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Transient adenosine release is modulated by NMDA and $GABA_B$ receptors

Michael D. Nguyen, **Ying Wang**, **Mallikarjunarao Ganesana**, and **B. Jill Venton***

Department of Chemistry and Neuroscience Graduate Program, University of Virginia, Charlottesville, VA, USA

Abstract

Adenosine is a neuroprotective agent that modulates neurotransmission and is modulated by other neurotransmitters. Spontaneous, transient adenosine is a recently discovered mode of signaling where adenosine is released and cleared from the extracellular space quickly, in less than three seconds. Spontaneous adenosine release is regulated by adenosine A_1 and A_{2a} receptors, but regulation by other neurotransmitter receptors has not been studied. Here, we examined the effect of glutamate and GABA receptors on the concentration and frequency of spontaneous, transient adenosine release by measuring adenosine with fast-scan cyclic voltammetry in the rat caudate-putamen. The glutamate NMDA antagonist, 3-(R-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, 6 mg/kg i.p.), increased the frequency of adenosine transients and the concentration of individual transients but NMDA (agonist, 50 mg/kg, i.p.) did not change the frequency. In contrast, antagonists of other glutamate receptors had no effect on the frequency or concentration of transient adenosine release, including the AMPA antagonist NBQX (15 mg/kg i.p.) and the mGlu2/3 glutamate receptor antagonist LY 341495 (5 mg/kg i.p.). The $GABA_B$ antagonist CGP 52432 (30 mg/kg i.p.) significantly decreased the number of adenosine release events while the $GABA_B$ agonist baclofen (4 mg/kg i.p.) increased the frequency of adenosine release. The $GABA_A$ antagonist bicuculline (5 mg/kg i.p.) had no significant effects on adenosine. NMDA and $GABA_B$ likely act presynaptically, affecting the overall cell excitability for vesicular release. The ability to regulate adenosine with NMDA and $GABA_B$ receptors will help control the modulatory effects of transient adenosine release.

Keywords

voltammetry; NMDA; $GABA_B$; baclofen; CPP; CGP 52432

*Corresponding author: PO Box 400319, Charlottesville, VA 22904, Phone: 434-243-2132, jventon@virginia.edu.

Author contributions

MDN and BJV conceived of the project. MDN and YW performed the experiments. MDN, YW, and MG analyzed the data. MDN and BJV wrote the paper.

Notes

The authors declare no competing financial interest.

Associated content

Supporting information: Supplemental methods (electrochemistry) and a supplemental figure (Fig. S1) of vehicle injection are provided. Figure S2 shows histology.

Introduction

Adenosine is an important neuromodulator in the brain, regulating neurotransmission. Generally, adenosine has an inhibitory effect on neurotransmission,[1] acting through A_1 adenosine receptors to depress release. For example, adenosine regulates glutamate, GABA, and dopamine neurotransmission.[2,3] Many neurotransmitters also regulate the release of adenosine, revealing a feedback loop between adenosine modulation of neurotransmission and neurotransmitter regulation of adenosine.[4–6] The ability of neurotransmitters to regulate adenosine often differs based on brain region and type of adenosine signaling. Our lab has recently discovered a new mode of adenosine signaling: spontaneous, transient release that lasts for only about three seconds.[7–10] These spontaneous transients are modulated by A_1 receptors [7] and A_{2a} receptors[11] in the caudate and prefrontal cortex but regulation by other neurotransmitter receptors has not been studied.

Glutamate is the primary excitatory neurotransmitter and adenosine modulates glutamate release. In the hippocampus, adenosine receptors inhibit glutamate efflux[12] through pre-synaptic A_1 receptors, while A_1-A_{2a} heteromers in glutamatergic terminals[13] exert dynamic inhibitory and excitatory control of glutamate neurotransmission.[14] Similarly, glutamate can also regulate the release of adenosine. For example, application of the glutamate agonist NMDA evokes adenosine release in cerebellum brain slices[15] and decreases neuromodulation due to adenosine acting at A_1 receptors.[16] The glutamate agonist, AMPA, evokes adenosine release in the basal forebrain and cortex.[17] A combination of NMDA and AMPA antagonists decreased high and low frequency stimulated adenosine release in striatal brain slices.[18] However, in neostriatal brain slices, A_{2a} agonists inhibited NMDA currents but not AMPA currents.[19] Dale's group found that train-evoked adenosine release is independent of NMDA and AMPA receptors, while single pulse, evoked adenosine is dependent on NMDA and AMPA receptors in the cerebellum.[20] Metabotropic glutamate (mGlu) receptors also interact closely with adenosine receptors,[21] as A_{2a} and metabotropic glutamate receptors are co-localized pre-synaptically.[22] Single pulse adenosine release is also dependent on mGlu4 receptors in cerebellar brain slices.[6] Thus, the regulation of adenosine by glutamate receptors depends on the type of adenosine release and brain region.

GABA, the precursor to glutamate, is an inhibitory neurotransmitter that is also regulated by adenosine. During normoxic conditions in the hippocampus, potassium-evoked GABA release is decreased through A_1 and A_{2a} receptors, while during ischemia only A_{2a} receptors decrease release.[23] Similarly, adenosine suppresses GABA induced currents through A_1 receptors in the dorsal horn of rats[24] and inhibits evoked GABA release in the hypothalamus.[25] The modulatory interactions between adenosine and GABA are bi-directional, as GABA also modulates evoked adenosine release. Single pulse adenosine release is dependent on $GABA_B$ receptors in cerebellar brain slices.[6] Thus the interplay between adenosine and GABA is dependent on cellular conditions, region, and release type.

Here, we examine the effects of glutamate and GABA receptors to regulate spontaneous, transient adenosine release in the caudate-putamen of anesthetized rats. Administering antagonists of NMDA and $GABA_B$ modulated the frequency of spontaneous adenosine release, while antagonists of AMPA, $GABA_A$, and group II metabotropic glutamate

receptors had no effect. However, the effects were opposite those expected if NMDA and $GABA_B$ receptors caused release; blocking NMDA with CPP caused larger, more frequent adenosine transients while blocking $GABA_B$ with CGP 52432 decreased the frequency of adenosine transients. Administering NMDA did not have the opposite effect of the NMDA antagonist, suggesting the receptor is already activated, but baclofen, a $GABA_B$ antagonist did increase the frequency of adenosine transients, an effect opposite that of the $GABA_B$ agonist. Thus, NMDA and $GABA_B$ appear to modulate adenosine through presynaptic mechanisms, a key finding that will enable future studies that control spontaneous, transient release to harness its neuromodulatory properties.

