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Adenosine Release during Seizures Attenuates $GABA_A$ Receptor-Mediated Depolarization

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Seizure-induced release of the neuromodulator adenosine is a potent endogenous anticonvulsant mechanism, which limits the extension of seizures and mediates seizure arrest. For this reason several adenosine-based therapies for epilepsy are currently under development. However, it is not known how adenosine modulates GABAergic transmission in the context of seizure activity. This may be particularly relevant as strong activation of GABAergic inputs during epileptiform activity can switch $GABA_A$ receptor ($GABA_AR$) signaling from inhibitory to excitatory, which is a process that plays a significant role in intractable epilepsies. We used gramicidin-perforated patch-clamp recordings to investigate the role of seizure-induced adenosine release in the modulation of postsynaptic $GABA_AR$ signaling in pyramidal neurons of rat hippocampus. Consistent with previous reports, $GABA_AR$ responses during seizure activity transiently switched from hyperpolarizing to depolarizing and excitatory. We found that adenosine released during the seizure significantly attenuated the depolarizing $GABA_AR$ responses and also reduced the extent of the after-discharge phase of the seizure. These effects were mimicked by exogenous adenosine administration and could not be explained by a change in chloride homeostasis mechanisms that set the reversal potential for $GABA_ARs$, or by a change in the conductance of $GABA_ARs$. Rather, A_1R-dependent activation of potassium channels increased the cell's membrane conductance and thus had a shunting effect on $GABA_AR$ currents. As depolarizing $GABA_AR$ signaling has been implicated in seizure initiation and progression, the adenosine-induced attenuation of depolarizing $GABA_AR$ signaling may represent an important mechanism by which adenosine can limit seizure activity.

Introduction

Epilepsy is a severe neurological disorder characterized by recurrent seizures, which reflect a failure of inhibitory systems to contain the generation and spread of neuronal hyperexcitability. Most seizures are terminated either as a result of the depletion of factors necessary for sustaining seizure activity (e.g., energy substrates, ions, neurotransmitters) or as a result of endogenous inhibitory mechanisms that are triggered by elevated network activity. Studying such mechanisms is therefore a promising strategy for developing anti-epileptic therapies (Löscher and Köhling, 2010). One endogenous anticonvulsant mechanism is the seizure-induced release of the neuromodulator adenosine, which regulates synaptic activity via four known guanine nucleotide-binding protein (G-protein)-coupled adenosine receptors (ARs): A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R (Boison and Stewart, 2009). In particular, activation of A_1Rs has been shown to inhibit network excitability, limit the extension of seizures, and mediate seizure arrest (Etherington and Frenguelli, 2004; Fedele et al., 2006; Li et al., 2007). The coupling of A_1Rs to G_i/G_o proteins underlies several mechanisms by which adenosine can modulate synaptic transmission (Dunwiddie and Masino, 2001). In the case of glutamatergic transmission, presynaptic activation of A_1Rs causes a reduction in release probability by inhibiting calcium (Ca^{2+}) influx through voltage-gated channels (Yawo and Chuhma, 1993; Wu and Saggau, 1994; Gundlfinger et al., 2007), or by interfering directly with the release process (Scanziani et al., 1992; Thompson et al., 1993). At the same time, adenosine has been shown to function postsynaptically where it activates a series of downstream potassium (K^+) channels, including G-protein-coupled, inward-rectifying K^+ channels (GIRKs), that can modulate the excitatory effects of postsynaptic glutamate receptors (Takigawa and Alzheimer, 2002).

Relatively less is known about how adenosine modulates inhibitory synaptic transmission. This may be particularly relevant in the context of epilepsy, where a breakdown in effective GABA type A receptor ($GABA_AR$)-mediated synaptic inhibition contributes to the ongoing dynamics of seizures (Wright et al., 2011). In the hippocampus, intense activation of $GABA_ARs$ during early stages of a seizure results in a large influx of chloride (Cl^-) (Isomura et al., 2003b; Fujiwara-Tsukamoto et al., 2006, 2007). The result is a collapse in the *trans*-membrane Cl^- gradient and pronounced depolarizing shift in the reversal potential of the $GABA_AR$ (E_{GABA_A}) (Thompson and Gähwiler, 1989; Staley et al., 1995; Kaila et al., 1997). This depolarizing shift in E_{GABA_A} is thought to establish the conditions for networks of GABAergic neurons to generate synchronized after-discharges that are char-

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acteristic of epileptiform activity (Michelson and Wong, 1991; Fujiwara-Tsukamoto et al., 2010).

Recordings from hippocampal neurons have shown that exogenous adenosine does not affect the presynaptic release of GABA (Yoon and Rothman, 1991; Thompson et al., 1992). Whether adenosine contributes to the modulation of $GABA_AR$ signaling in the acute setting of a seizure remains unexplored. Here we use gramicidin perforated patch-clamp recordings in an *in vitro* model of temporal lobe epilepsy to study how adenosine released during seizures influences postsynaptic $GABA_AR$ signaling in CA3 pyramidal cells. We show that as seizure activity drives a switch in GABAergic signaling from inhibitory to excitatory, adenosine acts postsynaptically to reduce the resulting $GABA_AR$-mediated depolarization.

Materials and Methods

Slice preparation. Rat organotypic hippocampal slice cultures were prepared using a method similar to that described by Stoppini et al. (1991). Briefly, 7-d-old male Wistar rats were killed by cervical dislocation in accordance with Schedule 1 methods of UK Animals Scientific Procedures Act 1986. The brains were extracted and placed in cold (4°C) Geys Balanced Salt Solution, supplemented with D-glucose (34.7 mM). All reagents were purchased from Sigma-Aldrich, unless stated. The hemispheres were separated and individual hippocampi were removed and immediately sectioned into 350-μm-thick slices on a McIlwain tissue chopper. Slices were rinsed in cold dissection media, placed onto Millicell-CM membranes, and maintained in culture media containing 25% EBSS, 50% MEM, 25% heat-inactivated horse serum, glucose, and B27 (Invitrogen), and incubated at 36°C in a 5% CO_2 humidified incubator. Recordings were performed after 7–14 d *in vitro*, when the hippocampal slices were comparable to postnatal day 14–21. Previous work in rat hippocampus has shown that E_{GABA_A} reaches mature levels within the first two postnatal weeks (Tyzio et al., 2007), and recordings from rat organotypic hippocampal slices have confirmed that GABAergic (Streit et al., 1989) and glutamatergic (De Simoni et al., 2003) synaptic transmission are mature at these stages. Consistent with this, we found that the resting E_{GABA_A} was hyperpolarizing (mean resting $E_{GABA_A} = -82.7 \pm 1.2$ mV, ranging from −72.5 mV− to −91.2 mV−, $n = 17$) compared with the resting membrane potential (mean resting membrane potential = −73.5 ± 1.2 mV, ranging from −62.1 mV− to −79.7 mV−, $n = 17$). These resting E_{GABA_A} values are slightly more hyperpolarized than previously reported in the CA3 region (Tyzio et al., 2007), and might reflect differences in the level of endogenous $GABA_A$ receptor activity under different recording conditions and/or a bias toward selecting larger, more mature pyramidal neurons in our preparation.

Electrophysiological recordings. To preserve the intracellular milieu of the cell and particularly the intracellular Cl^- concentration ($[Cl^-]_i$) we used gramicidin-perforated patch-clamp recordings (Ebihara et al., 1995; Kyrozis and Reichling, 1995; Akerman and Cline, 2006). Patch pipettes of 3–5 MΩ tip resistance were pulled from filamental borosilicate glass capillaries (1.2 mm outer diameter, 0.69 mm inner diameter; Harvard Apparatus), using a horizontal puller (Sutter P-97). The pipettes were filled with a high KCl internal solution containing gramicidin. The composition of internal solution contained (in mM): 135 KCl, 4 Na_2ATP, 0.3 Na_3GTP, 2 $MgCl_2$, and 10 HEPES. Osmolarity was adjusted to 290 mOsM and the pH was adjusted to 7.35 with KOH. Gramicidin (Calbiochem) was dissolved in dimethylsulfoxide (DMSO) to achieve a stock solution of 10 mg/ml. This was then diluted in internal solution on the day of experimentation to achieve a final concentration of 80 μg/ml. The resulting solution was vortexed for 1 min, sonicated for 30 s, and then filtered with a 0.45 μm pore cellulose acetate membrane filter (Nalgene).

