0–5°C artificial cerebral spinal fluid (aCSF) for 2 min for recovery. Four hundred-micrometer coronal slices of the caudate–putamen were prepared using a vibratome (LeicaVT1000S, Bannockburn, IL, USA), and transferred to oxygenated aCSF (95% oxygen, 5% CO_2), to recover for an hour before the experiment. aCSF (maintained at 35–37°C) flowed over the brain slices using a perfusion pump (Watson-Marlo 205U, Wilmington, MA, USA) at a rate of 2 mL/min for all experiments. Spontaneous adenosine transients were measured by inserting the electrode about 75 μm into the tissue and collected 1 h data after 10 min equilibrium. Mechanical stimulation experiments were performed by lowering the electrode 50 μm every 30 minutes. The electrode was placed in the caudate-putamen (AP +1.1 mm, ML + 1.5 mm, and DV −3.0 mm, scheme is shown in Figure S4). Only one slice was used per animal, so all n values are different animals.

In vivo experiment

All experiments were approved by Animal Care and Use Committee of the University of Virginia. C57BL/6 mice (6–8 weeks old, Jackson Lab), A_1 receptor knockout mice, and A_{2A} receptor knockout mice (6–8 weeks old, both KO obtained from Dr. S. Jamal Mustafa, West Virginia University)[71,76] were housed in a vivarium and given food and water *ad libitum*. Both of the KO mice are on the C57B background and that is the standard control mouse used for studies of their function.[71,76] Mice were anesthetized by flowing 4% isoflurane in 100% oxygen for induction and maintained with 1.5–3% in 100% oxygen via a facemask (Stoelting, Wood Dale, IL, USA), tail pinch to ensure that anesthesia is complete and sustained. Isoflurane levels were adjusted to until loss of righting reflex was observed. The mouse was laid on a heating pad maintaining the temperature around 37 °C. The surgical site was shaved, the skull was exposed, and a hole drilled that allowed the placement of the electrode in the caudate-putamen (AP +1.1 mm, ML + 1.5 mm, and DV −3.0 mm, scheme is shown in Figure S4). Bupivacaine (0.10 mL, APP Pharmaceuticals, Schaumburg, IL, USA) was applied under the skin for local anesthesia before drilling the skull. Spontaneous adenosine transient events were measured for 4 h total and mechanically-stimulated adenosine events were measured by lowering the electrode 100 μm every 15 minutes.

Statistics

Spontaneous adenosine transients were identified and characterized by an automated algorithm and adenosine transients were confirmed by an analyst to exclude any signals that were not adenosine, the duration results were determined at half-height of the primary peak.[50] All statistics were performed by using GraphPad 8 (GraphPad Software Inc., San Diego, CA, USA). All data are shown as mean ± SEM. Statistical significance was designated at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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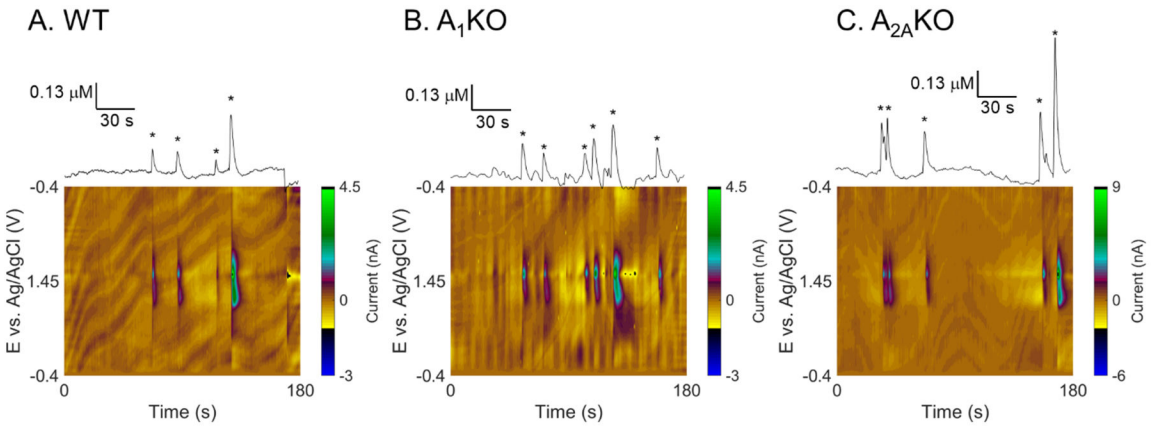

Figure 1.