Results and Discussion

Adenosine Detection with Fast-scan Cyclic Voltammetry

Adenosine release was measured rapidly using fast-scan cyclic voltammetry and spontaneous events were monitored *in vivo* in anesthetized rats. Carbon-fiber microelectrodes were inserted into the caudate-putamen of the rat brain and a waveform continuously applied to electrochemically detect adenosine. Applying the waveform causes a large background current, which is subtracted out; thus fast-scan cyclic voltammetry is a differential technique to measure rapid changes in concentration on a sub-second time scale.[26] Adenosine undergoes a set of two electron oxidations[27,28] at the microelectrode that results in a current that is proportional to the concentration of adenosine at the electrode.

Cyclic voltammograms for adenosine (Fig. 1A) show the characteristic electrochemical fingerprint for adenosine with a primary oxidation peak at 1.4 V and a secondary oxidation peak at 1.0 V. Using principal component analysis, a concentration vs time plot (Fig. 1B) for adenosine is calculated[7] and displayed directly above the color plots (Fig. 1C). Three spontaneous adenosine release events are starred. We have also used a recently written computer program to automate the identification of adenosine transients from these traces.[29] Three dimensional color plots of fast-scan cyclic voltammetry data are commonly plotted to show changes in analyte oxidation over time. The green/purple circles in the center of the 3-D plot are the primary oxidation peaks, while the green/purple circles directly below at 1.0 V are the secondary oxidation peaks for adenosine. The data show that large adenosine events are released and rapidly cleared from the extracellular space in less than four seconds. All data collection occurred after at least an hour of electrode implantation, in order to minimize any adenosine changes from damage due to insertion, as previous work has found immediate mechanosensitive adenosine release (within seconds) upon electrode movement.[9] Experiments were performed in anesthetized animals immobilized in a stereotaxic apparatus so the electrode would not move during the experiment.

Control Experiments

To verify that the frequency and concentration of adenosine transients did not change over time, a control experiment was performed with a vehicle injection of DMSO and saline. Adenosine transients in the hour before vehicle were compared to transients in the hour after vehicle administration. There was no significant difference in the number of transients (Fig. S1, n = 6 animals, paired *t*-test, $p = 0.31$) or underlying frequency distributions (Fig. S1B, n

= 6 animals, KS test, $p = 0.74$). There was also no significant difference in the concentration of individual adenosine transients (Fig. S1C, n = 250 events pre-drug and 221 events post-vehicle, unpaired *t*-test, $p = 0.71$) or the maximum cumulative concentration of transients (Fig. S1D, n = 6 animals, paired *t*-test, $p = 0.11$) following DMSO/saline injection. Thus, the frequency and concentration of adenosine transients do not change with time and any changes observed in drug experiments can be attributed to the drug.

Histology was performed to prove the electrode did little damage to the brain. Fig. S2 shows the path of an electrode with its placement in the caudate putamen. The track is not easy to see, proving our electrode does not cause much tissue damage. This medial area of the caudate was chosen because transients have been previously recorded here,[7] but the striatum is heterogeneous and adenosine release may vary within the caudate.[30]

Glutamatergic Regulation of Adenosine Release

NMDA Receptors—Antagonists were used to test the effect of different receptors to mediate adenosine release. CPP, a selective NMDA receptor antagonist, was injected at 6.25 mg/kg i.p., a dose previously established to be an effective antagonist in the brain.[31,32] Example data show the difference between pre-drug (Fig. 2A) and post-CPP transients (Fig. 2B). The color plot and concentration vs time traces for pre-drug data show five transients in an 80 second window, while eight transients are observed in the same time frame following CPP administration. The concentration of some adenosine events also appears larger after CPP. On average, although there was a trend towards more transients released after CPP administration, the number of transients did not significantly increase (Fig. 2C, n = 8 animals, paired *t*-test, $p = 0.28$). The box plot shows the median as the middle line, with the colored region being 25–75 % of values and the whiskers showing the range. However, when a histogram is made of the inter-event times, i.e. the time between consecutive transients, and the underlying distributions compared before and after drug administration, there was a significant difference in the frequency of adenosine release (Fig. 2D, n = 8 animals, KS test, $p = 0.035$). Similarly, the mean inter-event time decreased from 47 ± 2 s to 41 ± 3 s (n = 582 events pre-drug, n = 668 events post-drug, unpaired *t*-test, $p = 0.048$). The concentration per transient was calculated using all the transients and the mean concentration increased after CPP (Fig. 2E, n = 590 events pre-drug and 676 events post-drug, unpaired *t*-test, $p = 0.039$). To examine the cumulative concentration, the concentration of each transient was added for the one hour pre-drug or one hour CPP time period. Similar to the number of transients, the maximum cumulative concentration shows a trend of increasing after CPP, but the differences were not significant (Fig. 2F, n=8 animals, paired *t*-test, $p = 0.13$). The number of transients and cumulative concentrations were averaged per animal while for frequency distributions and concentration per transient, all adenosine transient event data were used. Thus, the larger n using all the transients made it easier to pull out significant differences in frequency and event concentration, but the same trends are observed in the measures that used only one value per animal.

For all antagonists that significantly affected adenosine release, we also administered the agonist to test its effect. Fig. 3 shows the data for NMDA (50 mg/kg i.p.), an NMDA receptor agonist. The dose of 50 mg/kg i.p. was chosen because it is low enough not to cause

death or seizures[33] but large enough to see effects in the brain[34]. The number of transients (Fig. 3A, n = 7 animals, paired *t*-test, *p*=0.85) did not change with NMDA. Similarly, the mean inter-event times (pre-drug: mean = 70 ± 6 s, n = 325 events; postdrug: mean = 73 ± 5 s, n = 319 events; unpaired *t*-test, *p* = 0.85) as well as the distribution of inter-event times (Fig. 3B, n = 7 animals, KS test, p = 0.40) did not change with NMDA. Surprisingly, the average concentration per transient did increase after NMDA (Fig. 3C, n = 347 events pre-drug and n =358 events post-drug, unpaired *t*-test, p < 0.01), similar to the NMDA antagonist. The cumulative concentration also did not change (Fig. 3D, n = 7 animals, paired t-test, p = 0.23). Thus, while the NMDA antagonist caused a change in frequency, the agonist did not.

AMPA Receptors—The AMPA receptor is another ionotropic, non-NMDA sensitive glutamate receptor that can mediate adenosine signaling in some brain regions[17]. NBQX is a selective antagonist of the AMPA receptor that provides potent anti-convulsant protection against seizures at 15 mg/kg i.p.[35] so we selected this dose. There were no significant differences in the number of adenosine transients after NBQX (Fig. 4A, n = 5 animals, paired *t*-test *p* = 0.99). In addition, the mean inter-event times (pre-drug: mean = 64 ± 4 s, n = 269 events; post-drug: mean = 64 ± 4 s, n = 269 events; unpaired *t*-test, *p* = 0.97) and the distribution of inter-event times remained unchanged following NBQX (Fig. 4B, n = 5 animals, KS test, *p* = 0.38). When examining the concentration of each individual transient, there was no change in concentration for each transient with NBQX (Fig. 4C, n = 274 events pre-drug, n=274 events post-drug, unpaired *t*-test, *p* = 0.32,). The maximum cumulative concentration also did not differ (Fig. 3D, n = 5 animals, paired *t*-test, *p* = 0.65). In summary, the AMPA antagonist, NBQX, caused no changes in the frequency or concentration of spontaneous, transient adenosine release.

