JPn Neuroscience
The Journal of Physiology

Adenosine effects on inhibitory synaptic transmission and excitation–inhibition balance in the rat neocortex

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Key points

- Adenosine might be the most widespread neuromodulator in the brain, but its effects on inhibitory transmission in the neocortex are not understood.
- Here we report that adenosine suppresses inhibitory transmission to layer 2/3 pyramidal neurons via activation of presynaptic A_1 receptors.
- We present evidence for functional A_{2A} receptors, which have a weak modulatory effect on the A_1-mediated suppression, at about 50% of inhibitory synapses at pyramidal neurons.
- Adenosine suppresses excitatory and inhibitory transmission to a different extent, and can change the excitation–inhibition balance at a set of synapses bidirectionally, but on average the balance was maintained during application of adenosine.
- These results suggest that changes of adenosine concentration may lead to differential modulation of excitatory–inhibitory balance in pyramidal neurons, and thus redistribution of local spotlights of activity in neocortical circuits, while preserving the balanced state of the whole network.

Abstract Adenosine might be the most widespread neuromodulator in the brain: as a metabolite of ATP it is present in every neuron and glial cell. However, how adenosine affects operation of neurons and networks in the neocortex is poorly understood, mostly because modulation of inhibitory transmission by adenosine has been so little studied. To clarify adenosine's role at inhibitory synapses, and in excitation–inhibition balance in pyramidal neurons, we recorded pharmacologically isolated inhibitory responses, compound excitatory–inhibitory responses and spontaneous events in layer 2/3 pyramidal neurons in slices from rat visual cortex. We show that adenosine (1–150 μM) suppresses inhibitory transmission to these neurons in a concentration-dependent and reversible manner. The suppression was mediated by presynaptic A_1 receptors (A_1Rs) because it was blocked by a selective A_1 antagonist, DPCPX, and associated with changes of release indices: paired-pulse ratio, inverse coefficient of variation and frequency of miniature events. At some synapses (12 out of 24) we found evidence for A_{2A}Rs: their blockade led to a small but significant increase of the magnitude of adenosine-mediated suppression. This effect of A_{2A}R blockade was not observed when A_1Rs were blocked, suggesting that A_{2A}Rs do not have their own effect on transmission, but can modulate the A_1R-mediated suppression. At both excitatory and inhibitory synapses, the magnitude of A_1R-mediated suppression and A_{2A}R–A_1R interaction expressed high variability, suggesting high heterogeneity of synapses in the sensitivity to adenosine. Adenosine could change the balance between excitation and inhibition at a set of inputs to a neuron bidirectionally, towards excitation or towards inhibition. On average, however, these bidirectional changes cancelled each other, and the overall balance of excitation and inhibition was maintained during application of adenosine. These results suggest that changes of adenosine concentration may lead to differential modulation of excitatory–inhibitory balance in pyramidal neurons, and thus redistribution of local spotlights of activity in neocortical circuits, while preserving the balanced state of the whole network.

DOI: 10.1113/jphysiol.2014.279901

(Received 20 June 2014; accepted after revision 28 November 2014; first published online 12 December 2014)
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Abbreviations A_1R, adenosine receptor type 1; $A_{2A}R$, adenosine receptor type 2A; Ado, adenosine; APV, D-(−)-2-amino-5-phosphonopentanoic acid; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine (A_1R antagonist); mEPSP, miniature excitatory postsynaptic potential; mIPSP, miniature inhibitory postsynaptic potential; PPR, paired-pulse ratio; SCH-58261, 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine ($A_{2A}R$ antagonist).

Introduction

Adenosine is a potent neuromodulator, and as an ATP metabolite it is abundant in the brain. Neurons and astrocytes release adenosine and ATP in an activity-dependent manner (Pascual *et al.* 2005; Wall & Dale, 2008; Halassa *et al.* 2009; Lovatt *et al.* 2012). In the extracellular space, adenosine-phosphates are broken down to form adenosine (Dunwiddie *et al.* 1997). Extracellular concentration of adenosine is thus an indicator of activity and energy demand: as activity and energy expenditure rise from physiological to pathological, levels of adenosine increase (Fredholm, 2014). Consequently, adenosine is implicated in a multitude of functions associated with physiological and pathological alterations of brain activity. These functions include regulation of the sleep–wake cycle and cortical slow oscillations (Bjorness & Greene, 2009; Halassa *et al.* 2009), cognition, learning and memory (Fuxe *et al.* 2007; Halassa *et al.* 2009; Kadowaki Horita *et al.* 2013) as well as the neuroprotective response to traumatizing events such as hypoxia, ischaemia and excitotoxicity (de Mendonca *et al.* 2000; Dunwiddie & Masino, 2001; Cunha, 2005; Gomes *et al.* 2011). Moreover, potential therapeutic effects of adenosine receptor activation (Fredholm, 2010) have been recently explored for management of neurological diseases including epilepsy (Fedele *et al.* 2006; Dale & Frenguelli, 2009; Masino *et al.* 2011), Parkinson's disease (Hurley *et al.* 2000; Kitagawa *et al.* 2007) and schizophrenia (Deckert *et al.* 2003; Hong *et al.* 2005).

Out of four types of G-protein coupled adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3), the A_1Rs and A_{2A}Rs are the most abundant in the brain (Fastbom *et al.* 1987; Svenningsson *et al.* 1997; Chaudhuri *et al.* 1998; Cremer *et al.* 2011). A_1Rs are widespread throughout the brain, including the cerebral cortex (Dixon *et al.* 1996; Fredholm *et al.* 2001). A_1R activation has a generally suppressive effect on excitatory transmission and cell excitability in the hippocampus (Dunwiddie & Haas, 1985; Thompson *et al.* 1992; Scanziani *et al.* 1992; Wu & Saggau, 1994; Gundlfinger *et al.* 2007) and neocortex (Murakoshi *et al.* 2001; Kerr *et al.* 2013; van Aerde *et al.* 2013; Bannon *et al.* 2014). A_{2A}Rs are expressed most densely within the striatum, nucleus accumbens, and olfactory bulb (Lopes *et al.* 1999; Murakoshi *et al.* 2001; Schiffmann *et al.* 2007), but are also present in the neocortex (Cunha *et al.* 1996; Dixon *et al.* 1996; Sebastião & Ribeiro, 1996; Fredholm *et al.* 2001; Lopes *et al.* 2004). Only few studies have found a facilitatory effect of $A_{2A}R$ activation on excitatory synaptic transmission in the hippocampus (Lopes *et al.* 2002; Dias *et al.* 2012) and neocortex (Bannon *et al.* 2014). Actions of A_{2A}Rs at cortical synapses are poorly understood, partially because A_{2A}Rs often do not directly modulate neural activity, but rather interact with receptors for other neuromodulators (Sebastião & Ribeiro, 2009). Expression of $A_{2A}R$ in only a portion of synapses, and the small magnitude of the effects further complicate the study of $A_{2A}R$-regulation of cortical synaptic transmission.

In the cortex, the effects of adenosine on synaptic transmission have been studied mostly at excitatory synapses. Regulation of inhibitory synapses by adenosine has received little attention, partially because early studies in the hippocampus found that adenosine suppresses excitatory, but not inhibitory transmission (Lambert & Teyler, 1991; Yoon & Rothman, 1991; Brundege & Dunwiddie 1996). Recent work in the neocortex provided a patchy picture: adenosine had no effect on inhibitory transmission to layer 2/3 pyramids in the prefrontal cortex of rats (Mathew *et al.* 2008), but did suppress inhibitory transmission in mice, to layer 5 pyramids in somatosensory cortex (Kruglikov & Rudy, 2008) and Cajal–Retzius neurons in immature visual cortex (Kirmse *et al.* 2008). It is not clear from these sparse data whether the effects of adenosine on inhibitory transmission are restricted to specific cell types, brain regions, or even species.

In the study reported here we asked: How does adenosine modulate inhibitory transmission to layer 2/3 pyramidal neurons in rat visual cortex? Does it change the excitatory–inhibitory balance? Which receptors mediate adenosine's effects on inhibitory transmission? To address these questions, we made whole-cell recordings from layer 2/3 pyramidal neurons in slices, and measured the effects of bath application of adenosine and antagonists of A_1 and A_{2A} receptors on pharmacologically isolated inhibitory synaptic responses, spontaneous events and compound excitatory–inhibitory responses.