Hippocampal slices were transferred to a recording chamber and continuously superfused with 95% O_2/5% CO_2 oxygenated artificial cerebrospinal fluid (aCSF), warmed to 30–32°C. The composition of aCSF contained (in mM): 120 NaCl, 3 KCl, 2 $MgCl_2$, 1.5 $CaCl_2$, 1.2 NaH_2PO_4, 23 $NaHCO_3$, and 11 D-glucose. The pH was adjusted to be between 7.35 and 7.40 using NaOH. Previous studies have shown that hippocampal organotypic slices can be used as a post-traumatic model of epileptogenesis (Dyhrfjeld-Johnsen et al., 2010; Berdichevsky et al., 2012). By 7 d *in vitro* the tissue has recovered from the acute trauma associated with the slicing process and the neurons exhibit hyperexcitability, probably due to increased connectivity (Berdichevsky et al., 2012). Seizures were induced by perfusion with nominally Mg^{2+}-free aCSF, and the perfusion media was switched back to normal aCSF after the start of a seizure episode. This transient exposure to 0 Mg^{2+} allowed us to induce reproducible single seizure episodes and prevented repeated seizure episodes that can occur with continuous exposure to 0 Mg^{2+}.

Seizure onset was characterized by a pronounced membrane potential depolarization upon which occurred high-frequency, low-amplitude discharges (see Fig. 1). For analysis purposes the onset of the seizure was defined as the time at which the membrane potential depolarized beyond a "threshold" set at 40 mV above the cell's resting membrane potential ("baseline"). The end of the ictal-like phase and start of the after-discharge phase was defined as the point at which the membrane potential returned to baseline for the first time after the start of the seizure. The after-discharge phase was composed of rhythmic bursts of activity occurring from a relatively hyperpolarized membrane potential. An individual after-discharge event was defined as a burst of activity in which the membrane potential depolarized above the threshold for seizure onset, and then returned to below the baseline value. The end of the after-discharge phase was defined as the end of the last after-discharge event. Postseizure hyperpolarization was measured from the end of the after-discharge phase and expressed relative to the baseline membrane potential. All experiments involving seizure activity (see Figs. 1–3) were performed using uncompensated current-clamp recordings, without any DC injection in the I = 0 mode.

For the seizure-free exogenous adenosine experiments, slices were perfused with normal aCSF containing tetrodotoxin (TTX; 1 μM) to prevent spiking activity. To measure hyperpolarizing $GABA_AR$ responses under seizure-free conditions, the resting membrane potential was held at −70 mV by somatic DC current injection. Depolarizing $GABA_AR$ responses were recorded from a resting membrane potential of −110 mV, established by injecting DC current through the recording pipette in the presence of 200 μM furosemide to block the activity of Cl^- transporter proteins. Neurons were visualized under a 60×, water-immersion objective (Olympus BX51WI) and pyramidal neurons from the CA3 region were identified based upon their location within the hippocampus and their triangular somatic morphology. Following formation of a tight seal between the pipette and the underlying plasma membrane (>1 GΩ), the perforation progress was evaluated by continuously monitoring the decrease in the access resistance. Recordings were started once the access resistance had stabilized between 308 and 0 MΩ (mean $R_a \sim 55$ MΩ), which usually occurred at ~20–50 min from gigaseal. During off-line analysis, all membrane potentials were subsequently corrected for the voltage drop across the access resistance. Recordings were made using an Axopatch 1D amplifier (Molecular Devices) and data acquired using WinWCP Strathclyde Whole Cell Analysis software (V.3.9.7; University of Strathclyde) and stored for off-line analysis. $GABA_ARs$ were activated by delivering short "puffs" of the $GABA_AR$-selective agonist, muscimol (45 μM), through a patch pipette placed in the vicinity of the cell and connected to a picospritzer (5–10 psi for 20–500 ms; General Valve). Puffs were delivered at 15 s intervals to allow recovery of chloride homeostasis (Staley and Proctor, 1999; Jin et al., 2005). At the end of the experiment the integrity of the perforated patch was confirmed by applying suction to rupture the underlying membrane and achieve whole-cell recording mode. This led to a rapid decrease in the access resistance and a shift in E_{GABA_A} due to diffusion of Cl^- from the pipette into the cell's cytoplasm.

All experiments comparing seizure activity, $GABA_AR$ responses, resting E_{GABA_A}, $GABA_AR$ conductance, and membrane conductance were conducted with a within-cell design, so that each parameter was compared before and after a treatment. To measure the amplitude of individual $GABA_AR$ potentials in current-clamp, the membrane potential immediately before the muscimol puff (measured over a 20 ms window) was subtracted from the membrane potential at the peak of the muscimol response (measured over a 5 ms window). During the postsei-

zure period $GABA_AR$ potentials were found to be depolarizing and then gradually decreased in amplitude and reversed polarity to become hyperpolarizing. To estimate the kinetics of this recovery of $GABA_AR$ responses we fitted an exponential to the amplitudes of depolarizing $GABA_AR$ responses during a 1 min time window before their reversal to hyperpolarizing. The recovery rate was calculated as the time constant of the exponential fit.

To measure resting E_{GABA_A}, muscimol-evoked currents were recorded in the voltage-clamp configuration at six different holding potentials (between −50 and −100 mV). For each cell, I–V curves were constructed for the "holding current" and the peak of the "total current" (reflecting the holding current plus the current through the activated $GABA_ARs$). E_{GABA_A} was defined as the intersection of these two I–V curves, which indicated the voltage at which there was no net current through the $GABA_AR$ (see Fig. 6*A*). To estimate peak $GABA_AR$ conductance (see Fig. 7*A*), a 1 s voltage ramp (from −100 to −30 mV) was applied to the membrane to provide a more accurate estimate of the holding current at every membrane potential. "Pure" $GABA_AR$ currents were then calculated by subtracting this holding current from the peak of the total current (in response to muscimol) for specific membrane potentials. In this manner I–V plots of the peak $GABA_AR$ current were constructed and the peak $GABA_AR$ conductance was calculated as the slope of the linear fit. Membrane conductance (see Fig. 5*A*) was calculated from the membrane current generated by 1 s hyperpolarizing voltage steps (−10 mV), delivered under voltage-clamp conditions.

To measure E_{GABA_A} during seizure activity, recordings were rapidly alternated between current-clamp and voltage-clamp modes. The current-clamp recordings allowed the monitoring of seizure activity, while the periods in voltage-clamp (2 s duration) were used to measure E_{GABA_A} with voltage ramp protocols (see Fig. 2*C–E*). Each period in voltage-clamp consisted of two consecutive voltage ramps (500 ms duration, ramping from −90 to −40 mV). The first ramp was applied under baseline conditions and the second ramp was applied during activation of $GABA_ARs$ by local application of GABA (100 μM), in the presence of the $GABA_B$ receptor blocker CGP55845 (5 μM). This made it possible to construct I–V plots of the holding current generated by the first ramp and the total current generated by the second ramp (reflecting the holding current plus the current through the activated $GABA_ARs$) (Fig. 2*D*). E_{GABA_A} was defined as the voltage at which the holding current and the total current intersected (Fig. 2*E*).

Drug manipulations. Pharmacological manipulations were performed by bath application of drugs using a perfusion system. Adenosine was dissolved directly into the aCSF on the day of experiment, whereas all other drugs were prepared in stock solutions, stored at −20°C and added to aCSF on the day of experiment to reach the required concentration. Tolbutamide, CGS 15943, and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were dissolved in DMSO. All other drugs were dissolved in normal aCSF. Adenosine, $BaCl_2$, furosemide, and tolbutamide were purchased from Sigma-Aldrich. Muscimol, TTX, tertiapin, apamine, CGS 15943, CGP55845, and DPCPX were purchased from Tocris Bioscience.

Data analysis and statistics. Digital signal processing and presentation were performed using custom-made programs in the MATLAB environment (MathWorks). Numeric results are presented as mean ± SEM. Statistical comparisons were performed using the paired *t* test or ANOVA, with *post hoc* Bonferroni's correction.

Results

Endogenous adenosine release modulates seizure duration and the postseizure recovery period

We set out to investigate the postsynaptic effects of adenosine released during epileptic-like seizure activity. A commonly used model of seizure activity is the removal of extracellular Mg^{2+}, which predisposes hippocampal slices to periods of synchronized hyperexcitability (Anderson et al., 1986; Mody et al., 1987; Gutiérrez et al., 1999; Avoli et al., 2002). We monitored seizure activity by performing current-clamp recordings from hippocampal CA3 pyramidal neurons using the gramicidin-perforated patch method. In accordance with previous work, we could distinguish two phases of the seizure episodes: (1) the initial ictal-like phase (also referred to as the "tonic" phase) and (2) the after-discharge phase (also referred to as the "clonic" phase) (Higashima et al., 1996; Trevelyan et al., 2007; Isomura et al., 2008). The ictal phase was characterized by a >40 mV membrane potential depolarization upon which occurred high-frequency, low-amplitude discharges (Fig. 1*A*). As the seizure progressed, the membrane potential slowly repolarized, reached preseizure resting membrane potential values, and then entered the after-discharge phase. The after-discharge phase was composed of rhythmic bursts of activity that emerged from a relatively hyperpolarized membrane potential background. Beyond the after-discharge phase, the membrane potential remained at relatively hyperpolarized values for several minutes and slowly recovered to preseizure values (Fig. 1*A*). Comparing two consecutive seizures within the same recordings confirmed that the seizures were highly reproducible. There was no difference between the first and second seizure in terms of seizure length (first seizure mean was 205.2 ± 52.8 s, second seizure mean was 225.1 ± 48.8 s; $n = 5$, $p = 0.58$) or the extent of membrane hyperpolarization postseizure (first seizure showed a mean hyperpolarization relative to baseline of −18.3 ± 3.2 mV, second seizure mean was −19.1 ± 1.5 mV; $n = 5$, $p = 0.71$). Using this model we were therefore able to explore the contribution of AR signaling during seizure episodes, using pharmacological manipulations in a within-slice paired design.