Metabotropic Glutamate Receptors—There are multiple metabotropic glutamate receptors and here we investigated group II metabotropic glutamate receptors (mGlu2/3) *in vivo* as mGlu2/3 have shown pre-synaptic inhibition in the CNS[36]. The specific mGlu2/3 antagonist, LY 341495, decreased the number of seizures in epileptic mice at 5 mg/kg i.p.[37] and thus we chose this dose. The mGlu2/3 antagonist did not alter the number of adenosine transients (Fig. 5A, n = 6 animals, paired *t*-test, *p* = 0.33). Similarly, the mean inter-event time (pre-drug: mean = 53±4 s, n = 387 events; post-drug: mean = 56 ± 4 s, n = 361 events; unpaired *t*-test, *p* = 0.52) or the underlying frequency distribution did not change following LY 341495 administration (Fig. 5B, n = 6 animals, KS test, *p* = 0.16). The concentration per transient also did not change after LY 341495 administration (Fig. 5C, n = 393 events pre-drug, n = 367events post-drug, unpaired *t*-test, *p* = 0.84) nor did the maximum cumulative concentration change (Fig. 5D, n = 6 animals, paired *t*-test, *p* = 0.36). Thus, mGlu2/3 receptors do not regulate the frequency or concentration of spontaneous adenosine release.

Glutamatergic receptor regulation of transient adenosine release—Adenosine and glutamate have a concomitant relationship in the brain, where inhibitory effects of adenosine modulate the effects of glutamate[38] and excitatory glutamatergic receptors cause release of adenosine. For example, NMDA application evokes adenosine that acts at inhibitory A_1 receptors.[39] However, not all studies have shown a regulation of adenosine by

glutamate receptors. Electrically-stimulated, transient adenosine release was partially dependent on ionotropic glutamate receptors in the striatum, but was independent of them in the hippocampus, nucleus accumbens, and cortex.[30] Similarly, train-evoked adenosine release was also independent of NMDA and AMPA receptors in cerebellar rat brain slices[20].

From these previous studies,[5,6,18] we hypothesized that if spontaneous transient adenosine release was due to activation of NMDA or AMPA receptors, their antagonists would decrease release. However, neither NMDA nor AMPA receptor antagonists decreased the amount of transient adenosine released. Interestingly, the agonist NMDA did increase the concentration of each release event, providing some evidence that NMDA activation might increase the concentration spontaneous adenosine release.

Instead of finding that ionotropic glutamate antagonists decreased adenosine release, we found the opposite effect: inhibiting NMDA receptors with CPP increased the frequency of transient release events and also increased the concentration per event. This result is surprising since blocking NMDA receptors is generally inhibitory and the observed effect was excitatory. One possible explanation is a feedback loop between adenosine and pre-synaptic NMDA receptors.[40] Glutamate and adenosine (or ATP, a precursor of adenosine) could be co-localized in vesicles and activation of pre-synaptic NMDA receptors by glutamate might signal to decrease the frequency of vesicular release, decreasing adenosine as well. Therefore blocking NMDA receptors would increase adenosine release. NMDA antagonists increase the paired-pulse facilitation response for glutamate, by blocking the feedback of glutamate on NMDA receptors.[41] In addition, A_1 and NMDA autoreceptors are co-localized[14,42] and A_1 receptors antagonists also increase the frequency of adenosine release.[7] NMDA itself did not have the opposite effect on frequency of the NMDA antagonist CPP, which may indicate that NMDA receptors are fully activated at basal conditions and cannot be further activated to decrease the frequency of adenosine release.

In contrast to NMDA antagonist, the AMPA antagonist NBQX had no effect on the frequency of transient adenosine release. Previous research had identified that AMPA receptors regulate adenosine release; for example, AMPA antagonists decreased single pulse stimulated adenosine.[5,6] However, other studies found that AMPA receptors had no effect on train-evoked adenosine release in the cerebellum[20] and adenosine A_{2a} agonists inhibited NMDA currents but not AMPA currents.[19] Our results agree with the latter studies, as NBQX had no effect on the frequency or concentration of spontaneous adenosine transients. Metabotropic glutamate receptors regulate signaling via G-protein coupled receptors. Metabotropic glutamate group II receptors (mGlu2/3) are generally located pre-synaptically and inhibit neurotransmitter release, although they are also localized on cholinergic interneurons in the striatum.[43] The mGlu antagonist LY 341495 has an affinity of 14–22 nM for mGlu 2/3 and is less potent for mGlu4 receptors (K_i = 22 μM).[44] LY 341495 had no effect on spontaneous adenosine release, indicating that mGlu 2/3 receptors do not regulate adenosine release. Future studies could examine other types of metabotropic glutamate receptors, such as mGlu4 receptors,[6] perhaps in brain slices where the blood brain barrier is not an issue.

GABA receptors

$GABA_A$ Receptors—There are two main types of GABA receptors: $GABA_A$ receptors which are ionotropic, ligand gated ion channels and $GABA_B$ receptors which are metabotropic receptors that indirectly open ion channels. The $GABA_A$ antagonist bicuculline did not affect the number of adenosine transients (Fig. 6A, n = 6 animals, paired *t*-test, $p = 0.42$). The dose of 5 mg/kg i.p. was chosen because it has been previously used to reverse locomotor effects [45] and did not cause seizures in our rats. Bicuculline also did not change the mean inter-event time (pre-drug: 78 ± 7 s, n = 265 events; post-drug: 70 ± 6 s, n = 295 events; unpaired *t*-test, $p = 0.33$) or the underlying frequency distributions of transients (Fig. 6B, n = 6 animals, KS test, $p = 0.54$). There was no change in either the concentration of each adenosine release event (Fig. 6C, n = 271 events pre-drug, n = 301 events post-drug, unpaired *t*-test, $p = 0.63$) or the maximum cumulative adenosine concentration (Fig. 6D, n = 6 animals, paired *t*-test, $p = 0.20$). Ionotropic $GABA_A$ receptors thus did not regulate transient adenosine release.