Examples of spontaneous adenosine release in brain slices. Top: concentration vs time trace, with stars indicating the peak was identified as spontaneous adenosine release by our automated algorithm. Bottom: 3-D color plot of spontaneous adenosine release in three different types of mice. A. Wild type, B: A_1KO, and C: A_{2A}KO.

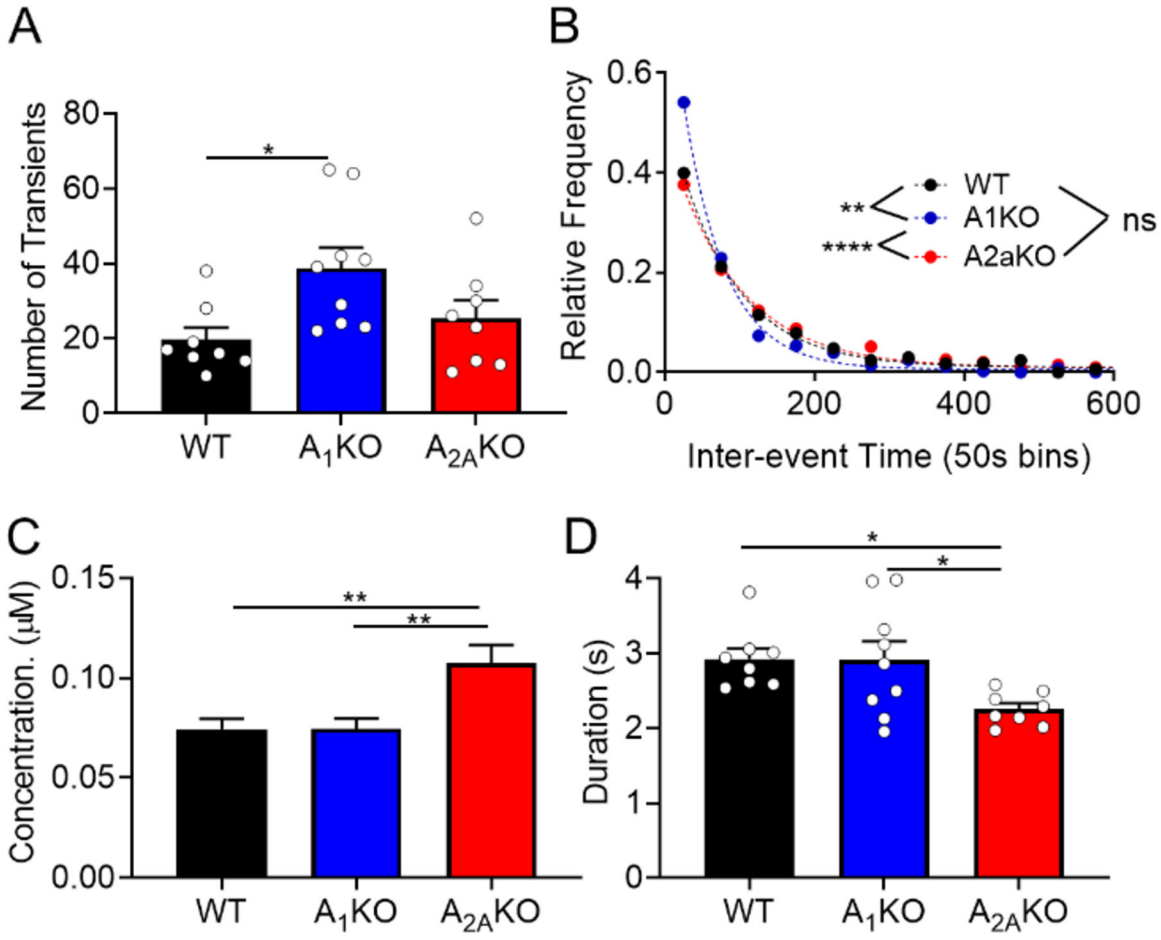

Figure 2.

Spontaneous adenosine measurement in brain slices. A. Number of spontaneous adenosine events varies by genotype (One-way ANOVA, overall p=0.023, n=8–9 brain slices, 1 slice per animal). B. Inter-event time histogram (50 s bins) of all adenosine transients (Kruskal-Wallis test, overall p<0.0001). C. Mean concentration of first 10 spontaneous adenosine release in every slice. (One-way ANOVA, Tukey's multiple comparisons, overall p=0.0004, 80 transients in each genotype, **p<0.01, n=8 brain slices, 1 slice per animal) D. Average duration of spontaneous adenosine release (One-way ANOVA, Tukey's multiple comparisons, overall p=0.026) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, error bars are SEM. WT=wild type C57BL/6 mice.