Methods

Slice preparation

All experimental procedures used in this study are in compliance with the US National Institutes of Health regulations and were approved by the Institutional Animal Care and Use Committee of the University of Connecticut. Details of slice preparation and recording were similar to those used in previous studies (Volgushev *et al.* 2000; Lee *et al.* 2012; Bannon *et al.* 2014). Wistar rats (16–28 days old, Charles River, Wilmington, MA, USA or Harlan, South Easton, MA USA) were anaesthetized with isoflurane, decapitated, and the brain was quickly removed and placed into an ice-cold oxygenated artificial cerebrospinal fluid solution (ACSF), containing (in mM): 125 NaCl, 25 $NaHCO_3$, 25 glucose, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, bubbled with 95% O_2–5% CO_2, pH 7.4. Coronal slices (350 μm thick) containing the visual cortex were prepared from the right hemisphere. Slices were allowed to recover for at least one hour at room temperature. For recording, individual slices were transferred to a recording chamber mounted on an Olympus BX-50WI microscope equipped with infrared differential interference contrast (IR-DIC) optics. In the recording chamber slices were perfused with oxygenated ACSF at 28–32°C.

Intracellular recording in brain slices

Layer 2/3 pyramidal cells from visual cortex were selected for recording in the whole cell configuration. In total, we included in the analysis results from $n = 93$ cells for synaptic stimulation experiments and $n = 27$ cells for miniature/spontaneous activity experiments.

Identification of pyramidal neurons using DIC microscopy was reliable, as demonstrated in our previous work with biocytin labelling and morphological reconstruction of recorded neurons (Volgushev *et al.* 2000). Intracellular pipette solution contained (in mM): 130 potassium gluconate, 20 KCl, 10 Hepes, 10 sodium phosphocreatine, 4 Mg-ATP, 0.3 Na_2-GTP, (pH 7.4 with KOH). All drugs were bath applied. For experiments recording GABAergic postsynaptic potentials, the glutamatergic blockers 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM; Sigma, St Louis, MO, USA) and (2*R*)-amino-5-phosphonopentanoate (APV, 20 μM; Tocris, Bristol, UK) were applied to block AMPA receptors and NMDA receptors, respectively. For experiments measuring miniature EPSPs (mEPSPs), tetrodotoxin (TTX, 0.1–0.5 μM; Tocris) was added to the extracellular solution at least 25 min before recordings were started. TTX was dissolved in water to make a 0.5 mM stock before being added to the bath. In some experiments (where mentioned), picrotoxin (100 μM; Sigma) was used to block inhibitory transmission. Picrotoxin was dissolved in the ACSF directly. Adenosine (Sigma) was dissolved in ACSF to make a 1 mM stock before being applied to the bath. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; Sigma) was dissolved in >99.9% DMSO to make a 1 mM stock. SCH-58261 (Tocris) was dissolved in >99.9% DMSO to make a 1 mM stock. The final concentration of DMSO in bath was <0.05%.

With the ionic composition of extracellular and pipette solutions used in this study, the equilibrium potential for chloride, and thus reversal potential of $GABA_A$ receptor-mediated postsynaptic potentials (PSPs) is expected to be approximately at −50 mV. Indeed, reversal potential of pharmacologically isolated (DNQX and APV in the bath) inhibitory postsynaptic currents (IPSCs) was between −50 mV and −55 mV (Fig. 1*C* and *D*). Thus at resting membrane potential (typically between −65 and −75 mV) synaptic stimulation induced depolarizing GABAergic IPSPs and inward IPSCs. The GABAergic nature of evoked responses was confirmed by their blockade by PTX (100 μM) in $n = 8$ experiments (Fig. 1*B*, lower trace). In all experiments, synaptic responses were recorded at resting membrane potential (around −70 mV) and at depolarized potentials (∼ −45 mV) (Fig. 1*B*, top trace). Only responses that expressed reversal were considered GABAergic and used for the analysis.

Synaptic stimulation

Two pairs of stimulating electrodes (S1 and S2) were placed in layer 4, below the layer 2/3 recording site, either in layer 4 (Fig. 1*A*) or in layer 5. In both cases, we used a paired-pulse stimulation protocol with a 50 ms inter-pulse interval. Paired stimuli were applied to S1 and S2 in alternating sequence once per 7.5 s; as a result each input was stimulated with paired pulses every 15 s. Layer 4 stimulation (Fig. 1*A*) was used in experiments with pharmacologically isolated IPSPs or IPSCs. This stimulation was similar to that used in our previous study of adenosine's effects on excitatory transmission (Bannon *et al.* 2014), thus facilitating comparison of results. Stimulation intensity was adjusted to evoke minimal responses. Layer 5 stimulation was used to induce compound excitatory/inhibitory postsynaptic currents. These responses were recorded in voltage clamp mode at different holding potentials (−40 mV, −50 mV, −60 mV and −70 mV) and used for calculation of excitatory and inhibitory conductances (see below). Stimulation intensity was adjusted to obtain clearly biphasic responses at $V_{hold} = -40$ mV. Stimulation intensity in these experiments was typically ∼5–10 stronger than that in experiments with pharmacologically isolated IPSPs evoked by stimulation in layer 4.

Data analysis

Data analysis was made by using custom-written programs in MatLab environment (MathWorks, Natick, MA, USA). All inputs included in the analysis fulfilled the criteria of (1) stability of IPSP/IPSC amplitudes during the control period, (2) stability of the membrane potential throughout the recording, and (3) stability of the onset latency and kinetics of the rising slope of the responses. IPSP (IPSC) amplitudes were measured as the difference between the mean membrane potential (current) during two time windows (0.5–2 ms width), the first window placed before the response onset and the second window placed on the rising slope of the postsynaptic response, just before the peak (see examples in Figs 1*C* and 3*A*). The amplitude of the response to the second pulse in the paired-pulse stimulation protocol was measured using windows of the same duration, but shifted by the length of the inter-pulse interval (50 ms).

Excitatory and inhibitory conductances during compound responses were calculated by solving the conductance model equation (Monier *et al.* 2008). The following equations were used:

$$I_{\mathrm{inj}} = C_{\mathrm{m}}\frac{\mathrm{d}V_{\mathrm{m}}}{\mathrm{d}t} + I_{\mathrm{inh}} + I_{\mathrm{exc}} + I_{\mathrm{leak}}, \tag{1}$$

where I_{inj} denotes the injected current, C_{m} the membrane capacitance, I_{exc} the excitatory current produced by changes of excitatory conductance during the compound responses, I_{inh} the current produced by changes of inhibitory conductance, I_{leak} the leak current. In voltage clamp mode I_{leak} equals to the holding current before the stimulus application, and was directly measured in a window placed before the stimulus. When applied to voltage clamp measurement, the derivative terms are zero, thus eqn (1) yields:

$$I_{\mathrm{inj}} = I_{\mathrm{inh}} + I_{\mathrm{exc}} + I_{\mathrm{leak}}, \tag{2}$$

where excitatory and inhibitory currents are defined by:

$$I_{\mathrm{exc}} = G_{\mathrm{exc}}(V_{\mathrm{hold}} - E_{\mathrm{exc}}), \tag{3}$$

$$I_{\mathrm{inh}} = G_{\mathrm{inh}}(V_{\mathrm{hold}} - E_{\mathrm{inh}}), \tag{4}$$

where G_{exc} denotes the excitatory conductance, V_{hold} the holding potential, E_{exc} the reversal potential of excitatory component (0 mV), G_{inh} the inhibitory conductance, E_{inh} the reversal potential of inhibitory component (−50 mV). Substituting I_{exc} and I_{inh} in eqn (2) using eqns (3) and (4) leads to:

$$\begin{aligned} I^1_{\mathrm{inj}}(t) = {} & G_{\mathrm{exc}}(t)\left(V^1_{\mathrm{hold}} - E_{\mathrm{exc}}\right) \\ & + G_{\mathrm{inh}}(t)\left(V^1_{\mathrm{hold}} - E_{\mathrm{inh}}\right) + I_{\mathrm{leak}}. \end{aligned} \tag{5}$$