$GABA_B$ Receptors—The $GABA_B$ antagonist, CGP 52432, has been used previously in brain slices to increase glutamate receptor-dependent adenosine release.[6] CGP 52432 has anti-depressant properties at 30 mg/kg in mice,[46] thus we used that dose. The example color plots and traces show about 6 transient pre-drug (Fig. 7A) in 80 s, while only 2 transients are observed in the same period of time after CGP 52432 (Fig. 7B). CGP 52432 significantly decreased the number of adenosine transients (Fig. 7C, n = 8 animals, paired *t*-test, $p = 0.0059$). The mean inter-event times increased with $GABA_B$ antagonist, indicating longer times between consecutive transients, from 64 ± 4 s (n = 383 transients) pre-drug to 89 ± 8 s (n = 270 transients, unpaired *t*-test, $p = 0.046$). However, the underlying distributions were not significantly different (Fig. 7D, n = 8 animals, KS test, $p = 0.30$). The $GABA_B$ antagonist CGP 52432 did not significantly change the average adenosine transient concentration (Fig. 7E, n = 391 events pre-drug, n = 278 events post-drug, unpaired *t*-test, $p = 0.72$). CGP 52432 did not also significantly decrease the maximum cumulative concentration (Fig. 7F, n = 8 animals, paired *t*-test, $p = 0.26$). The $GABA_B$ receptor antagonist regulated the number and frequency of adenosine transients, but not the concentration of release.

Because the $GABA_B$ antagonist had a significant effect on the frequency of spontaneous release, we also administered the $GABA_B$ agonist baclofen (4.0 mg/kg, i.p.) to a separate set of animals. The dose of 4.0 mg/kg was chosen because it has been previously used to facilitate behavioral flexibility[47] and suppress ethanol self-administration in rats.[48] The example color plots and traces show about 2 transients pre-drug (Fig. 8A) in an 80 s window, while 4 transients are observed in the same period of time after baclofen (Fig. 8B). The $GABA_B$ agonist has the opposite effect of the antagonist: the mean inter-event time decreased (pre-drug: mean 74 ± 5 s, n=362 events; post-drug: mean =47 ± 2, n = 578 events; unpaired *t*-test, $p < 0.0001$) and the distribution of inter-event times significantly changed after baclofen (Fig. 8D, n = 8 animals, K-S test, $p = < 0.01$). While the number of transients (Fig. 8C, n = 8 animals, paired *t*-test, $p = 0.12$) and cumulative concentration (Fig. 8F, n = 8 animals paired *t*-test, $p=0.087$) did not significantly change after baclofen, they also trended in the opposite direction as the antagonist, with more transients and a larger cumulative

concentration. The amount released per transient was not significantly changed (Fig. 8E, n = 369 events pre-drug, n = 586 events post-drug, unpaired *t*-test, $p = 0.80$). Overall, these experiments show that $GABA_B$ receptors can be either inhibited or activated to produce opposite effects on the number of adenosine transients.

Regulation of spontaneous adenosine by GABAergic receptors—GABA is the primary inhibitory neurotransmitter in the brain and $GABA_B$ receptors are inhibitory metabotropic receptors. $GABA_B$ receptors are located pre- and post-synaptically and are localized on glutamatergic terminals[49] and GABAergic terminals.[50,51] Previously, $GABA_B$ antagonists slightly increased single pulse evoked release, but had little effect on train evoked adenosine release.[6] Therefore, we expected that if $GABA_B$ receptors had an effect, the antagonist would increase the concentration or frequency of spontaneous adenosine release. The $GABA_B$ antagonist, CGP 52432, did not affect concentration but did affect the frequency; however, CGP 52432 had the opposite effect as expected, decreasing the frequency of spontaneous adenosine release. The $GABA_B$ agonist baclofen had the opposite effect of the antagonist, increasing the frequency of spontaneous release. Our studies were performed in the striatum, whereas the previous single-spike evoked release was performed in the cerebellum so there are differences by brain region as well as stimulation type. Our results suggest a feedback loop, similar to that observed in the NDMA studies, but of opposite effect (Fig. 9B). Pre-synaptically located $GABA_B$ receptors can regulate GABA concentrations[52] and if blocking $GABA_B$ receptors raised GABA levels, this could cause general inhibition and decrease of adenosine release. The inhibition after $GABA_B$ antagonist may lead to fewer action potentials and fewer adenosine release events.

$GABA_A$ receptors are ionotropic receptors that gate chloride channels. Although there is no evidence of co-localization of GABA and adenosine receptors, cross-talk between $GABA_A$ and A_1 receptors has been demonstrated.[53] Adenosine decreases the hyperpolarization effects of $GABA_A$ through A_1 receptors[54] and alters the response of $GABA_A$ receptors from inhibitory to excitatory after seizures.[55] Those studies all focused on the effects of adenosine modulating GABA release. Here, we examined the effect of blocking $GABA_A$ receptors on adenosine release and found that the $GABA_A$ antagonist, bicuculline, had no effect on transient adenosine release (Fig. 7). Thus, spontaneous adenosine release is not regulated by ionotropic $GABA_A$ receptors, only metabotropic $GABA_B$ receptors.

Presynaptic regulation of Adenosine by Glutamate and GABA Receptors

The regulation of adenosine release is complex and can vary from brain region to brain region [30,56]. Indeed, even the striatum itself is heterogeneous, especially for dopamine and GABA release, and our studies examined regulation of adenosine release in only one location, the medial striatum.[57,58] The main result is that blocking NMDA and $GABA_B$ regulates spontaneous adenosine release, particularly the frequency of release, in the medial caudate. Adenosine A_1 and A_{2a} receptors have been shown to self-regulate spontaneous adenosine release and the mechanism of action is thought to be presynaptic.[7,11,53] However, the exact release site of spontaneous adenosine is not known; it could be from neurons or it could be from astrocytes. While NMDA and $GABA_B$ antagonists have opposite effects of what might be expected if activating those receptors caused release, the results are consistent

with those receptors acting pre-synaptically to control glutamate and GABA concentrations and overall cell excitability (Fig. 9). However, these receptors, particularly NMDA receptors, are localized on many cells, including post-synaptic cells, glial cells, and interneurons. Not all pre-synaptic receptors modulate adenosine release, as the pre-synaptic mGlu2/3 receptors[34] had no effect on the frequency of release. Receptors that are primarily localized post-synaptically, like AMPA[38] and $GABA_A$[49] receptors, did not regulate transient adenosine release. The regulation by pre-synaptic receptors as well as the fast time course of release suggest that spontaneous adenosine release is vesicular,[54] although vesicular release could be adenosine or ATP, which is quickly broken down to adenosine.[55] Spontaneous adenosine regulates neurotransmission [3] and oxygen levels,[11] so antagonists of NMDA or $GABA_B$ could be used to modulate adenosine release frequency and thus regulate the rapid modulatory effects of adenosine.