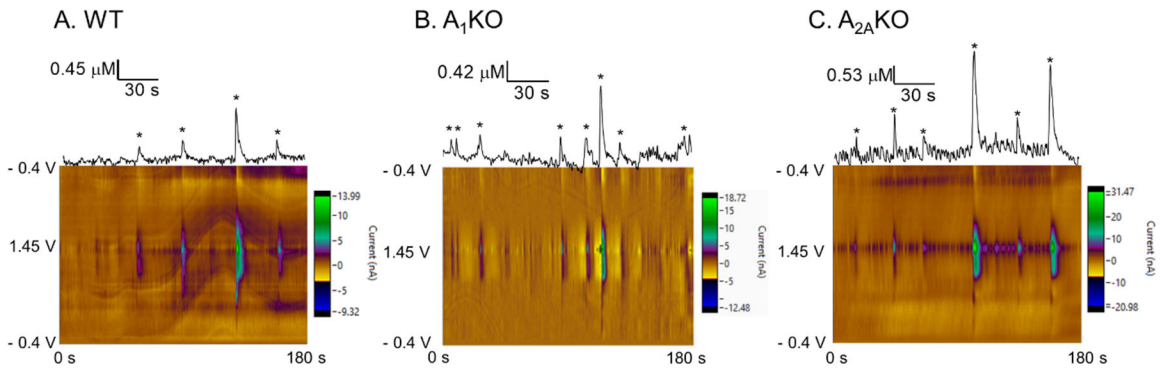

Figure 3.

Examples of spontaneous adenosine release *in vivo*. Top concentration vs time traces, with stars indicating the peak was identified as spontaneous adenosine release by our automated algorithm. Bottom: 3-D color plots of spontaneous adenosine release. A. Wild type, B: A_1KO, and C: A_{2A}KO.

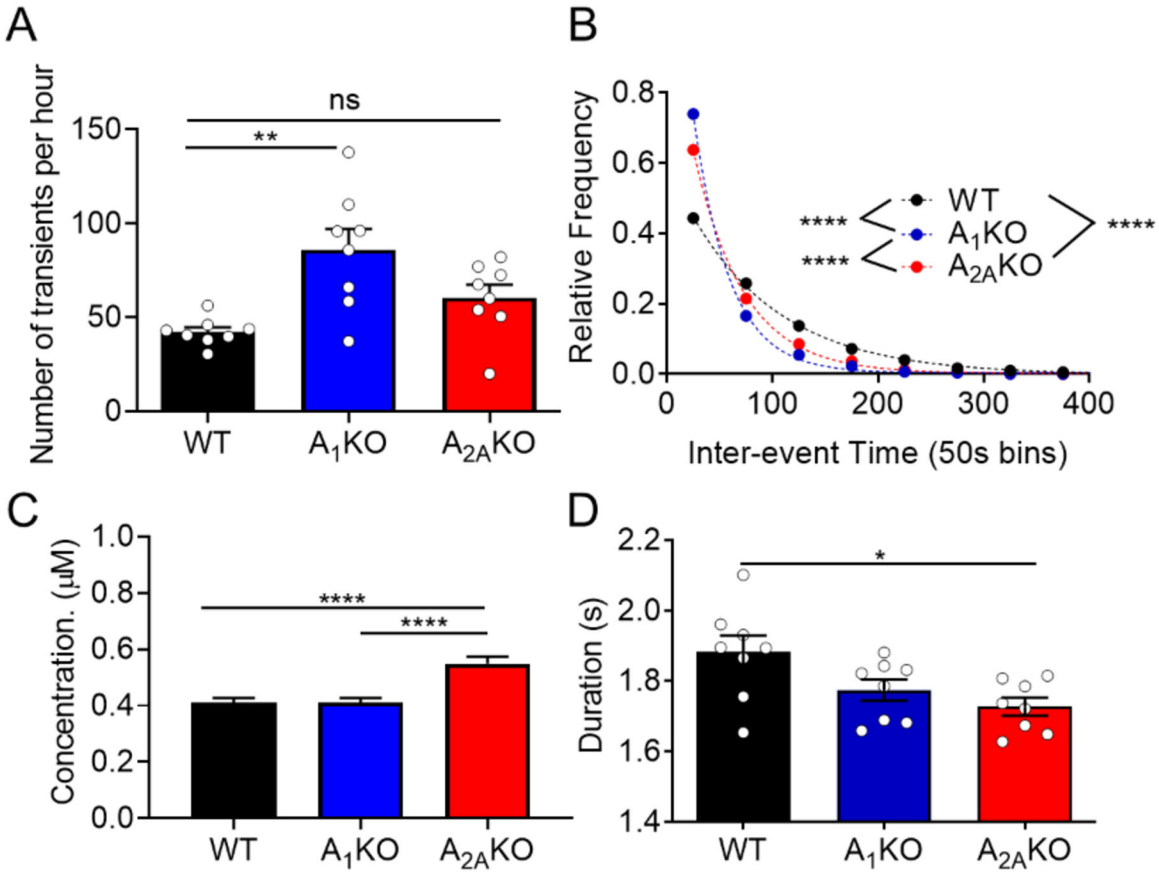

Figure 4.