For calculation of the time course of conductance changes, for each time point t a linear system consisting of four sets of eqn (5) each containing two unknowns G_{exc} and G_{inh} was composed for four different holding potentials ($V^1_{\mathrm{hold}} \ldots V^4_{\mathrm{hold}}$):

$$\begin{aligned} I^1_{\mathrm{inj}}(t) = {} & G_{\mathrm{exc}}(t)\left(V^1_{\mathrm{hold}} - E_{\mathrm{exc}}\right) \\ & + G_{\mathrm{inh}}(t)\left(V^1_{\mathrm{hold}} - E_{\mathrm{inh}}\right) + I_{\mathrm{leak}}, \end{aligned}$$

$$\ldots$$

$$\begin{aligned} I^4_{\mathrm{inj}}(t) = {} & G_{\mathrm{exc}}(t)\left(V^4_{\mathrm{hold}} - E_{\mathrm{exc}}\right) \\ & + G_{\mathrm{inh}}(t)\left(V^4_{\mathrm{hold}} - E_{\mathrm{inh}}\right) + I_{\mathrm{leak}}. \end{aligned}$$

The system was solved using internal MatLab function *linsolve*. Solution of the system of equations for each time point t gave us time course of conductance changes, $G_{\mathrm{exc}}(t)$ and $G_{\mathrm{inh}}(t)$. These traces were used to calculate the amplitudes of excitatory and inhibitory conductances.

The amplitudes of excitatory and inhibitory conductance were calculated as the mean conductance value in the time window placed over the peak of the $G_{\mathrm{exc}}(t)$ and $G_{\mathrm{inh}}(t)$ (Figs 2*A* and 9*A*). We have used two approaches to validate the conductance measurements. First, we have reconstructed total current responses at the recorded holding potential using the estimated conductances, and compared the reconstructed responses to those recorded. The reconstructed (black traces in Fig. 2*A* and *C*) and recorded (red traces in Fig. 2*A* and *C*) traces overlap almost completely, demonstrating that reconstructed responses reproduced the recorded responses well. Second, we have calculated conductances using pharmacologically isolated IPSCs recorded in the presence of 10 μM DNQX and 20 μM APV. As expected, calculated conductances showed a strong inhibitory but negligible excitatory component (Fig. 2*C* and *D*).

For statistical analysis we used Student's t tests or one-way ANOVAs with *post hoc* comparisons (Dunnett's and Tukey's HSD). Error bars represent the standard error of the mean (± SEM).

Results

Adenosine reduces the amplitude of evoked IPSPs and increases the paired-pulse ratio

To examine the effects of adenosine on inhibitory synaptic transmission to layer 2/3 pyramidal neurons we recorded IPSPs evoked by paired-pulse electric stimuli in control conditions and during bath application of adenosine at different concentrations. To facilitate comparison of the effects of adenosine on inhibitory transmission with results of our previous study on excitatory transmission (Bannon *et al.* 2014), we used the same experimental setting, and bath applied adenosine at increasing concentrations, from 5 μM to 150 μM (Fig. 3*A* and *B*). Already with the lowest tested concentration of 5 μM, adenosine induced a clear decrease of the IPSP

amplitude to 71.3 ± 3.9% of baseline ($P < 0.001$) (Fig. 3*C*). Increasing the concentration of adenosine in the bath led to a progressive reduction of the IPSP amplitude. At 20 μM, adenosine had a robust effect, reducing the IPSP amplitude to 55.9 ± 3.7% of baseline (Fig. 3*C*, $P < 0.001$). This concentration was selected for further experiments.

The reduction in IPSP amplitude during application of adenosine was accompanied by an increase in the paired-pulse ratio (PPR; Fig. 3*C*). The PPR is an index of release that is inversely related to the release probability (Stevens, 1993; Voronin, 1993; Dobrunz & Stevens, 1997; Murthy *et al.* 1997; Oleskevich *et al.* 2000; Zucker & Regehr, 2002). The PPR significantly increased during application of 5 μM adenosine to 109.7 ± 3.8% of baseline ($P < 0.05$). Higher concentrations of adenosine led to stronger increases of the PPR, to 117.1 ± 6.0% at 20 μM and 119.7 ± 7.2% at 50 μM. The increasing paired-pulse ratio that accompanies the decrease of IPSP amplitude during adenosine application suggests that adenosine had a presynaptic effect on inhibitory transmission, decreasing release probability at inhibitory synapses.

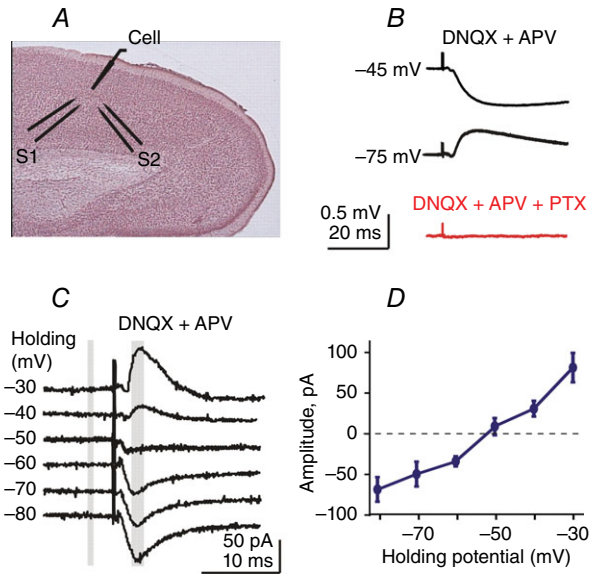

Figure 1. Pharmacologically isolated inhibitory responses in layer 2/3 pyramidal neurons
A, a scheme of location of recording and stimulation electrodes in a slice of rat visual cortex. Recordings were made from layer 2/3 pyramidal neurons. The two bipolar stimulation electrodes, S1 and S2, were placed in layer 4 below the recording site. *B*, pharmacologically isolated IPSPs (10 μM DNQX and 20 μM APV in the recording medium) reverse between −45 mV and −75 mV and are blocked by the $GABA_A$ antagonist picrotoxin (PTX, 100 μM). *C*, series of IPSCs recorded at different holding potentials from −30 mV to −80 mV. Vertical grey bars show time windows for amplitude measurement. *D*, IPSC amplitude plotted against holding potential, average data for $n = 10$ cells. The reversal potential of IPSC is between −50 mV and −55 mV, which corresponds to the chloride equilibrium potential which is around −50 mV with our extracellular and intracellular solutions.

A_1R blockade prevents effects of adenosine on evoked IPSPs

Which receptors mediate these effects of adenosine on inhibitory synaptic transmission? A_1Rs are expressed at high levels in the neocortex (Dixon *et al.* 1996; Fredholm *et al.* 2001). Our previous data show that suppression of excitatory transmission by adenosine is mediated by A_1Rs (Bannon *et al.* 2014). However, given the unclear effect of adenosine on inhibitory transmission (Yoon & Rothman, 1991; Sebastião & Ribeiro, 1996; Kruglikov & Rudy, 2008; Mathew *et al.* 2008), it is necessary to elucidate the roles of A_1Rs and $A_{2A}Rs$ during adenosine application. Therefore, we first examined whether A_1Rs are involved in suppression of inhibitory transmission by adenosine. We applied 20 μM adenosine in the presence of the selective A_1R antagonist DPCPX (50 nM) in the bath (Fig. 4*A* and *B*). In the presence of DPCPX, the adenosine-induced reduction in IPSP amplitude was completely abolished: the IPSP amplitude remained at 96.6 ± 2.7% of 4baseline (Fig. 4*C*, $P > 0.05$). Increasing the adenosine concentration to 100 μM still did not lead to a reduction in IPSP amplitude (93.1 ± 5.5%, Fig. 4*C*, $P > 0.05$). There were also no significant changes in the paired-pulse ratios of IPSPs in 20 μM and 100 μM adenosine in the presence of DPCPX.