To test whether endogenous adenosine is released at levels that modulate a seizure episode, we investigated the effect of 1 μM CGS 15943, an antagonist of the four known ARs. Blockade of ARs had a significant effect on the seizures, increasing total seizure duration from 272.8 ± 43.8 s to 666.4 ± 121.8 s ($n = 10$, $p < 0.01$, paired *t* test; Fig. 1*B*). This effect was primarily due to an increase in the after-discharge period from 185.7 ± 43.8 s to 565.7 ± 129.7 s ($n = 10$, $p < 0.01$, paired *t* test). Meanwhile the duration of the ictal phase was not significantly affected by AR antagonism, as it had a duration of 87.0 ± 19.6 s under control conditions and 100.6 ± 27.3 s following application of CGS 15943 ($n = 10$, $p = 0.61$, paired *t* test). To further investigate the time course of adenosine's release and effects we compared the membrane potential (averaged in 5 s time bins) from the start of each seizure, under control conditions, and during AR antagonism. There was no difference between the two conditions at the start of the seizure. However, over the course of the seizure the membrane potential became more hyperpolarized under control conditions, than during AR antagonism, and this was first evident 25 s from the start of the seizure (absolute membrane potential −42.2 ± 1.6 mV vs −37.9 ± 1.0 mV, $n = 10$, $p < 0.05$, paired *t* test). Blockade of ARs also reduced the degree of postseizure membrane hyperpolarization. For instance, the maximum postseizure hyperpolarization under control conditions was −22.9 ± 1.6 mV relative to baseline, and this was reduced to −15.5 ± 1.9 mV when ARs were blocked ($n = 10$, $p < 0.01$, paired *t* test; Fig. 1*C*). The adenosine-induced effects persisted for several minutes into the postseizure period, a time course that matches with previous reports of AR antagonism on the recovery of evoked field potentials (Etherington and Frenguelli, 2004; Etherington et al., 2009). More generally, our data demonstrate that ARs are strongly activated during seizures and function to attenuate seizure activity. This is consistent with earlier evidence that adenosine acts mainly as a seizure-suppressing substance (During and Spencer, 1992; Dunwiddie, 1999; Boison, 2006).

AR activation attenuates $GABA_A$ receptor-mediated depolarization during epileptiform activity

Previous work has demonstrated strong stimulation of $GABA_A$Rs during periods of hypersynchronous activity, such as ictal-like episodes. It has been argued that intense activation of $GABA_A$Rs leads to a pronounced influx and accumulation of intracellular Cl^-, which generates a transient-positive shift in E_{GABA_A} (Isomura et al., 2008; Wright et al., 2011) that is often followed by a transient increase in extracellular potassium (Kaila et al., 1997; Viitanen et al., 2010). This rapid depolarizing shift in E_{GABA_A} can create a situation where $GABA_A$R activity excites pyramidal cells and thus actually promotes their activity further (Köhling et al., 2000; Fujiwara-Tsukamoto et al., 2010). Our gramicidin recordings enabled us to record $GABA_A$R-mediated signals without disturbing intracellular chloride concentration dynamics during seizure episodes. As shown in Figure 2, local somatic application of the selective $GABA_A$R agonist, muscimol (45 μM), hyperpolarized the membrane potential of CA3 pyramidal neurons in the period before the seizure. However, during the after-discharge phase of the seizure and immediately following the seizure, $GABA_A$R activation strongly depolarized neurons (Fig. 2*B*). Importantly, $GABA_A$R responses during the after-discharge phase were sufficient to trigger action potentials. In the postseizure period, the depolarizing $GABA_A$R responses decreased gradually, were no longer able to trigger action potentials, and eventually reversed polarity and reverted to hyperpolarizing (Fig. 2*B*). To further investigate the mechanisms underlying the seizure-associated alteration in $GABA_A$R signaling, we performed a separate series of experiments to measure E_{GABA_A} during seizure activity. By rapidly switching from current-clamp to voltage-clamp mode during gramicidin recordings we were able to estimate E_{GABA_A} using voltage ramp protocols (Fig. 2*C–E*). E_{GABA_A} was measured at two points along the somatodendritic axis by activating $GABA_A$Rs at both somatic and dendritic sites (apical dendrites, ~150 μm from the soma) using two separate muscimol puffer pipettes. As shown in Figure 2*C* and E, ictal-like activity induced a pronounced depolarizing shift in both the somatic and dendritic E_{GABA_A}. The fact that the dendritic E_{GABA_A} shifted suggests that dialysis of cations via the gramicidin pores was unlikely to contribute significantly to these effects. Furthermore, the time course of the E_{GABA_A} changes closely matched the alterations in $GABA_A$R potentials described by Figure 2*A*. These results are consistent with a seizure-induced increase in intracellular Cl^-, which shifts E_{GABA_A} to depolarizing values and establishes the conditions for $GABA_A$R activation to trigger action potential activity. Consistent with a recovery of the Cl^- gradient in the postseizure period, E_{GABA_A} gradually returned to baseline values and $GABA_A$R responses reverted to hyperpolarizing.

To test whether endogenous adenosine modulates these seizure-induced changes in $GABA_A$R transmission, we used a within-cell design to measure $GABA_A$R-mediated potentials during control seizures and during seizures in which ARs were blocked with CGS 15943 (1 μM; Fig. 3). The amplitude of depolarizing $GABA_A$R potentials was measured during the 2 min period before the muscimol response reversed polarity (Fig. 3*A*,*B*). AR blockade had a strong and consistent effect on depolarizing $GABA_A$R responses recorded during the seizure episode. Blockade of ARs increased the amplitude of depolarizing $GABA_A$R responses to 155.5 ± 20.9% of control values ($n = 10$, $p < 0.05$, paired *t* test; Fig. 3*C*,*D*), indicating that seizure-induced adenosine release could attenuate depolarizing GABAergic potentials during seizures. Consistent with this hypothesis, when we examined the number of after-discharge events per seizure, we found that AR blockade increased their mean number from 45.8 ± 9.22 under control conditions to 87.30 ± 11.31 ($n = 10$, paired *t* test, $p < 0.05$). As depolarizing $GABA_A$R responses can contribute to prolongation of seizures and the generation of after-discharges (Higashima et al., 1996, 2000; Velazquez and Carlen, 1999; Fujiwara-Tsukamoto et al., 2003; Isomura et al., 2003a, 2008), the attenuation of depolarizing $GABA_A$R activity by adenosine may be an important aspect of adenosine's anticonvulsant action. We also examined the modulatory effect of AR activation after $GABA_A$R responses had reverted to hyperpolarizing. Hyperpolarizing $GABA_A$R responses were measured during a 2 min period after the muscimol response reversed polarity (Fig. 3*A*,*B*). Similar to the effects on depolarizing $GABA_A$R responses, antagonism