Conclusions

This study shows that spontaneous, transient adenosine release is modulated by glutamate and GABA receptors, specifically NMDA and $GABA_B$ receptors. These receptors regulate the frequency of the release through general excitation and inhibition. The frequency of spontaneous, transient adenosine is not dependent on metabotropic glutamate (mGlu2/3), $GABA_A$, or AMPA receptors. The regulation of spontaneous, transient release is different compared to previous studies of electrically-stimulated adenosine release; thus regulation of adenosine release differs by release mode. By understanding which receptors regulate fast acting adenosine, future studies and drug therapies will be able to manipulate and control the rapid modulatory properties of adenosine.

Methods

Chemicals and Drugs

All drugs were administered i.p to rats and purchased from Tocris Biosciences (Ellisville, MO, USA) unless otherwise noted. The NMDA receptor antagonist, CPP (3-((*R*)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid) was dissolved in 200 μL saline and injected at 6.25 mg/kg. The NMDA receptor agonist, NMDA, was dissolved in 500 μL saline and injected at 50 mg/kg. The AMPA antagonist NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide) was dissolved in saline and DMSO (500 μL each) and injected at 15 mg/kg. The group II metabotropic glutamate receptor antagonist LY 341495 ((2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid) was dissolved in saline and DMSO (500 μL) and injected at 5 mg/kg. The $GABA_B$ receptor antagonist CGP 52432 (3-[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl)phosphinic acid) was dissolved in 2.5 mL DMSO and 1 mL saline and injected at 30 mg/kg. The $GABA_B$ receptor agonist, baclofen, was dissolved in 0.3 mL saline and injected at 4.0 mg/kg. The $GABA_A$ receptor antagonist bicuculline was dissolved in 500 μL of saline and injected at 5 mg/kg.

Animal Experiments

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Virginia. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) between 250–350 grams anesthetized with urethane (1.5 mg/kg, i.p.), shaved, and placed in a stereotaxic frame. Bupivacaine (250 μL, Sensorcaine, MPF, APP Pharmaceuticals, LLC; Schaumburg, IL, USA) was injected subcutaneously at the surgical site for analgesia prior to incision. Holes were drilled in the skull for the placement of the electrode in the caudate-putamen (in mm from bregma): AP: +1.2, ML: +2.0, DV: –4.5. The Ag/AgCl reference electrode was placed on the contralateral side. Body temperature was regulated with a heating pad and thermistor probe (FHC; Bowdoin, ME, USA). In order to make sure the rat was deeply anesthetized throughout, anesthesia was checked every hour by toe pinch.

A carbon-fiber electrode was placed in the caudate-putamen and equilibrated for at least 30 minutes with the waveform applied. If few transients were seen during that 30 minutes of equilibration (less than 10/hour), the electrode was removed and a new electrode was inserted. After transients were verified, the electrode was then allowed to equilibrate for another 30 min, and then an hour of pre-drug data was collected. A drug was injected i.p and one hour of data was collected after drug injection. While the post-drug period might include a few minutes where the drug is still working into the bloodstream and across the blood brain barrier, there is not a good way to measure pharmacodynamics and most of the post-drug collection should be influenced by the drug. After *in vivo* testing, electrodes were calibrated *in vitro* using flow injection analysis with 1.0 μM adenosine in PBS buffer.[11]

Statistics

All statistics were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). The data are presented as mean ± SEM. A Kolmogorov-Smirnov (KS) test was used to determine underlying distributions between inter-event times (time between consecutive transients). A t-test was used to compare means between 2 groups. All data were considered significant at the 95% confidence level. Principal components analysis, with confirmation by an analyst, was used to identify adenosine transients, as previously described [7].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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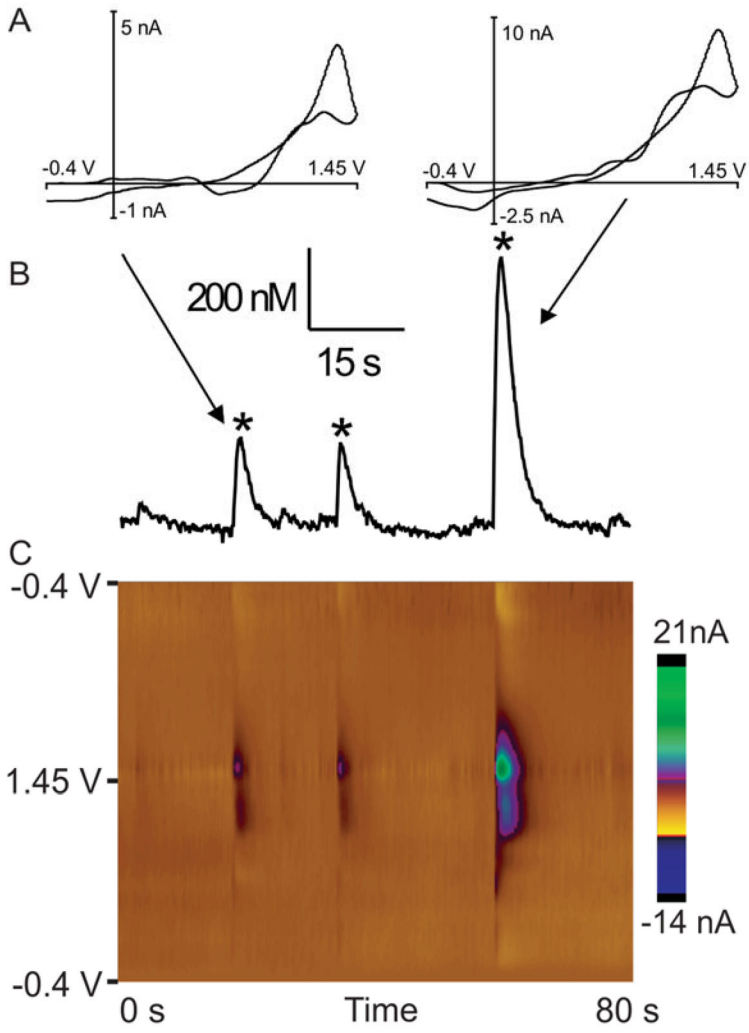

Figure 1. Spontaneous, transient adenosine release

A) Cyclic voltammograms (CV) of spontaneously release adenosine *in vivo*. The primary oxidation of adenosine occurs at 1.4 V and the secondary peak is at 1.0 V. B) A concentration vs time trace collected *in vivo* over an 80 second window. The concentrations were calculated from the current using *in vitro* values with principal components analysis. C) 3-D color plot of three example spontaneous events *in vivo*. Adenosine oxidation is green/purple.