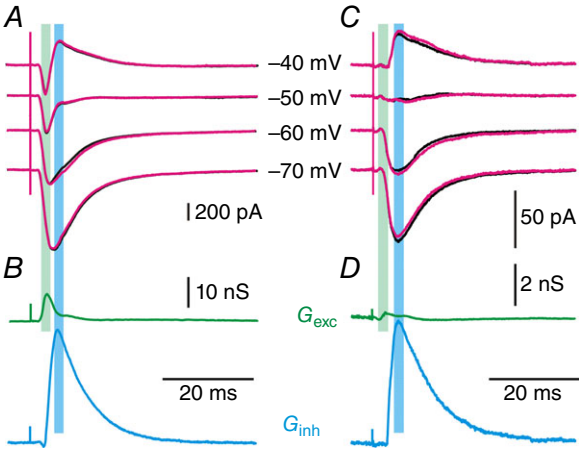

Figure 2. Calculation of excitatory and inhibitory conductance during compound responses and isolated IPSCs
A, compound responses recorded at holding potentials from −40 mV to −70 mV. Magenta traces are recorded responses. Black traces are responses that were reconstructed using calculated excitatory and inhibitory conductance from *B*. *B*, excitatory (G_{exc}) and inhibitory (G_{inh}) conductance estimated using responses from *A*. Green and blue vertical bars in *A* and *B* show time windows for calculating amplitudes of excitatory and inhibitory conductances. *C*, pharmacologically isolated IPSCs (10 μM DNQX and 20 μM APV) recorded at holding potentials from −40 mV to – 70 mV. *D*, excitatory (G_{exc}) and inhibitory (G_{inh}) conductance estimated using responses from *C*. Note that excitatory conductance is negligible. Conventions in *C* and *D* are as in *A* and *B*.

These results show that adenosine suppresses inhibitory synaptic transmission by activation of presynaptic A_1Rs.

A_{2A}Rs may reduce the suppressive effect of A_1R activation on evoked IPSPs

Because A_{2A}Rs are present in the neocortex (Cunha *et al.* 1996; Dixon *et al.* 1996) and may interact with A_1Rs, exerting a facilitatory effect at excitatory synapses (Bannon *et al.* 2014), we asked if A_{2A}Rs may also modulate transmission at inhibitory synapses. Although A_{2A}Rs have lower affinity for adenosine, both A_1Rs and A_{2A}Rs should be activated in 100 μM adenosine (Li & Henry, 1998; Ciruela *et al.* 2006), as used in the experiments shown in Fig. 4. Blockade of A_{2A}Rs should then reveal an effect of their activation on inhibitory transmission. However, application of the selective A_{2A}R antagonist SCH-58261 (30 nM) in the presence of 100 μM adenosine and 50 nM DPCPX did not lead to further change in IPSP amplitude (88 ± 4.5%, of baseline, which is 95.2 ± 3.7% compared to 100 μM Ado in DPCPX; Fig. 4*C*).

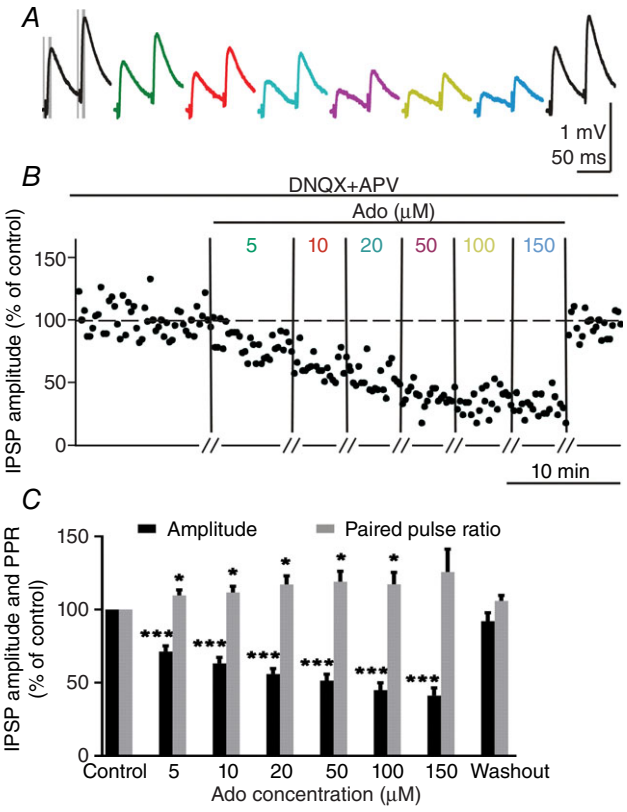

Figure 3. Adenosine reduces evoked IPSP amplitude and increases paired-pulse ratio (PPR) in a reversible and concentration-dependent manner
A, pharmacologically isolated IPSPs in a layer 2/3 pyramid evoked by paired stimuli (50 ms interpulse interval) in control conditions, during application of adenosine at increasing concentration and during washout. In the control response, time windows for amplitude measurement are marked in grey. The same windows were used on all other responses for amplitude measurement. *B*, the time course of amplitude changes of the responses to the first pulse in a pair (IPSP1). *C*, changes of the amplitude of IPSP1 and paired-pulse ratio (PPR) induced by an increasing concentration of adenosine. IPSP amplitudes were normalized by the amplitude of the IPSP1 in control for each input, and then averaged for $n = 13$ cells (18 inputs). Adenosine reduces IPSP amplitude and increases PPR in a concentration-dependent and reversible manner. Significance denoted as: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

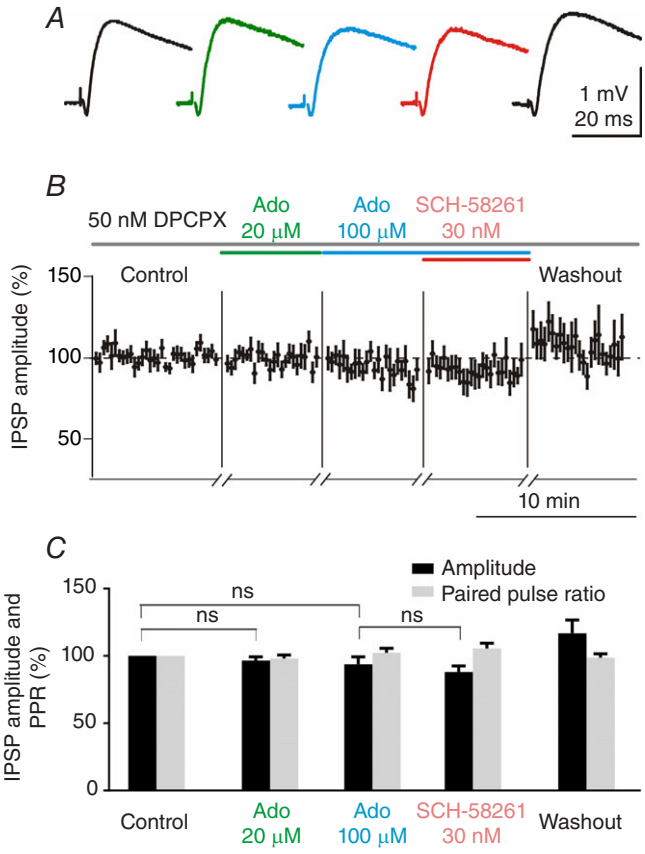

Figure 4. A_1 receptor antagonist DPCPX blocks adenosine's effects on synaptic transmission
A, pharmacologically isolated IPSPs in a layer 2/3 pyramid recorded in the presence of 50 nM DPCPX in the recording medium. IPSP in control (black), during application of 20 μM adenosine (green), 100 μM adenosine (cyan), addition of 30 nM of the selective A_{2A}R blocker SCH-58261 (red), and after washout of adenosine and SCH-58261. DPCPX (50 nM) was present in all solutions throughout the experiment, including washout. *B*, averaged time course of amplitude changes of IPSP1 in $n = 15$ inputs with a scheme of drug application. Note that time scale is interrupted, because timings in different neurons were aligned 'backwards', by the end of each drug application. *C*, averaged changes of the IPSP1 amplitude (black bars) and paired-pulse ratio (grey bars), as percentage of control ($n = 15$ inputs from 8 cells; same cells as in *B*). Note that 50 nM DPCPX prevents the suppressive effects adenosine on IPSPs. Note also that in the presence of DPCPX, application of the A_{2A}R antagonist 30 nM SCH-58261 in the presence of 100 μM adenosine did not change the IPSP amplitude.