Spontaneous adenosine release *in vivo*. A. Number of spontaneous adenosine events per hour (One-way ANOVA, overall p=0.0026, n=8 animals). B. Inter-event time histogram (50 s bins) of all adenosine transients (Kruskal-Wallis test, overall p<0.0001, n=8 animals) C. Mean concentration of first 60 spontaneous adenosine release (One-way ANOVA, overall p<0.0001, n=8 animals). D. Average duration of spontaneous adenosine release (One-way ANOVA, Tukey's multiple comparisons, overall p=0.017, n=8 animals), *p<0.05, **p<0.01, ****p<0.0001, error bars are SEM. WT=wild type C57BL/6 mice

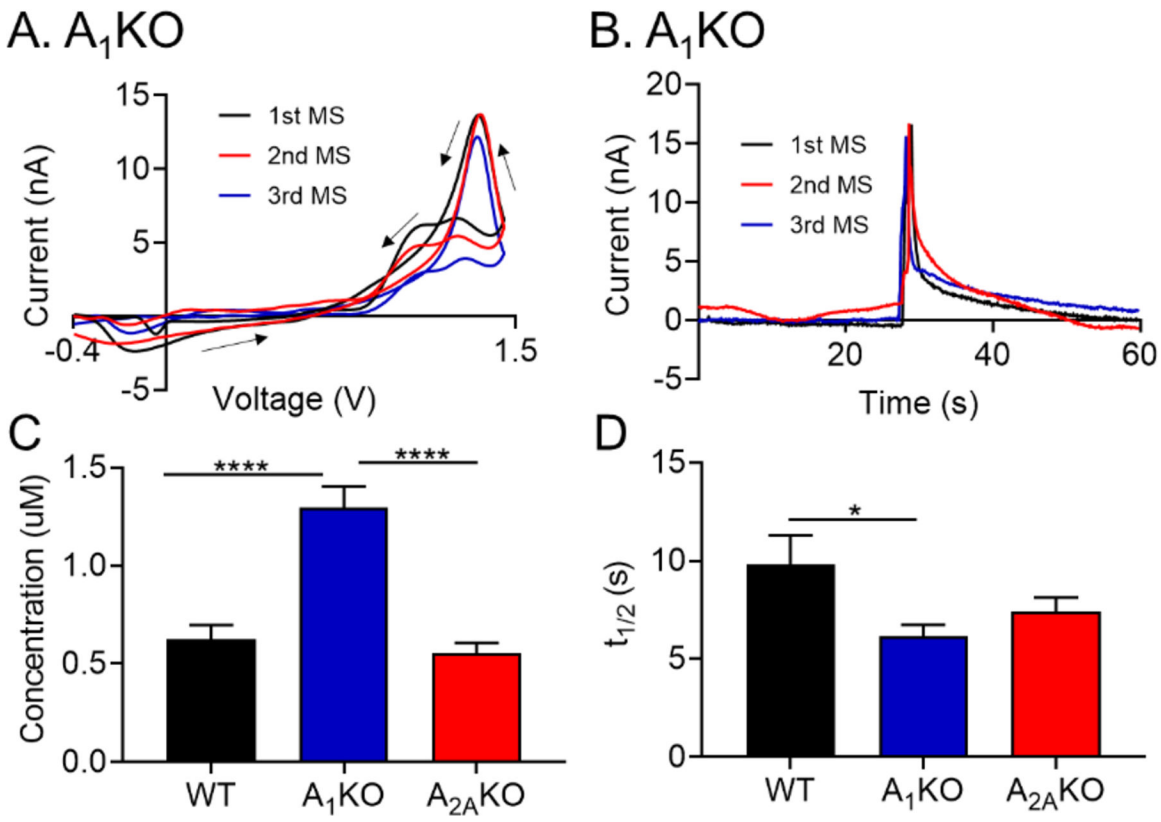

Figure 5.