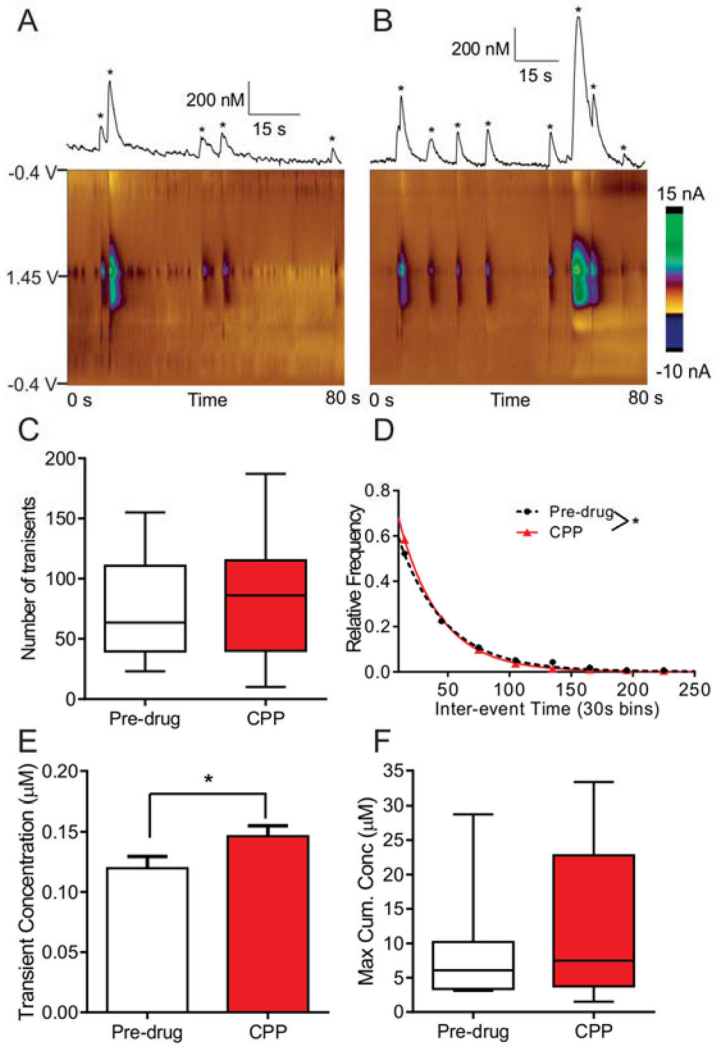

Figure 2. The effect of NMDA antagonist CPP on adenosine release

A) Example pre-drug release of adenosine. In this 80s time period, 5 adenosine transients (starred) were observed. B) Example adenosine trace after CPP (6.25 mg/kg, i.p.), where 8 transients, many larger in concentration, are now observed. C) Number of transients (n = 8 animals, paired *t*-test, $p = 0.28$). For all box plots, the line shows the median, the box the 25–75 % values, and the whiskers the range. D) Inter-event time histogram (30 s bins) pre-drug and after CPP were significantly different (n = 8 animals, KS test, $p = 0.035$). E) Event concentration was significantly different after CPP (n = 590 pre-drug, 676 post-drug, unpaired *t*-test, $p = 0.039$). Error bars are SEM. F) Maximum cumulative concentration is not statistically different (n = 8 animals, paired *t*-test, $p = 0.13$).

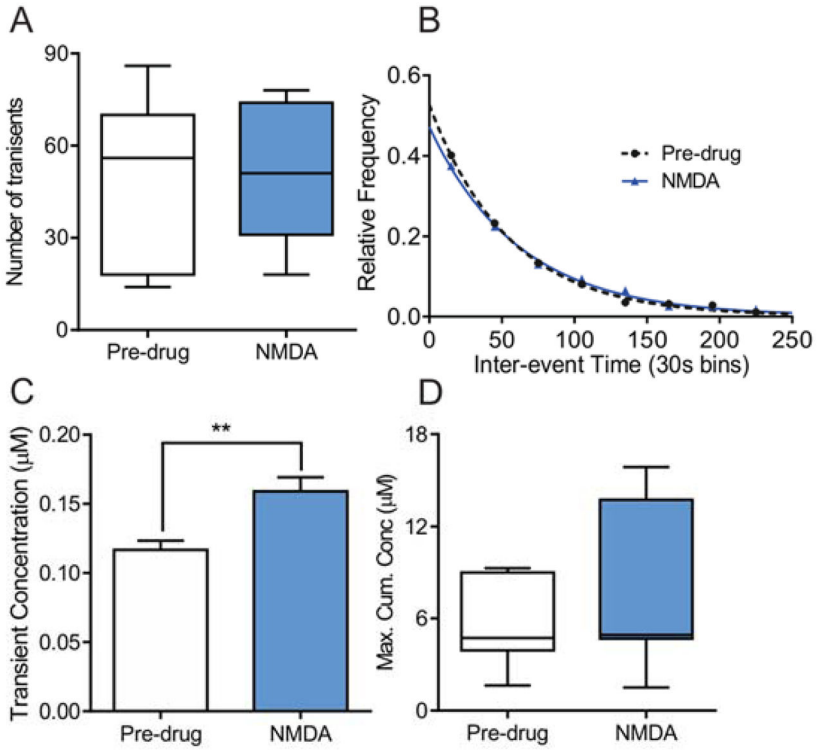

Figure 3. The effect of NMDA agonist NMDA on adenosine release

A) The number of transients before and after NMDA (50 mg/kg, i.p.) was not different (n = 7 animals, paired *t*-test, $p = 0.85$). B) The underlying distributions of inter-event times were not different (n = 7 animals, KS test, $p = 0.40$). C) The event concentration significantly increased after NMDA (n = 347 events pre-drug and 358 events post-drug, unpaired *t*-test, $p < 0.01$). D) The maximum cumulative concentration also did not change (n = 7 animals, paired *t*-test, $p = 0.23$)