We next asked if A_{2A}Rs modulate inhibitory synaptic transmission indirectly, via interaction with A_1Rs, as they do in some other brain areas (Rebola *et al.* 2005; Ciruela *et al.* 2011). In this case, failure of A_{2A}R blockade to change IPSP amplitude in the experiments shown in Fig. 4 could be due to the fact that A_1Rs were blocked. To test for this possibility, we applied 100 μM adenosine followed by application of 30 nM of the A_{2A}R antagonist SCH-58261, this time without blocking the A_1Rs (Fig. 5*A* and *B*). Application of 100 μM adenosine led to significant reduction of the IPSP amplitude to 58.3 ± 4.2% of baseline (Fig. 5*C*, $P < 0.001$). Addition of 30 nM SCH-58261 led to a small yet significant further reduction in the IPSP amplitude to 49.6 ± 4.6 % of control, or to 83.9 ± 4.5% of the 100 μM Ado condition ($n = 24$, $P < 0.01$). Out of 24 inputs studied in this series, IPSP amplitude significantly decreased upon application of SCH-58261 in 12 cases, increased in 3 cases, and did not show significant changes in the remaining 9 inputs (Fig. 5*D*). Subsequent addition of the A_1R antagonist DPCPX, and thus blockade of both A_1 and A_{2A} receptors, was expected to relieve IPSP suppression. Indeed, the IPSP amplitude increased after addition of 50 nM DPCPX. However, the recovery was not complete, returning to only 71.1 ± 4.8% of baseline (Fig. 5*C*). In the experiments shown in Fig. 5, recovery of IPSP amplitude after application of DPCPX and the resulting partial blockade of A_1Rs could be due to both a direct decrease in A1R-mediated suppression and an indirect effect of the reduced number of A_1Rs available for A_{2A}R-modulation. Figure 6 illustrates the dynamic nature of this modulation: in the continuous presence of 100 μM adenosine in the recording medium, IPSP amplitudes decreased during the blockade of A_{2A}Rs by 30 nM SCH-58261 to 79 ± 4.9% of control ($P < 0.001$, $n = 14$), but recovered upon SCH-58261 washout (108 ± 6.6% of control, $P = 0.26$ compared to control, $n = 14$). Out of 14 IPSPs tested in this series, nine were significantly reduced after SCH-58261 application, one was significantly increased, and the remaining four did not change (Fig. 6*B*).

The results presented in Figs 4–6 suggest that functional A_{2A}Rs are present at inhibitory synapses in the neocortex. The dependence of SCH-58261's effects on the activation of A_1Rs suggests that A_{2A}Rs do not regulate inhibitory transmission directly, but by modulating activity of A_1Rs.

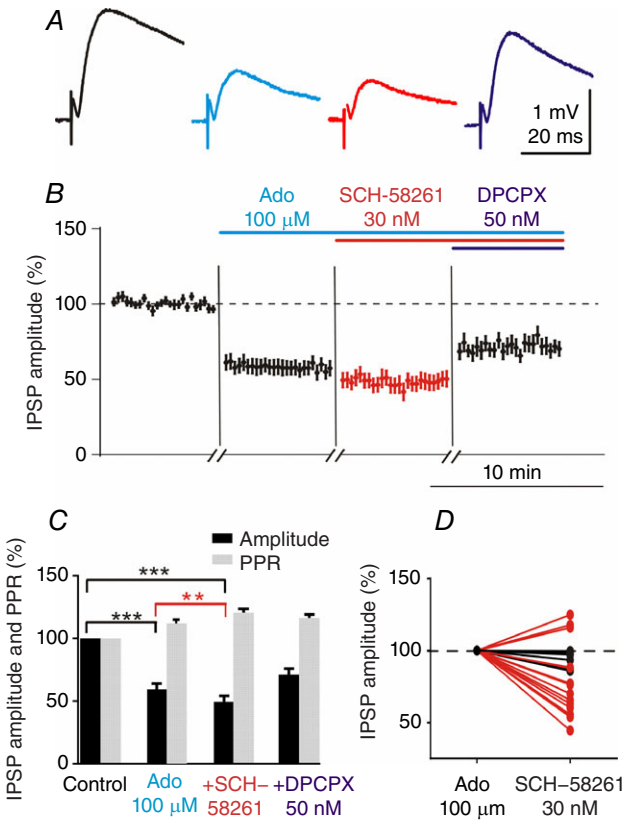

Figure 5. Blockade of A_{2A} receptors by SCH-58261 enhances the suppression of IPSP by adenosine
A, IPSPs evoked in a layer 2/3 pyramidal neuron in control (black), during application of 100 μM adenosine (cyan), addition of 30 nM of the A_{2A}R antagonist SCH-58261 (red), and 50 nM of the A_1R antagonist DPCPX (dark blue). *B*, averaged time course of amplitude changes of IPSP1 in $n = 24$ inputs with a scheme of drug application. Note that time scale is interrupted, because timings in different neurons were aligned 'backwards', by the end of each drug application. *C*, averaged change of the IPSP1 amplitude (black bars) and paired-pulse ratio (grey bars), as percentage of control ($n = 24$ inputs from 13 cells; same cells as in *B*). *D*, changes in IPSP1 amplitude after the application of 30 nM SCH-58261 in the presence of 100 μM adenosine for $n = 24$ individual inputs. For each input, IPSP amplitude was normalized to the 100 μM adenosine condition. Inputs in which IPSP amplitude changed significantly after SCH-58261 application are highlighted in red (within-subjects comparison of 30–50 individual IPSPs from each condition; $P < 0.05$, paired *t* test). Note that application of the A_{2A}R antagonist SCH-58261 in the presence of 100 μM adenosine led to a further reduction in the IPSP amplitude.

Suppression of inhibitory transmission by adenosine involves presynaptic mechanisms

To address the locus of adenosine's actions at inhibitory synapses, we examined whether application of 20 μM adenosine (leading to a reduction in IPSP amplitude) is associated with changes of indices of presynaptic release: PPR, the inversed coefficient of variation (CV^{-2}) of response amplitude, and the frequency of miniature IPSPs. Figure 7*A* shows that the decrease in IPSP amplitude during adenosine application was associated with an increase in the PPR. Changes in the IPSP amplitude and changes in PPR were significantly negatively correlated ($r^2 = 0.3378$, $P < 0.001$). Since release probability is inversely related to the PPR, this correlation implies that the decrease in IPSP amplitude was associated with

a decrease in release probability. The decrease in the amplitude of IPSPs during adenosine application was accompanied by a decrease in CV^{-2}, and changes in CV^{-2} were positively correlated with response amplitude changes ($r^2 = 0.1844$, $P < 0.05$, Fig. 7*B*). Since CV^{-2} is directly related to release probability (Voronin, 1993; Stevens, 1993), the decrease of the CV^{-2} indicates that the reduction in IPSP amplitude was associated with a decrease in release probability.