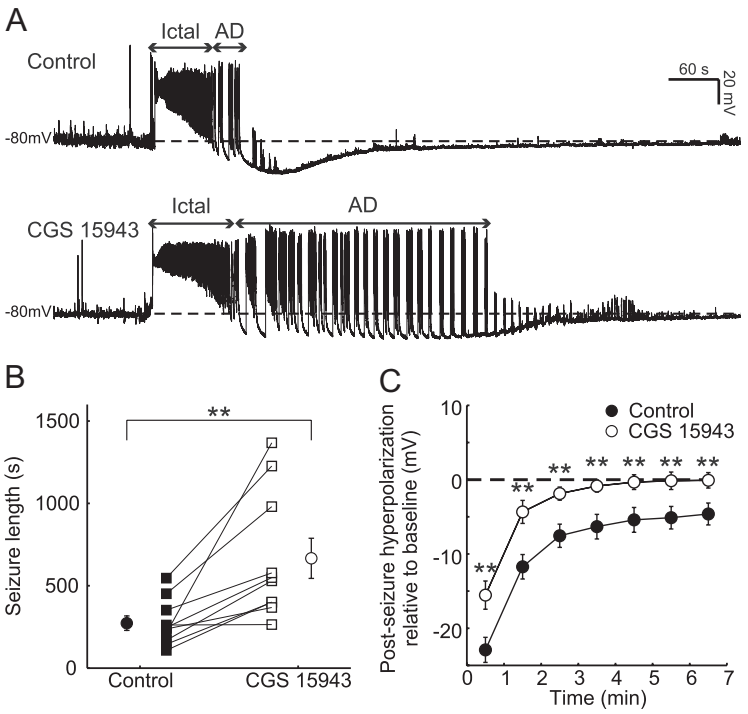

Figure 1. Endogenous adenosine modulates seizure activity and postseizure recovery. ***A***, Example seizures recorded from a CA3 pyramidal neuron under control conditions (upper trace) and during blockade of ARs with 1 μM CGS 15943 (lower trace). Recordings were performed using uncompensated current-clamp recordings, without DC current injection. Arrows indicate the ictal-like (Ictal) and after-discharge (AD) phases of the seizure. Horizontal dashed lines indicate preseizure resting membrane potential. Note that AR blockade results in an increase in total seizure duration and a faster recovery of the membrane potential to baseline levels following the end of the after-discharge phase. ***B***, Population data ($n = 10$ cells) for seizure length under control conditions (black symbols) and in the presence of the AR antagonist (open symbols). Square symbols indicate individual experiments; circles indicate population means. ***C***, Population data ($n = 10$ cells) for postseizure membrane potential recovery under control conditions (black circles) and in the presence of the AR antagonist (open circles). Values indicate the degree of hyperpolarization compared with preseizure baseline, where zero minutes represents the point of maximum hyperpolarization following the end of the after-discharge phase. Error bars indicate SEM, $^{**}p < 0.01$, paired *t* test.

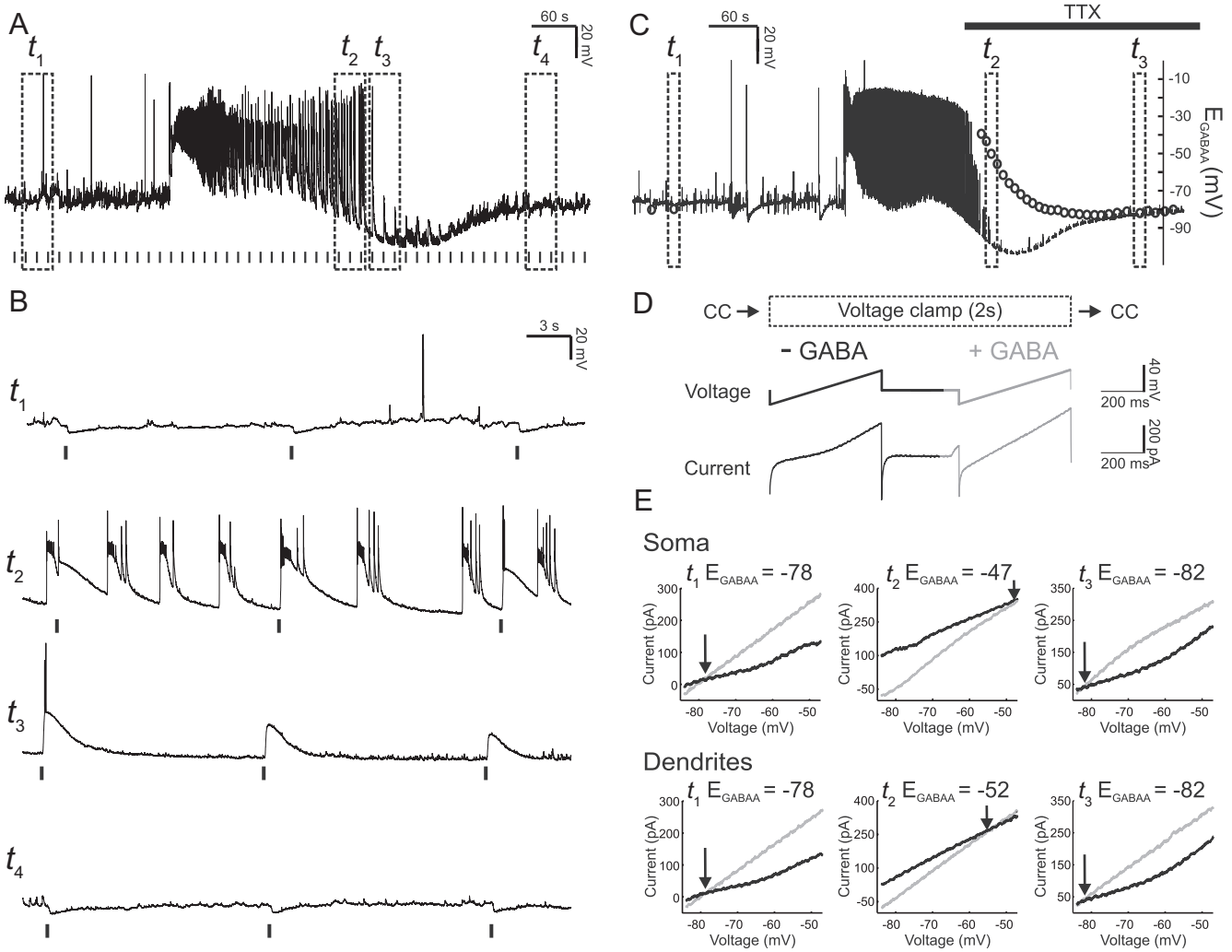

Figure 2. Seizure activity induces depolarizing and excitatory $GABA_AR$ signaling. ***A***, Example seizure recorded from a CA3 pyramidal neuron. Postsynaptic $GABA_AR$ responses were elicited with a muscimol puff every 15 s (vertical bars) during the seizure episode. Dashed boxes indicate time windows before the seizure (t_1) during the after-discharge phase (t_2), immediately after the seizure (t_3), and during the postseizure recovery period (t_4). ***B***, Expanded traces from the time windows represented by the boxes in ***A***. $GABA_AR$ responses before the onset of the seizure are hyperpolarizing (t_1). During the after-discharge period $GABA_AR$ responses become depolarizing and can trigger spikes (t_2). Immediately after the seizure, $GABA_AR$ responses are still depolarizing but show decreased amplitude and are less likely to trigger spikes (t_3). During the postseizure recovery period $GABA_AR$ responses return to being hyperpolarizing (t_4). ***C***, Recording in which somatic and dendritic E_{GABA_A} were measured during seizure activity. Dashed boxes indicate example E_{GABA_A} measurements before the seizure (t_1), shortly after the ictal phase (t_2), and later during the recovery period (t_3). Somatic E_{GABA_A} values are represented by the circles. To measure E_{GABA_A} without contamination from ongoing spiking activity, TTX was applied ~2 min after seizure onset (horizontal black bar). ***D***, E_{GABA_A} was measured by rapidly switching the recording mode from current-clamp (CC) to voltage-clamp and then applying two consecutive voltage ramps, the first under baseline conditions (black line; −GABA) and the second during activation of $GABA_AR$s (gray line; + GABA) either on the soma or on the apical dendrites (~150 μm from the soma). After 2 s in voltage-clamp the cell was returned to current-clamp. E_{GABA_A} measurements were made every 10 s. ***E***, I–V plots for somatic (top) and dendritic (bottom) measurements for the three time points represented by the boxes in ***C***. E_{GABA_A} was defined as the voltage at which the holding current (generated by the first ramp; black line) and the total current (reflecting the holding current plus the current through the activated $GABA_AR$s; gray line) intersected. Note that both somatic and dendritic E_{GABA_A} were at hyperpolarized values before the seizure (t_1), switched to depolarizing values during the seizure (t_2), and recovered to preseizure values (t_3).

of ARs increased the amplitude of the hyperpolarizing $GABA_AR$ responses to 150 ± 13.7% of control values ($n = 10$, $p < 0.05$, paired t test) (Fig. 3*C*,*D*). These results demonstrate that seizure-induced adenosine release attenuates not only the excitatory GABAergic potentials, but can also persist and modulate inhibitory $GABA_AR$-mediated signaling.