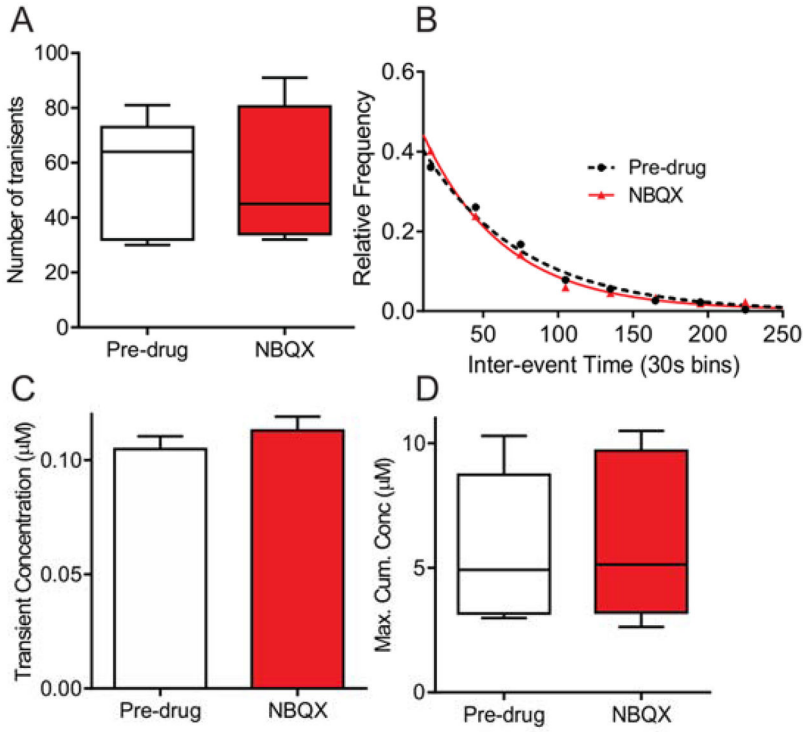

Figure 4. The effect of AMPA antagonist NBQX on transient adenosine release

A) Number of transients before and after NBQX (15 mg/kg, i.p.) are not different (n = 5 animals, paired *t*-test, $p = 0.99$). B) The underlying distributions of inter-event times are not different (n = 5 animals, KS test, $p = 0.38$). C) The event concentration does not change after NBQX (n = 274 both pre-drug and post-drug, unpaired *t*-test, $p = 0.32$). D) The maximum cumulative concentration also does not change (n = 5 animals, paired *t*-test, $p = 0.65$)

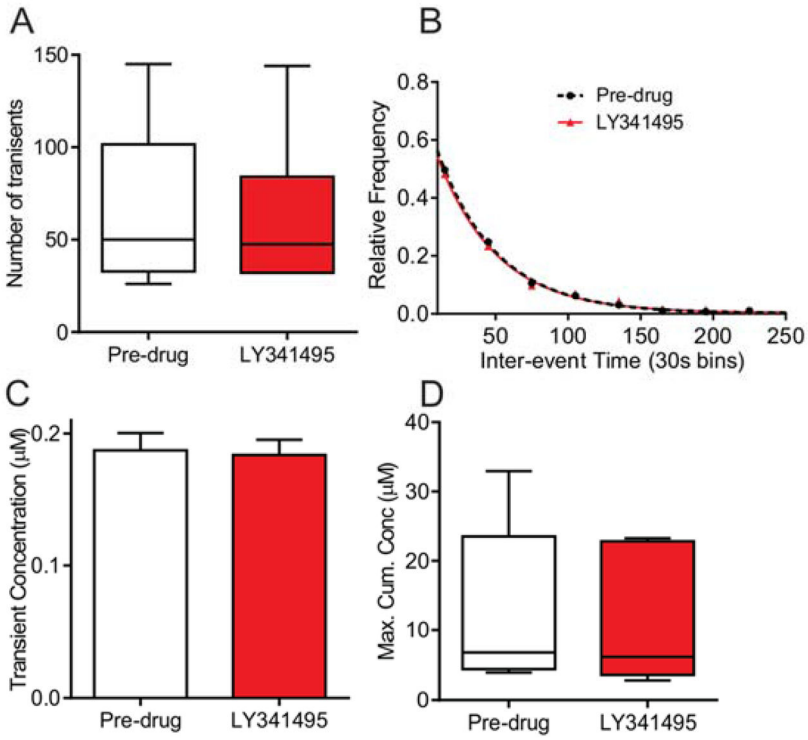

Figure 5. The effect of mGlu2/3 receptor antagonist LY 341495 on transient adenosine release

A) Number of transient release events did not change following mGlu2/3 antagonism with LY 341495 (5 mg/kg, i.p., n = 6 animals, paired *t*-test, $p = 0.33$). B) The underlying distribution of inter-event times did not change (n = 6 animals, KS test, $p = 0.16$). C) The event concentration did not change (n = 393 events pre-drug, 367 events post-drug, unpaired *t*-test, $p = 0.84$). D) The maximum cumulative concentration did not change (n = 6 animals, paired *t*-test, $p = 0.36$).

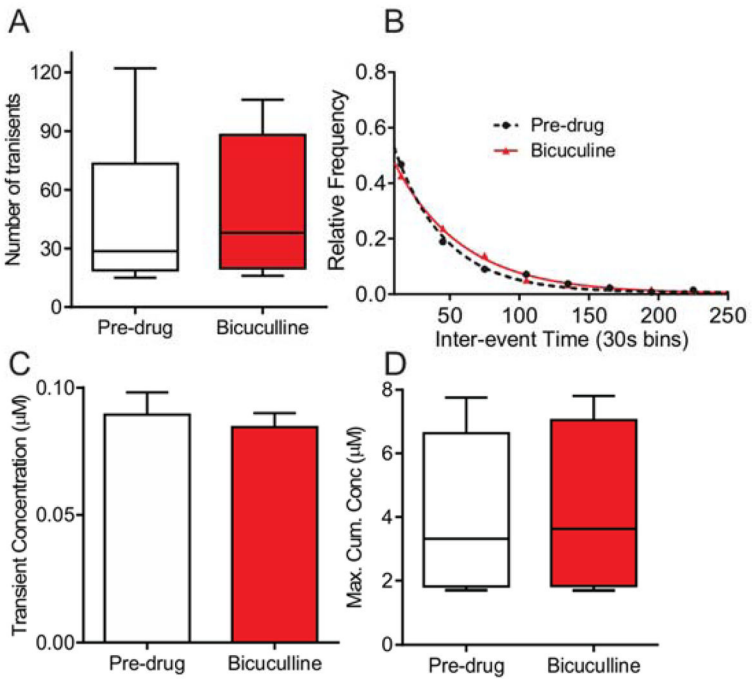

Figure 6. The effects of $GABA_A$ receptor antagonist bicuculline on spontaneous adenosine release

A) Number of transients did not change after the $GABA_A$ antagonist bicuculline (5 mg/kg, i.p., n = 6 animals, paired *t*-test, $p = 0.42$). B) The inter-event histograms were also not different (n = 6 animals, KS test, $p = 0.54$). C) The event concentration did not change after bicuculline (n = 271 transients pre-drug and 301 post-drug, unpaired *t*-test, $p = 0.63$). D) The maximum cumulative adenosine concentration did not change (n = 6 animals, paired *t*-test, $p = 0.20$).