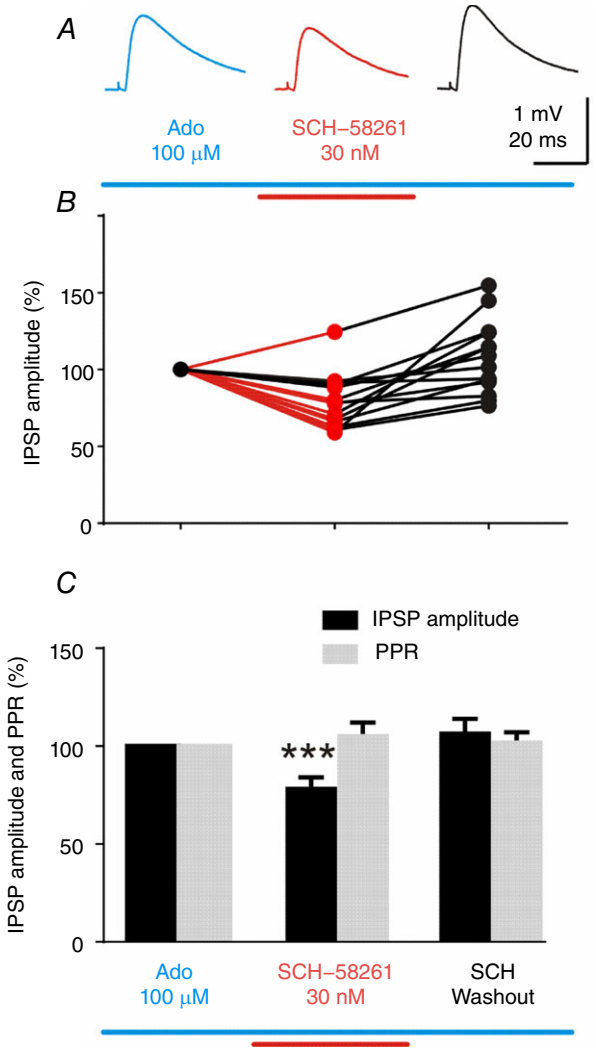

Figure 6. Reversible suppression of IPSP by blockade of A_{2A} receptors
A, IPSPs evoked in a layer 2/3 pyramidal neuron in 100 μM adenosine (cyan), after addition of 30 nM of the A_{2A}R antagonist SCH-58261 (red), and after washout of SCH-5836 (black). Note that 100 μM adenosine was present in the bath throughout the experiment. *B*, changes in IPSP1 amplitude after the application of 30 nM SCH-58261 in the presence of 100 μM adenosine for $n = 14$ individual inputs. For each input, IPSP amplitude was normalized to the 100 μM adenosine condition. Inputs in which IPSP amplitude changed significantly after SCH-58261 application are highlighted in red (within-subjects comparison of 30–50 individual IPSPs from each condition; $P < 0.05$, paired *t* test). *C*, averaged change of the IPSP1 amplitude (black bars) and paired-pulse ratio (grey bars), as percentage of control ($n = 14$; same data as in *B*).

Table 1. Effect of 20 μm adenosine on miniature EPSPs and miniature IPSPs

	Frequency	Median amplitude
mEPSP	80.4 ± 4.5%**	95.0 ± 1.2%**
mIPSP	83.2 ± 5.4%*	96.8 ± 1.5%

Effects of treatment on frequency and median amplitude of mEPSP ($n = 20$) and mIPSP ($n = 14$), expressed as a percentage of values from control condition. $^{**}P < 0.01$, $^{*}P < 0.05$. For controls, mEPSP frequency = 9.0 ± 0.9 events s^{-1}, mEPSP median amplitude = 0.29 ± 0.02 mV, mIPSP frequency = 0.42 ± 0.07 events s^{-1}, mIPSP median amplitude = 0.28 ± 0.01 mV. Data for mEPSPs are from Bannon *et al.* 2014.

We recorded miniature IPSPs (mIPSPs) in the presence of 0.1 μM TTX. Miniature IPSPs were isolated pharmacologically by adding blockers of glutamatergic transmission DNQX (10 μM) and APV (20 μM) to the recording medium. The GABAergic nature of the remaining miniature events was confirmed at the end of experiment by application of the $GABA_A$ receptor blocker picrotoxin (100 μM), which essentially abolished the detectable events (only 1 event per 8–10 s could be detected in these conditions, Fig. 8*A*, lower trace, and *D*).

Application of 20 μM adenosine led to a significant reduction in mIPSP frequency to 83.2 ± 5.4% of the baseline, from 0.42 ± 0.07 to 0.35 ± 0.07events s^{-1} (Fig. 8*E*, *F* and Table 1, $P < 0.05$). The median amplitude of mIPSPs did not change significantly during adenosine application (0.27± 0.01 *vs.* 0.28 ± 0.01 mV in control, $P > 0.05$, Fig. 8*G* and *H*, and Table 1). The decrease in the mIPSP frequency indicates that adenosine reduces release probability at inhibitory synapses.

Thus, changes in all three indicators of presynaptic release, the PPR, the CV^{-2} and frequency of mIPSPs, indicate that adenosine reduces the release probability at inhibitory synapses, and therefore the suppressive effects of adenosine on cortical inhibition are at least partially mediated by presynaptic mechanisms.

Postsynaptic effects of adenosine: hyperpolarization and decrease in the input resistance

In addition to its presynaptically mediated effects, adenosine application led to a decrease in the input resistance and hyperpolarization of the cell membrane in layer 2/3 pyramidal neurons. During application of 20 μM adenosine the input resistance decreased to 89.9 ± 3.8% of the baseline (from 278 ± 33 MΩ to 243 ± 27 MΩ, $P < 0.05$, $n = 21$ neurons, data not shown). The resting membrane potential was hyperpolarized by 1.2 ± 0.6 mV

during adenosine application from −72.7 ± 1.9 mV to −73.9 ± 1.6 mV ($P < 0.01$, data not shown). Both the membrane hyperpolarization and the decrease in input resistance were abolished when adenosine was applied in the presence of 30 nM DPCPX, suggesting the postsynaptic presence of A_1Rs. These postsynaptic effects of adenosine correspond to the results obtained in our prior work on the effects of adenosine on excitatory synaptic transmission to layer 2/3 pyramidal neurons (Bannon *et al.* 2014). The decrease in the input resistance and hyperpolarization of the membrane might have opposite effects on IPSP amplitudes measured in this study. A ∼10% decrease in input resistance might result in a decrease in IPSP amplitude, while membrane hyperpolarization, leading to an increase in the driving force relative to the chloride reversal potential (around −50 mV in our conditions) by ∼5%, might result in an increase in IPSP amplitude. Note that these changes, though possible, are much smaller than the observed reduction in IPSP amplitude (to 55.9% of control, Fig. 3*C*) and thus cannot alone explain adenosine-mediated suppression of IPSPs.

Having established how application of adenosine affects transmission at inhibitory synapses, we can now compare the effects of adenosine on excitatory and inhibitory transmission, and ask how adenosine may influence the excitation–inhibition balance. We used three approaches to address this question: analysis of miniature PSPs, the concentration dependence of adenosine's effects on evoked IPSPs and EPSPs, and changes in the ratio of excitatory and inhibitory conductances during compound responses.

Adenosine reduces the frequency of both excitatory and inhibitory spontaneous PSPs

Table 1 shows that application of 20 μM adenosine reduced the frequency of mIPSPs and mEPSPs similarly, to about ∼80% of control. There was no significant difference between mEPSPs and mIPSPs in the amount of adenosine-induced reduction in their frequency (to 80.4 ± 4.5% *vs.* 83.2 ± 5.4% of the respective baselines, $P > 0.05$). Thus, adenosine reduced release probability at excitatory and inhibitory synapses to a similar extent.

Excitatory synapses are, however, more numerous in the neocortex (DeFelipe & Farinas, 1992; Markram *et al.* 2004). To estimate the relative contribution of excitatory and inhibitory events in our preparation, we first recorded all miniature events and then added blockers of glutamate receptors to isolate inhibitory events. In the presence of 0.1 μM TTX in the bath, the averaged frequency of miniature events was 5.6 ± 1.0 events s^{-1} ($n = 5$ cells). Application of glutamatergic transmission blockers DNQX (10 μM) and APV (20 μM) led to a dramatic decrease in the frequency of miniature events (Fig. 8*A* middle trace; Fig. 7*C*) to 0.55 ± 0.2 events s^{-1} (9.8 ± 4.1% of TTX-only condition, Fig. 8*D*). The remaining events were mIPSPs because they were blocked by application of 100 μM of the $GABA_A$ antagonist picrotoxin (Fig. 8*A*, lower trace, and *D*). Similar results were found in an additional series of experiments which were performed without TTX in the recording medium, and thus included both action potential-dependent and -independent synaptic events. The frequency of spontaneous event in control was 8.3 ± 0.7 events s^{-1}, but decreased to 0.8 ± 0.1 events s^{-1} (9.6 ± 2.3% of control)after blockade of glutamate receptors with DNQX (10 μM) and APV (20 μM) ($n = 8$ cells, data not shown). Thus the ratio of excitatory/inhibitory events in our preparation was about 9:1, consistent with a larger number of excitatory synapses at neocortical neurons.