Adenosine attenuates postsynaptic $GABA_A$ receptor signaling by increasing membrane conductance via activation of K^+ channels, downstream of the A_1R

The fact that AR activation during a seizure can attenuate both depolarizing and hyperpolarizing $GABA_AR$ responses could be explained via an adenosine-induced change in the postsynaptic cell's membrane conductance, which would generate a "shunting" effect (Takigawa and Alzheimer, 2002). First, to establish if AR activation is sufficient to generate the observed effects, we tested whether exogenously applied adenosine (100 μM) is sufficient to attenuate the $GABA_AR$-mediated responses in seizure-free slices (see Materials and Methods; Fig. 4). Bath application of adenosine induced a hyperpolarization of the membrane potential of CA3 pyramidal neurons from −76.5 ± 0.9 mV to −89.7 ± 0.9 mV ($n = 6$, $p < 0.001$, paired t test), consistent with the downstream activation of K^+ channels (Fredholm et al., 2005; Clark et al., 2009). To maintain the same driving force for the $GABA_AR$, we compensated for the adenosine-induced hyperpolarization by current injection through the patch pipette. The baseline membrane potential was maintained at either −110 mV to compare depolarizing $GABA_AR$ responses, or at −70 mV to

compare hyperpolarizing $GABA_AR$ responses (Fig. 4*A*,*C*). In the presence of adenosine, depolarizing $GABA_AR$ responses were significantly smaller. The amplitude was reduced to 68.1 ± 4.7% of control values ($n = 5$, $p < 0.01$, paired t test), which was reversed following washout of the adenosine (97.7 ± 5.9% of control, $p < 0.01$, paired t test compared with adenosine; Fig. 4*B*). Under conditions that generated hyperpolarizing $GABA_AR$ responses, we saw a similar reduction in GABAergic signaling. Adenosine caused a reduction in the amplitude of $GABA_AR$ responses to 69.5 ± 5.7% of control values ($n = 6$, $p < 0.01$, paired t test) and this effect was reversed following washout of the adenosine (104.7 ± 7.8% of control, $p < 0.01$, paired t test compared with adenosine; Fig. 4*D*). These results demonstrate that activation of ARs is sufficient to attenuate postsynaptic $GABA_AR$ signaling when it is either depolarizing or hyperpolarizing.

To test whether a shunting mechanism can account for the effect of adenosine upon $GABA_AR$ responses, we used voltage-clamp and current-clamp recordings to measure the effect of adenosine on membrane conductance and the role of various downstream K^+ channels. Adenosine has been reported to activate several K^+ channels via A_1R signaling (Hosseinzadeh and Stone, 1998; Fredholm et al., 2005; Clark et al., 2009; Kawamura, Jr. et al., 2010) including: GIRKs (Fredholm et al., 2005; Clark et al., 2009), ATP-sensitive K^+ channels (KATPs) (Hosseinzadeh and Stone, 1998; Kawamura, Jr. et al., 2010), and small conductance Ca^{2+}-activated K^+ channels (SKs) (Clark et al., 2009). As predicted, voltage-clamp recordings showed that the application of adenosine resulted in a significant shift in the membrane conductance of CA3 pyramidal neurons from 4.5 ± 0.4 nS to 9.9 ± 1.0 nS. This represented an increase to 225.6 ± 14.9% of control values ($n = 23$, $p < 0.001$, paired t test; Fig. 5*A*). Furthermore, the effect of adenosine on membrane conductance was substantially reduced by coadministration of the A_1R antagonist DPCPX (5 μM; 118.4 ± 8.3% of control, $n = 6$, $p < 0.01$), by coadministration of the nonselective K^+ channel blocker Ba^{2+} (1.5 mM; 113.1 ± 5.4% of control, $n = 6$, $p < 0.01$), or by coadministration of a mixture of K^+ channel blockers designed to block the major downstream targets of A_1Rs (GIRKs, 400 nM tertiapin; KATPs, 1 mM tolbutamide; and SKs, 400 nM apamine; 141.2 ± 8.7% of control, $n = 10$, $p < 0.01$; Fig. 5*A*).

Importantly, the same pharmacological manipulations abolished the effect of adenosine on $GABA_AR$ responses. The effects of $GABA_AR$ activation upon the membrane potential were measured in current-clamp, with the baseline membrane potential maintained at −70 mV. As before, adenosine alone significantly reduced $GABA_AR$ responses amplitude to 59.3 ± 3.2% of control ($n = 25$, $p < 0.0001$, paired t test compared with control). However, this effect of adenosine was prevented by coadministration of the A_1R antagonist, DPCPX (94.2 ± 3.1% of control; $n = 5$, $p < 0.01$, ANOVA with *post hoc* Bonferroni's correction). The effect of adenosine upon $GABA_AR$ responses was also abolished by coadministration of the nonselective K^+ channel blocker Ba^{2+} (88.2 ± 11.5% of control; $n = 6$, $p < 0.01$, ANOVA with *post hoc* Bonferroni's correction), or by coadministration of the mixture of K^+ channel blockers designed to block the major downstream targets of A_1Rs (84.9 ± 4.5% of control; $n = 5$, $p < 0.05$, ANOVA with *post hoc* Bonferroni's correction; Fig. 5*B*,*C*).

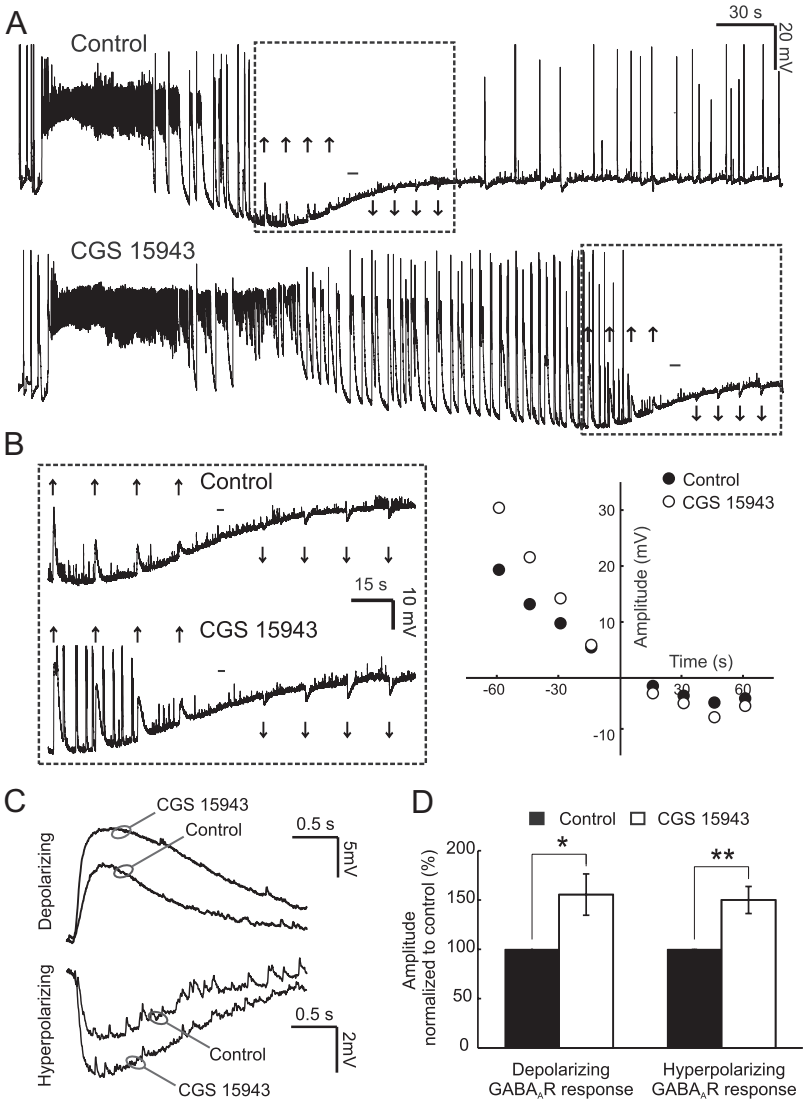

Figure 3. AR activity attenuates postsynaptic GABAergic potentials during seizures. ***A***, Example seizures recorded under control conditions (upper trace) and in the presence of the AR antagonist CGS 15943 (1 μM) (lower trace). Upward arrows indicate depolarizing postsynaptic $GABA_AR$ responses, downward arrows indicate hyperpolarizing $GABA_AR$ responses, and the hyphen indicates the reversal point, when there is no net response to a muscimol puff. Rectangles indicate a 2 min time window, centered about the reversal for the $GABA_AR$ response. ***B***, Traces (left) are expanded versions of the recording in ***A*** and illustrate the amplitude and direction of $GABA_AR$ responses. The plot (right) shows the amplitude of $GABA_AR$ responses in this recording (*y*-axis), in relation to the time when the $GABA_AR$ response switched back from depolarizing to hyperpolarizing (at 0 s on the *x*-axis); black circles represent $GABA_AR$ responses amplitude under control conditions, while open circles represent $GABA_AR$ responses amplitude under AR blockade. ***C***, Averaged traces for the cell shown in ***A*** confirm that blocking ARs results in an attenuation of both the depolarizing and hyperpolarizing $GABA_AR$ responses. ***D***, Population data ($n = 10$ cells) for the amplitude of depolarizing and hyperpolarizing postsynaptic $GABA_AR$ responses. Data are shown for the AR antagonist (open bars), normalized to control values (black bars). Error bars indicate SEM, *$p < 0.05$, **$p < 0.01$, paired t test.