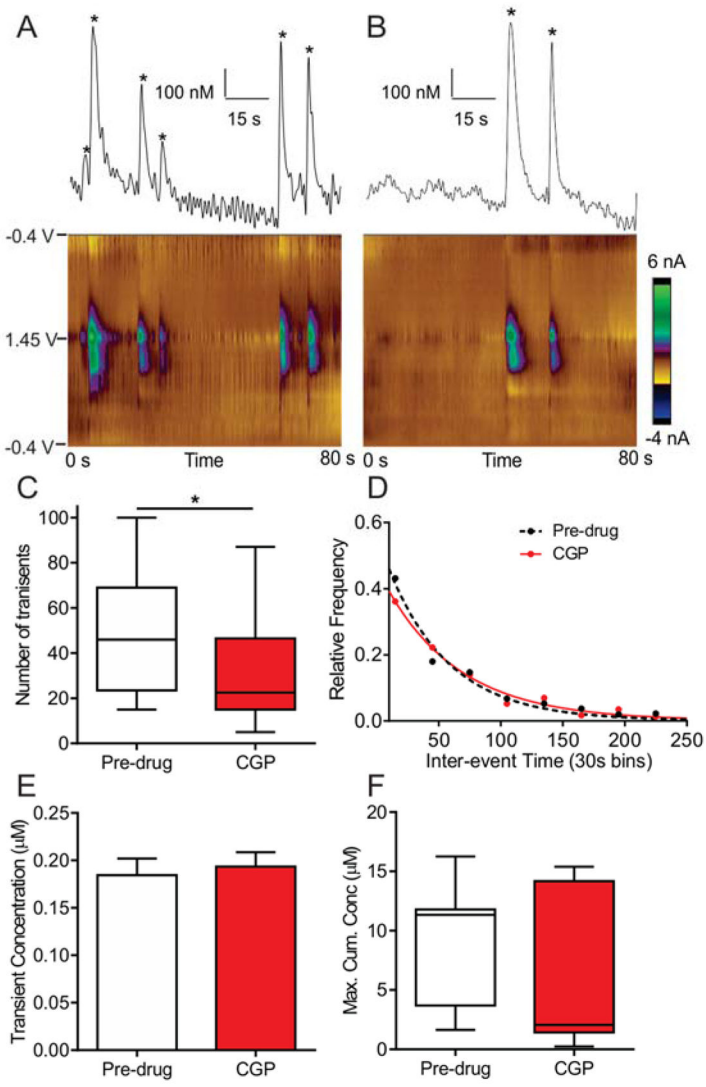

Figure 7. The effects of $GABA_B$ receptor antagonist CGP 52432

A) Example pre-drug data showing 6 adenosine transients in an 80 s period (starred). B) Example data after the $GABA_B$ antagonist CGP 52432 (30 mg/kg, i.p.) in the same rat, where only 2 transients were observed. The magnitude of the transients is similar after CGP 52432. C) The number of transients decreased significantly after CGP 52432 (n = 8 animals, paired *t*-test, $p = 0.0059$). D) The underlying distribution for the inter-event time histograms were not significantly different (n = 8 animals, KS test, $p = 0.30$), however the mean inter-event times are different (64 ± 4 s, n = 383 events pre-drug, 89 ± 8 s, n = 270 events post-drug, unpaired *t*-test, $p = 0.046$). E) The $GABA_B$ antagonist did not change the average event concentration (n = 391 events pre-drug, 278 events post-drug, unpaired *t*-test, $p = 0.72$). F) The maximum cumulative concentration also did not change (n = 8 animals, paired *t*-test, $p = 0.26$).

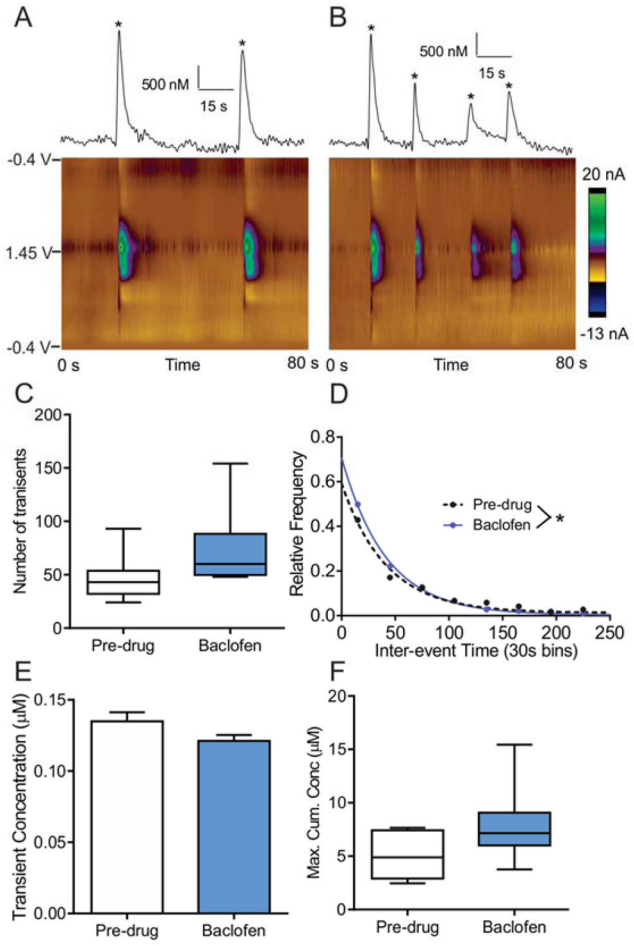

Figure 8. The effects of $GABA_B$ receptor agonist baclofen

A) Example pre-drug release of adenosine. In this 80 s time period, 2 adenosine transients (starred) were observed. B) Example adenosine trace after baclofen (4.0 mg/kg, i.p.), where 4 transients are observed. C) Number of transients (n = 8 animals, paired *t*-test, $p = 0.12$). For all box plots, the line shows the median, the box the 25–75 % values, and the whiskers the range. D) Inter-event time histogram (30 s bins) pre-drug and after baclofen were significantly different (KS test, $p < 0.01$). E) Event concentration was not significantly different after baclofen (n=369 events pre-drug, 586 events post-drug, unpaired *t*-test, $p = 0.80$). Error bars are SEM. F) Maximum cumulative concentration was not statistically different (n = 8 animals, paired *t*-test, $p = 0.087$).

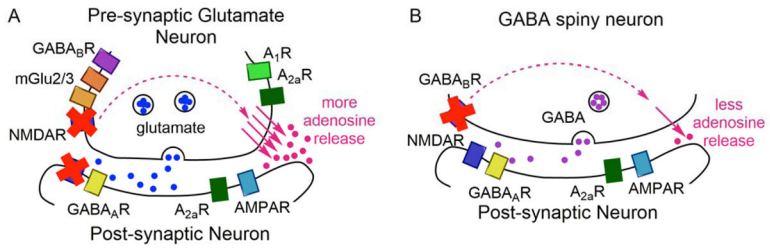

Figure 9. Possible presynaptic mechanisms to regulate spontaneous transient adenosine release

A) Cartoon of receptor positions on glutamate neurons. Blocking NMDA receptors with CPP increases the frequency of transient adenosine release, likely through a pre-synaptic feedback loop that causes more vesicular release. B) Cartoon of GABA spiny neuron. Blocking $GABA_B$ receptors with CGP 52432 decreases the frequency of adenosine release, and the $GABA_B$ agonist baclofen increases frequency of release, likely through a feedback loop that increases GABA and general inhibition. $GABA_B$ receptors are also located on glutamatergic neurons.