Differential concentration dependence of suppression of evoked IPSPs and EPSPs by adenosine

Figure 9 *A* and *B* summarizes the concentration dependence of the suppression of IPSP and EPSP amplitudes by adenosine. Comparison of these dependences reveals several important points. First, even at 1 μM adenosine induces a small but significant suppression of IPSP amplitudes to 86.8 ± 3% of the baseline ($n = 18$, $P < 0.05$). In contrast, EPSPs were

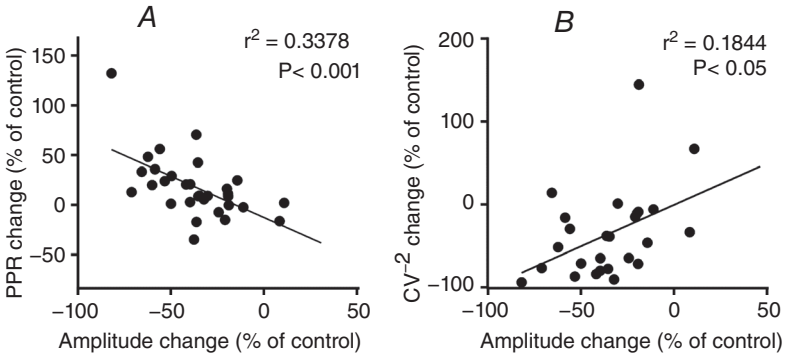

Figure 7. Suppression of IPSPs by adenosine involves presynaptic mechanisms
A, changes in paired-pulse ratio (PPR) plotted against changes in the IPSP1 amplitude induced by application of 20 μM adenosine ($n = 31$ inputs from 16 cells). *B*, changes in CV^{-2} plotted against changes in the IPSP1 amplitude from the same experiments.

not significantly suppressed by this low concentration (89.4 ± 8.8% of baseline, $n = 14$, $P = 0.247$), which could be in part because of a larger variability of the effects of 1 μM adenosine on EPSPs. IPSPs and EPSPs are suppressed about equally by 5 μM adenosine: IPSP amplitudes were reduced to 71.3 ± 3.9% ($n = 18$, $P < 0.001$) and EPSPs to 68.8 ± 6.7% of the baseline ($n = 14$, $P < 0.001$). Second, when the concentration of adenosine increases, suppression of IPSPs saturates at lower levels than suppression of EPSPs. At concentrations of 10, 20, 50 and 100 μM of adenosine IPSPs were suppressed significantly less than EPSPs (Fig. 9*B*).

Thus, at low concentrations adenosine preferentially suppresses evoked IPSPs, but leaves EPSPs unchanged. At high concentrations, the relative strength of adenosine's effects on excitation and inhibition reverses and suppression of EPSPs becomes stronger than suppression of IPSPs. These concentration-dependent differential effects of adenosine on excitatory and inhibitory synaptic transmission, combined with high variability of the effects of adenosine on responses at individual excitatory and inhibitory inputs (Fig. 9*A*), may result in bidirectional local changes of the balance between excitation and inhibition within a small range of synaptic circuits.

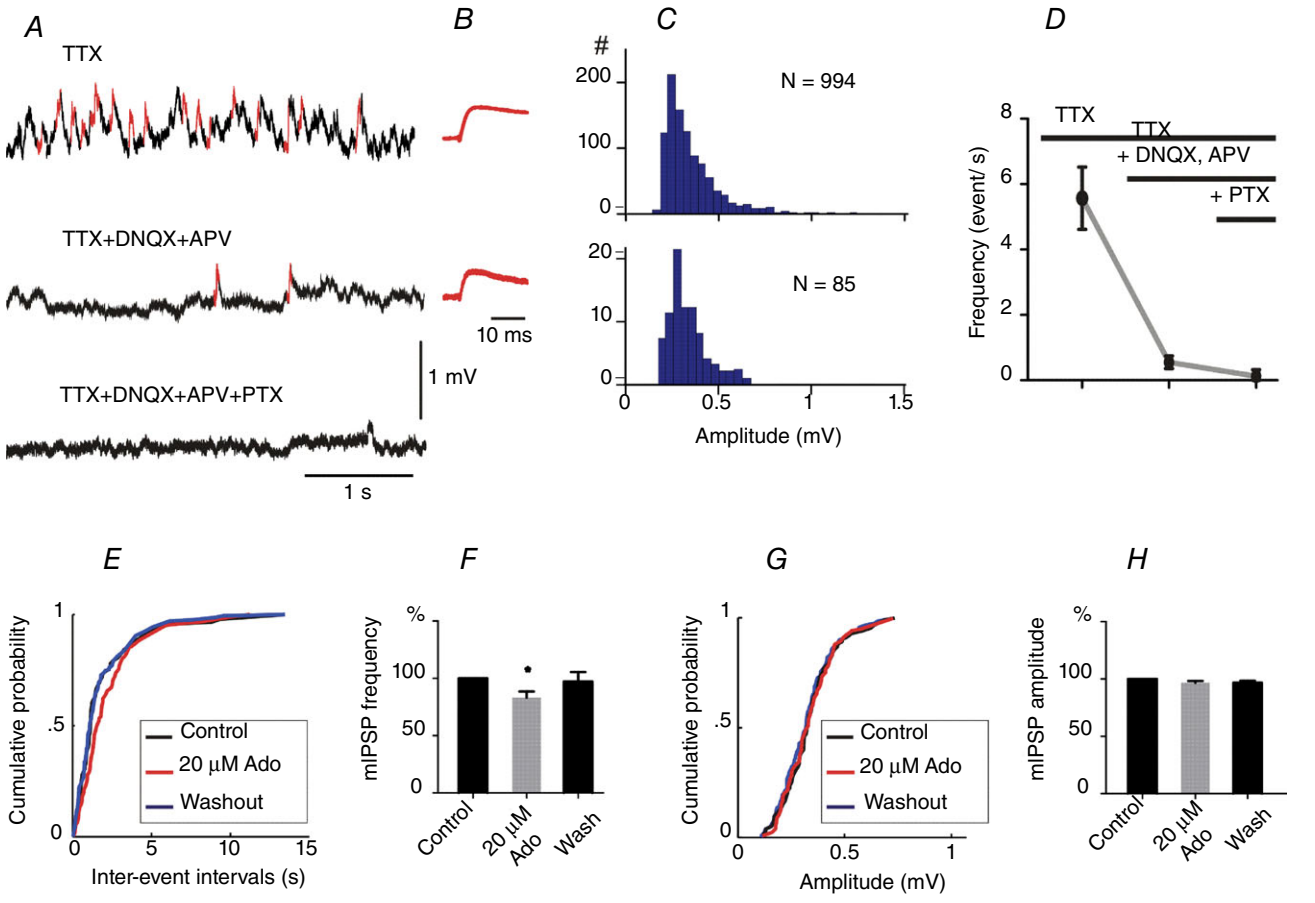

Figure 8. Adenosine reduces the frequency of miniature IPSPs
A, pharmacological isolation of inhibitory miniature IPSPs. Traces of membrane potential from one cell recorded with TTX (0.1 μM) in the bath. Detected miniature events are indicated in red. Application of antagonists of glutamate receptors, DNQX (10 μM) and APV (20 μM), isolates inhibitory events, which are blocked by $GABA_A$ antagonist picrotoxin (PTX, 100 μM). *B*, averaged excitatory and inhibitory miniature events (trace on top) and isolated inhibitory miniature events (lower trace) from the recordings illustrated in *A*. Total length of each recording used for detection of miniatures was 150 s. *C*, amplitude distributions of miniatures recorded in TTX (top) and pharmacologically isolated mIPSPs (bottom). Note different *Y*-axes. *D*, averaged frequency of mEPSPs and mIPSPs (in TTX), mIPSPs (after addition of DNQX + APV), and after the application of PTX for $n = 5$ cells. *E*, cumulative probability distributions of first-order intervals between mIPSPs in control (black), in 20 μM adenosine (red) and after washout (blue) in one neuron. *F*, averaged mIPSP frequency in control, during the application of 20 μM adenosine and after adenosine washout for $n = 14$ cells. *G*, cumulative probability distributions of mIPSP amplitudes in control (black), in 20 μM adenosine (red) and after washout (blue) in the neuron from *E*. *H*, averaged mIPSP median amplitude in control, during the application of 20 μM adenosine and after adenosine washout for $n = 14$ cells. Note that adenosine reduces the mIPSP frequency but not their amplitude.