Mechanically-stimulated adenosine in brain slices. A. Example CV of stimulated adenosine release in A_1KO mice brain slice, where the electrode was lowered 50 μm every 15 minutes three times. MS=mechanical stimulation. The black arrows mean the direction of the CV scanning. B. Current vs Time of the primary oxidation peak for same stimulations in A_1KO mice as 5A. C. Average concentration of each stimulation (One-way ANOVA, Tukey's multiple comparisons, overall $p<0.0001$, n=8 slices, 1 slice per animal). D. Average duration ($t_{1/2}$) varies by genotype. (One-way ANOVA, Tukey's multiple comparisons, overall $p=0.025$, n=8 slices, 1 slice per animal.) *$p<0.05$, ****$p<0.0001$, error bars are SEM.

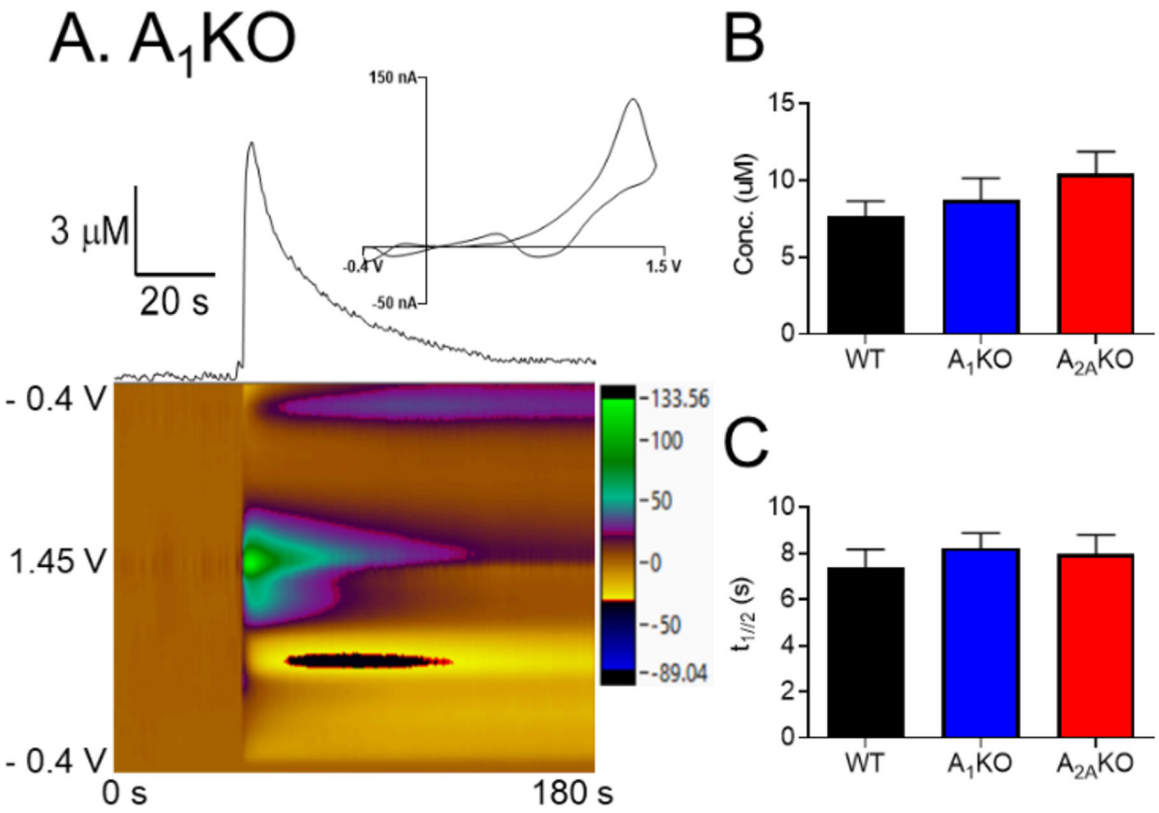

Figure 6.

Mechanically-stimulated adenosine measurement *in vivo*. A. Example data of mechanical stimulation in A_1KO mice. Bottom figure is the color plot of the measurement by FSCV (x-axes is time, y-axes is potential and the color differences represent current). Top left is concentration vs time curve of the primary peak, top right is the cyclic voltammogram of adenosine at 30 s. B. Comparison of mechanically-stimulated adenosine concentration shows no difference by genotype (One-way ANOVA, Tukey's multiple comparisons, overall p=0.31, n=8 animals). C. Comparison of $t_{1/2}$ shows no differences genotypes (One-way ANOVA, Tukey's multiple comparisons, overall p=0.74, n=8 animals).