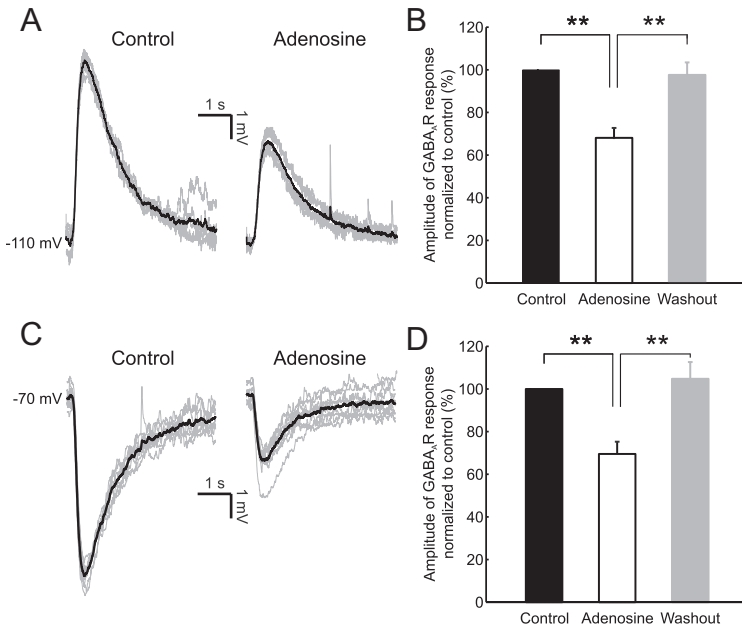

Figure 4. Exogenous adenosine application mimics the inhibitory effect on $GABA_AR$-mediated signaling. Depolarizing and hyperpolarizing $GABA_AR$ responses were elected by local application of muscimol under control conditions and in the presence of 100 μM adenosine. ***A***, Depolarizing $GABA_AR$ responses recorded from a CA3 pyramidal neuron under control conditions (left) and in the presence of adenosine (right). Cells were maintained at a resting membrane potential of −110 mV by somatic DC current injection (see Materials and Methods). Individual (gray traces) and mean (black traces) $GABA_AR$ potentials are shown. ***B***, Population data ($n = 5$ cells) for the amplitude of depolarizing postsynaptic $GABA_AR$ responses in the presence of adenosine (open bars) and after adenosine washout (gray bars), normalized to control values (black bars). Error bars indicate SEM, $^{**}p < 0.01$, paired *t* test. ***C***, Hyperpolarizing $GABA_AR$ responses recorded from a CA3 pyramidal neuron under control conditions (left) and in the presence of adenosine (right). Cells were maintained at a resting membrane potential of −70 mV by somatic DC current injection. ***D***, Population data ($n = 6$ cells) for the amplitude of hyperpolarizing postsynaptic $GABA_AR$ responses, using the same conventions as above. Error bars indicate SEM, $^{**}p < 0.01$, paired *t* test.

There was no significant difference between the DPCPX, Ba^{2+}, and K^+ channel blockers mixture for membrane conductance or $GABA_AR$-mediated responses amplitude ($p > 0.05$ in all cases, ANOVA with *post hoc* Bonferroni's correction). These results suggest that adenosine suppresses postsynaptic $GABA_AR$-mediated responses by increasing the cell's membrane conductance via activation of K^+ channels that are downstream of A_1R signaling.

To better understand how the changes in membrane conductance and $GABA_AR$ response relate to extracellular adenosine concentration we tested the effects of 1, 10, and 100 μM adenosine (Fig. 5*C*,*D*). Exogenous application of 1 μM adenosine had no statistically significant effect on either the membrane conductance (106.4 ± 6.4% of control, $p = 0.37$, $n = 5$, paired *t* test) or on the amplitude of $GABA_AR$ potentials (96.7 ± 3.4% of control, $p = 0.44$, $n = 3$, paired *t* test). In contrast, application of 10 μM adenosine increased the membrane conductance to 157.4 ± 15.0% of control ($p < 0.01$, $n = 8$, paired *t* test) and decreased the amplitude of $GABA_AR$ potentials to 78.7 ± 5.2% of control ($p < 0.01$, $n = 7$, paired *t* test). Finally, application of 100 μM adenosine increased the membrane conductance to 228.4 ± 19.7% of control ($p < 0.001$, $n = 14$, paired *t* test, Fig. 5*C*) and decreased the $GABA_AR$ response amplitude to 53.4 ± 3.5% of control ($p < 0.001$, $n = 14$, paired *t* test, Fig. 5*D*). The importance of extracellular adenosine concentration was also evident from the correlation between the adenosine-induced increase in membrane conductance and the decrease in $GABA_AR$ response amplitude (Pearson correlation, $r^2 = 0.49$, $p < 0.001$, $n = 24$; Fig. 5*E*). In terms of kinetics, previous studies using rapid agonist application in dissociated neurons have reported that A_1R-induced activation of K^+ channels has a latency of ~0.1 s and is maximal within 5 s (Sodickson and Bean, 1998). In our preparation we found that the peak of the adenosine-induced response was reached after 40.5 ± 6.5 s ($n = 10$), presumably reflecting the slower diffusion of adenosine into the slice.

$GABA_AR$ reversal potential and conductance are not altered by AR activation

Other mechanisms that could contribute to the adenosine-induced effect upon postsynaptic $GABA_AR$-mediated responses include alterations in Cl^- homeostasis mechanisms (Kaila et al., 1997; Dzhala et al., 2005, 2010; Jin et al., 2005; Huberfeld et al., 2007) and/or changes in the conductance of the $GABA_AR$ (Roseti et al., 2008, 2009). To investigate whether AR activation affects Cl^- homeostasis we measured E_{GABA_A} under control conditions and following bath application of adenosine. Muscimol-evoked $GABA_AR$ currents were recorded at six different holding voltages (from −50 to −100 mV) and E_{GABA_A} was calculated from the resulting I–V plots (see Materials and Methods; Fig. 6*A*). Under control conditions the mean E_{GABA_A} was −82.7 ± 1.2 mV and this remained unchanged following activation of ARs, when the mean E_{GABA_A} was −82.2 ± 1.0 mV ($n = 17$, $p = 0.48$, paired *t* test; Fig. 6*B*). These results suggest that adenosine signaling does not modulate Cl^--regulating mechanisms that set the E_{GABA_A}. To further explore a potential role of adenosine in modulating Cl^- homeostasis mechanisms during seizure activity, we analyzed our seizure data to assess the rate at which $GABA_AR$ responses recovered from depolarizing back to hyperpolarizing during the postseizure period (Fig. 3). In agreement with previous studies of chloride homeostasis, we found that the recovery of depolarizing $GABA_AR$ responses could be estimated using an exponential fit (Brumback and Staley, 2008; Nardou et al., 2011). Under control conditions we found that the recovery rate had a time constant of 34.7 ± 2.8 s and that this was not affected by AR blockade with CGS 15943, when the mean time constant was 31.6 ± 3.0 ($p = 0.22$, $n = 8$, paired *t* test).Together, these results show that changes in Cl^- homeostasis mechanisms are unlikely to contribute to the attenuation of the $GABA_AR$-mediated potentials.

Reducing either the postsynaptic density of $GABA_AR$ receptors and/or their permeability would result in a decreased $GABA_AR$ conductance, which could also potentially contribute to the adenosine-induced attenuation of $GABA_AR$-mediated responses. To test this possibility we used voltage-clamp recordings and measured the $GABA_AR$ conductance across a range of membrane potentials (see Materials and Methods). I–V plots of the $GABA_AR$-mediated currents were constructed and conductance was calculated as the slope of linear fits of the data (Fig. 7*A*). Under control conditions the peak $GABA_AR$ conductance was 14.0 ± 1.8 nS, on average, and remained unchanged following 10 min activation of ARs when the mean was 14.9 ± 1.8 nS ($n = 9$, $p = 0.23$, paired *t* test; Fig. 7*B*). These

results show that changes in the $GABA_AR$ conductance are unlikely to contribute to the adenosine-induced attenuation of the $GABA_AR$-mediated potentials.

Discussion

We investigated the role of the endogenous anticonvulsant adenosine in modulating GABAergic signaling during epileptiform activity. Intense activation of the GABAergic system during seizure activity leads to a transient switch in the action of $GABA_ARs$ from inhibitory to excitatory. Seizure activity also raises the levels of extracellular adenosine. We found that the resulting AR activation reduced the amplitude of excitatory GABAergic potentials during seizures and that the suppression of GABAergic signaling lasted beyond the period in which the E_{GABA_A} was shifted positively. These effects of endogenously released adenosine were mimicked by exogenous adenosine administration. In examining the mechanism of adenosine's effects, we established that adenosine does not change chloride homeostasis mechanisms that set E_{GABA_A}, or the conductance of $GABA_ARs$. Rather, A_1R-dependent activation of K^+ channels can account for the suppression of depolarizing and hyperpolarizing GABAergic potentials via an increase in postsynaptic conductance and a resulting shunting effect. As depolarizing $GABA_AR$-mediated signaling has been implicated in seizure initiation and progression, the adenosine-induced attenuation of depolarizing $GABA_AR$ signaling may represent an important mechanism by which adenosine can limit seizure activity.

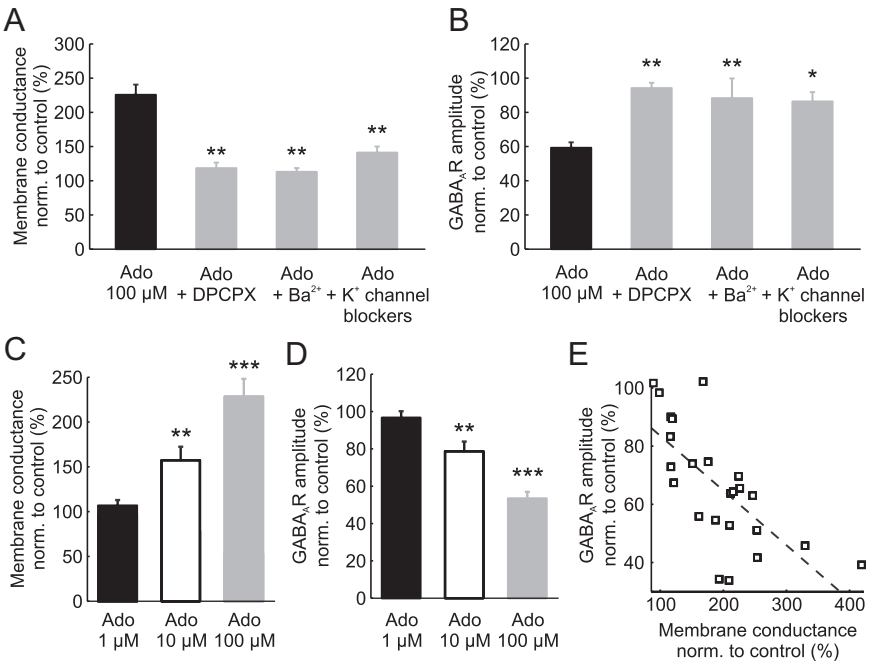

Figure 5. Adenosine attenuates postsynaptic $GABA_AR$ potentials via A_1R activation of K^+ channels that increase membrane conductance. ***A***, Adenosine (Ado; 100 μM; black bar, $n = 23$) increased the membrane conductance to ~220% of control values. Coadministration of adenosine with the selective A_1R antagonist DPCPX (5 μM; $n = 6$) prevented the adenosine-induced increase in membrane conductance. Similar results were obtained when adenosine was coadministered with either a nonspecific blocker of K^+ channels (Ba^{2+} 1.5 mM; $n = 6$), or with a mixture of K^+ channel blockers containing the KATP channel blocker (tolbutamide 1 mM), a GIRK blocker (tertiapin 400 nM), and a small conductance Ca^{2+}-activated K^+ channel blocker (apamine 400 nM; $n = 10$). ***B***, Population data for the amplitude of postsynaptic $GABA_AR$ responses for each of the groups in ***A***. When adenosine was delivered alone it reduced the amplitude of $GABA_AR$ responses by ~40%. This effect was blocked when adenosine was applied in the presence of DPCPX, Ba^{2+}, or the K^+ channel blocker mixture. All measurements were normalized to control values in the absence of any drugs. Statistical tests reflect comparisons with the adenosine-only group. Error bars indicate SEM, $*p < 0.05$, $**p < 0.01$, ANOVA with *post hoc* Bonferroni's correction. ***C***, ***D***, The effect of 1, 10, and 100 μM adenosine on membrane conductance (***C***) and $GABA_AR$ response amplitude (***D***). Results from statistical tests indicate within cell comparisons before and after adenosine administration. Error bars indicate SEM, $**p < 0.01$, $***p < 0.001$, paired *t* test. ***E***, The increase in membrane conductance showed a significant negative correlation with the $GABA_AR$ response amplitude (Pearson correlation, $r^2 = 0.49$, $p < 0.001$, $n = 24$). Dashed line indicates linear fit of the data; open squares represent individual experiments.

Studies using microdialysis probes implanted in the hippocampus of epileptic patients have estimated that seizures lasting 40–200 s trigger substantial (up to 65 μM) increases in extracellular adenosine (During and Spencer, 1992). This adenosine surge correlates with seizure arrest and adenosine levels remain elevated during the postseizure period. In rat hippocampal slices, short seizures are sufficient to elevate extracellular levels of adenosine to ~5 μM and blocking A_1Rs increases seizure duration and intensity (Etherington et al., 2009; Lopatář et al., 2011). Seizures in our study had a mean duration of ~250 s, which would be predicted to trigger adenosine release. Consistent with this prediction, blockade of ARs doubled seizure duration. These results confirm that adenosine has a net antiepileptic effect and they are consistent with *in vivo* studies in which focal augmentation of adenosine in the hippocampus of epileptic rats can reduce seizure frequency and duration, and even prevent epileptogenesis (Güttinger et al., 2005a,b; Li et al., 2007, 2009; Boison, 2009; Szybala et al., 2009).

Adenosine had its principal effects during the after-discharge phase of the seizures we recorded. Synchronized after-discharge activity is characteristic of epileptiform activity and it has been shown that a transient change in E_{GABA_A} is central to this process. During the ictal phase of seizures, intense activation of $GABA_ARs$ in combination with membrane depolarization results in a substantial Cl^- influx into principal neurons, which overwhelms Cl^- homeostasis mechanisms. The resulting positive shift in E_{Cl} (and therefore E_{GABA_A}) sets the conditions for networks of GABAergic interneurons to generate the synchronized excitation that underlies an after-discharge (Isomura et al., 2003b; Zsiros et al., 2007). The collapse in the Cl^- gradient also affects the drive on K^+-Cl^- cotransporter proteins, which results in transient increases in extracellular K^+ that can also influence ongoing activity (Kaila et al., 1997; Viitanen et al., 2010). Consistent with this scenario, we found that ictal activity shifts E_{GABA_A} to depolarizing values and that activation of $GABA_ARs$ during the after-discharge period is able to generate depolarizing excitatory responses and trigger action potentials. The exact time point at which E_{GABA_A} becomes depolarizing during a seizure will depend on the balance of ionic conductances and the ability of homeostatic mechanisms to clear Cl^-. To address this, future experiments could use imaging techniques to measure temporal and spatial changes in chloride that occur during a seizure.

Our pharmacological experiments showed that blocking ARs during seizures increased the excitatory actions of GABAergic signaling. Consistent with this, we found that when ARs were active under control conditions, seizures had less after-discharge events and a shorter after-discharge period. Other actions of adenosine may affect presynaptic GABAergic transmission dur-

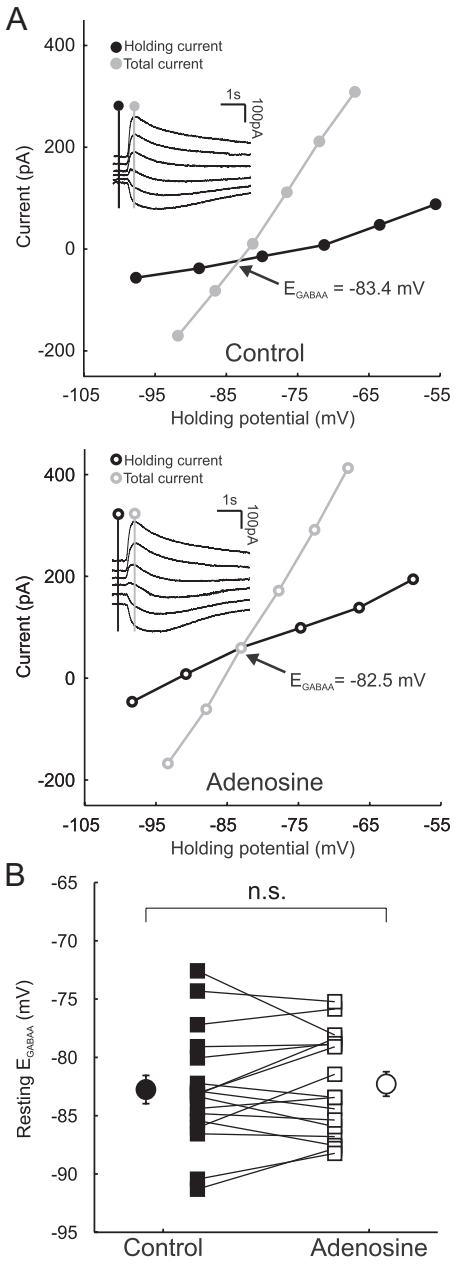

Figure 6. AR activation does not alter Cl^- homeostasis. ***A***, Data from a representative CA3 pyramidal neuron in which resting E_{GABA_A} was measured under control conditions (top) and in the presence of 100 μM adenosine (bottom). Inserts, Example of $GABA_AR$ currents evoked by muscimol puffs at different holding potentials. Holding current (reflecting membrane current) and total current (reflecting membrane current plus the muscimol-evoked current) were measured at the points indicated by the vertical black and gray lines, respectively. I–V plots were constructed and E_{GABA_A} was calculated as the potential at which the total current was equal to the holding current. ***B***, Resting E_{GABA_A} values for individual cells ($n = 17$) under control conditions (black squares) and in the presence of adenosine (open squares). Population averages are also shown (black circles, control; open circles, adenosine). Error bars indicate SEM; $p > 0.05$, paired *t* test.

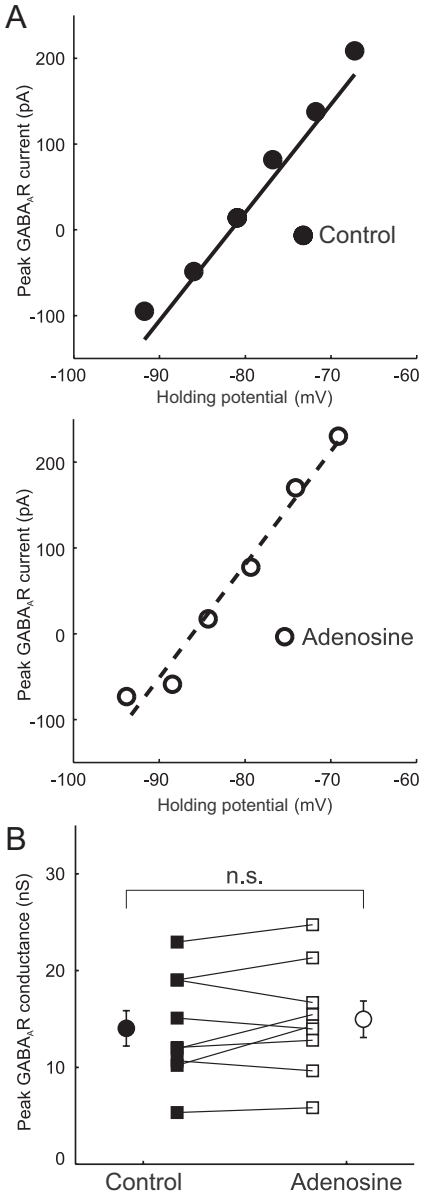

Figure 7. AR activation has no effect on $GABA_AR$ conductance. ***A***, Data from a representative CA3 pyramidal neuron in which the $GABA_AR$ conductance was measured under control conditions (top) and in the presence of 100 μM adenosine (bottom). The peak $GABA_AR$ current was calculated as described in the text and the peak $GABA_AR$ conductance was calculated as the slope of the linear fit. ***B***, Peak $GABA_AR$ conductance for individual cells ($n = 9$) under control conditions (black squares) and in the presence of adenosine (open squares). Population averages are also shown (black circles, control; open circles, adenosine). Error bars indicate SEM; $p > 0.05$, paired *t* test.

ing seizure activity. For instance, adenosine may affect the activity of interneurons either directly, or indirectly via reducing their glutamatergic inputs, which would ultimately decrease the amount of GABA released. Nevertheless, it is known that GABAergic interneurons can remain active during seizures and can in fact maintain seizure activity and after-discharge events when glutamatergic transmission is blocked (Fujiwara-Tsukamoto et al., 2006, 2010). Adenosine's effect on the membrane potential could also have complex effects upon excitability of the network, such as by increasing de-inactivation of excitatory cationic voltage-dependent conductances. However, in the context of seizure activity at least, the net effect of adenosine is inhibitory; when ARs were active the postsynaptic membrane potential was more hyperpolarized and the neurons showed less excitability in the form of after-discharges.

The acute effect of adenosine upon $GABA_AR$-mediated signaling could involve changes to Cl^- homeostasis mechanisms or changes to the expression and/or properties of $GABA_ARs$. Indeed, previous studies have reported seizure-induced alterations in E_{GABA_A} that are mediated by changes in the activity of Cl^-

transporter proteins, including NKCC1 and KCC2 (Rivera et al., 2004; Jin et al., 2005; Dzhala et al., 2010; Khirug et al., 2010). However, we found that adenosine had no direct effect on steady-state E_{GABA_A} or the recovery of depolarizing $GABA_AR$ responses back to hyperpolarizing. AR activation also did not alter the conductance of the $GABA_AR$, indicating that adenosine release over the timescale relevant to a seizure does not have an acute effect upon the properties of the $GABA_AR$. These observations are consistent with previous recordings following short-term activation of ARs (Yoon and Rothman, 1991; Thompson et al., 1992; Kruglikov and Rudy, 2008) and with the conclusion that adenosine's effects upon $GABA_AR$ responses during seizure activity must be via an alternative mechanism.

The parsimonious explanation for adenosine's effects upon $GABA_AR$ responses is that they are mediated by a postsynaptic shunting effect that develops under conditions where GABAergic transmission transiently changes from inhibitory to excitatory. In support of such a shunting mechanism, as E_{GABA_A} and the postsynaptic membrane potential shifted throughout the seizure period, AR activation consistently reduced the amplitude of $GABA_AR$-mediated potentials. We found that adenosine reduced $GABA_AR$ responses via an A_1R-dependent activation of K^+ channels that hyperpolarize the membrane potential and increase membrane conductance. Adenosine has been reported to activate three types of K^+ channels that are downstream of A_1R signaling: GIRKs, KATPs, and SK channels, all of which would be expected to increase membrane conductance (Hosseinzadeh and Stone, 1998; Fredholm et al., 2005; Clark et al., 2009; Kawamura, Jr. et al., 2010). Indeed, although we investigated the interaction between adenosine and GABAergic transmission in the context of a model of post-traumatic epilepsy (Berdichevsky et al., 2012), it would seem likely that adenosine's effect upon GABAergic transmission would be a general principle for pathological situations in which GABA becomes transiently depolarizing and adenosine is released at sufficient levels to increase membrane conductance.

The exact mechanisms by which extracellular adenosine increases during seizure activity are still the focus of investigation, and multiple sources appear to contribute in neurons and/or glia, such as the action of equilibrative transporter proteins, local breakdown of ATP, and activity-dependent exocytosis (Dale and Frenguelli, 2009). Direct measurements of the dynamics of extracellular adenosine accumulation are somewhat limited in terms of their spatial and temporal resolution, but previous work indicates that levels of extracellular adenosine are related to seizure duration and intensity (During and Spencer, 1992; Etherington et al., 2009). Consistent with this idea, the effects we observed were evident only while adenosine levels were elevated and could be rapidly reversed following adenosine washout. We also found a relationship between adenosine concentration and response, such that higher concentrations of adenosine resulted in greater increases in membrane conductance and attenuation of $GABA_AR$ responses. GABAergic responses were reduced when adenosine was applied at a concentration of 10–100 μM in the aCSF, which is within the range of extracellular concentrations that have been reported during seizures and other pathological states (During and Spencer, 1992; Pearson et al., 2006). Previous studies have also suggested that elevated adenosine levels persist well into the postseizure period, until the adenosine has been cleared from the extracellular space (During and Spencer, 1992; Etherington et al., 2009; Lopatář et al., 2011). Consistent with this, we found that adenosine signaling in the postseizure period continued to attenuate $GABA_AR$ signaling once it had returned to hyperpolarizing.

Recordings from hippocampal neurons have revealed that adenosine does not have a direct effect upon the presynaptic release of GABA, although GABAergic synapses in other systems do show evidence of presynaptic modulation (Yoon and Rothman, 1991;Thompson et al., 1992; Kruglikov and Rudy, 2008; Han et al., 2011). Our observations extend this work by demonstrating that adenosine modulates the postsynaptic effects of GABAergic inputs during the dynamic events of a seizure. The increase in extracellular adenosine that results from intense network activity appears to be well placed to attenuate the deleterious effects of depolarizing $GABA_AR$ signaling that typically emerge under the same conditions. Adenosine does this without "shutting down" GABAergic transmission, unlike its effect upon the glutamatergic system (Thompson et al., 1993). The net effect is that synaptic inhibition is still functional during the recovery period, as activity throughout the network begins to be reinstated.

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