Adenosine can shift the excitation–inhibition balance bidirectionally

To compare the effects of adenosine on excitatory and inhibitory responses of the same neuron and assess possible changes in excitation–inhibition balance, we used compound responses containing both excitatory and inhibitory components. In these experiments, we placed the stimulation electrodes in layer 5, which allowed recruitment of a more heterogeneous population of presynaptic fibres than local layer 4 stimulation (Kirkwood & Bear, 1994) used in previous experiments (see Fig. 1). Evoked compound responses were recorded in voltage clamp mode at different holding potentials (Fig. 10*A*). At hyperpolarized holding potentials (Fig. 10*A*, trace at −70 mV) responses consisted of inward currents only, but at less negative potentials the late components of responses were reversed, revealing their compound nature expressed as a sequence of inward–outward currents (Fig. 10*A*, trace at −40 mV). The compound excitatory–inhibitory current responses were used to calculate changes in excitatory and inhibitory conductance induced by synaptic stimulation (Monier *et al.* 2008). A brief increase in excitatory conductance at the beginning of a compound response was followed by large amplitude, long-lasting increase in inhibitory conductance (Fig. 10*B*). The higher amplitude and longer duration of the inhibitory conductance increase in compound responses is consistent with predominance of inhibitory components in responses evoked in the visual cortex by electric stimulation *in vivo* (Douglas & Martin, 1991; Pei *et al.*

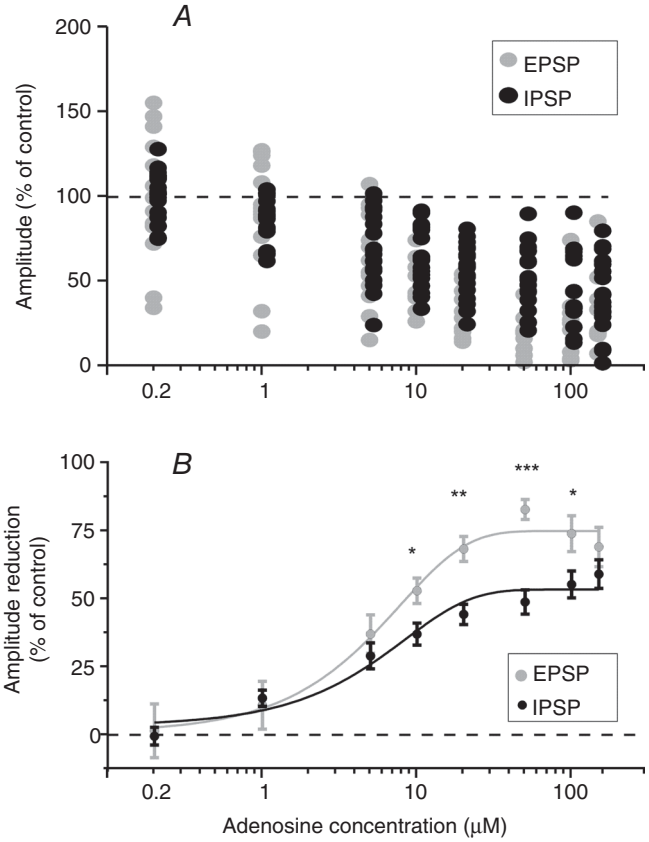

Figure 9. Comparison of the concentration dependence of IPSP and EPSP suppression by adenosine
A, changes of the amplitude of EPSPs (grey symbols) and IPSPs (black symbols) during application of different concentrations of adenosine. Each point represents amplitude changes of one input at one concentration. Data points for IPSPs are slightly shifted to the left for visibility. Data for 0.2 μM–5 μM adenosine are from $n = 14$ inputs for EPSPs and $n = 18$ inputs for IPSPs; data for 10 μM–150 μM adenosine are for $n = 10$ inputs for EPSPs and $n = 18$ inputs for IPSPs. *B*, concentration dependence of reduction in EPSP (grey) and IPSP (black) amplitude by adenosine. Data from *A*. Continuous curves show sigmoid fit to the data points. Data for EPSPs for 10 μM–150 μM adenosine are from Bannon *et al.* 2014.

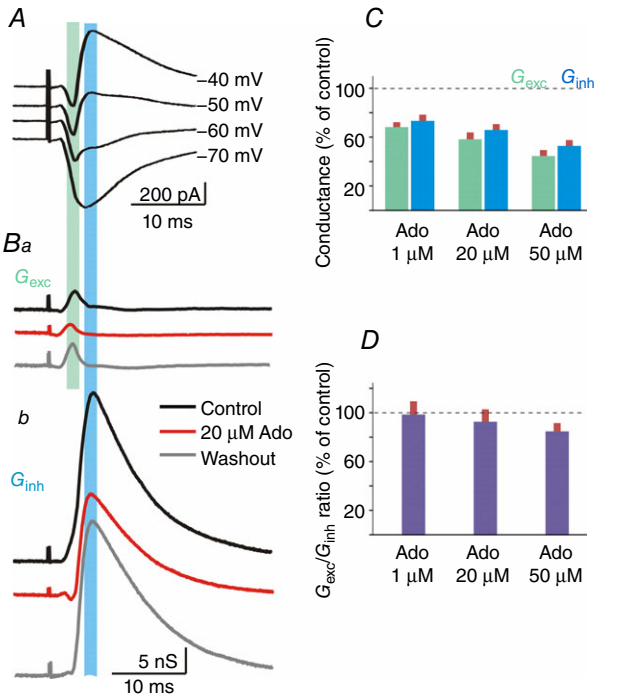

Figure 10. Adenosine reduces the excitatory and inhibitory conductance in compound responses
A, compound responses recorded at holding potentials from −40 mV to –70 mV in control, before the application of adenosine. *Ba* and *b*, excitatory (G_{exc}, *Ba*) and inhibitory (G_{inh}, *Bb*) conductance during compound responses. Black traces show excitatory and inhibitory conductance estimated using control responses from *A*. Red traces show excitatory and inhibitory conductance during the application of 20 μM adenosine. Grey traces show conductances measured after adenosine washout. Green and blue vertical bars in *A* and *B* show time windows for calculating amplitudes of excitatory and inhibitory conductances. *C*, averaged changes of excitatory and inhibitory conductance during application of adenosine at concentrations of 1 μM ($n = 12$ inputs), 20 μM (16 inputs) and 50 μM (11 inputs). Conductance changes in each input were first normalized to control and then these percentage values were averaged over inputs. *D*, changes of G_{exc}/G_{inh} ratio in the same cells.

1991; Borg-Graham *et al.* 1998; Chung & Ferster, 1998). Application of 20 μM adenosine led to a clear reduction in both excitatory and inhibitory conductances (Fig. 10*B* and *C*). In $n = 16$ responses, excitatory conductance was reduced to 59.1 ± 5.6% of control ($P < 0.001$) and inhibitory conductance was reduced to 66.8 ± 4.8% of control (Fig. 10*C*, $P < 0.01$). These changes were reversible. After washout of adenosine, excitatory and inhibitory conductances partially recovered towards their original values (94 ± 8.6 and 97.2 ± 6.3% of control, respectively). As a measure of the excitation–inhibition balance we used the ratio of excitatory to inhibitory conductance. In 12 out of 16 responses, application of 20 μM adenosine led to significant changes in the balance between excitation and inhibition. The ratio of excitatory to inhibitory conductance was significantly ($P < 0.05$) increased in six responses and decreased in another six responses. In the remaining four responses the ratio did not change. Interestingly, the bidirectional changes of the balance in individual responses 'cancelled' each other, and on the average there was no significant change of the excitation–inhibition balance (93.6 ± 10.3% of control, $P > 0.05$, Fig. 10*D*).

Because of the different concentration dependences of adenosine's suppression of excitatory and inhibitory transmission (Fig. 9), we expected that the excitation/inhibition ratio measured in compound responses will also change in concentration-dependent way. Indeed, 1 μM adenosine, while suppressing both inhibitory and excitatory conductances, changed their ratio in individual neurons, but not on average (99.5 ± 10.9% of control, $n = 12$). In contrast, application of 50 μM adenosine resulted in a small but significant shift of the average balance towards inhibition (85.7 ± 6.8%, $P < 0.05$, $n = 11$).

Thus, adenosine may indeed change the balance of excitation and inhibition at specific sets of synapses, such as those contributing to compound responses in our experiments. These changes may occur in both directions: either excitatory or inhibitory inputs may be suppressed disproportionally, leading to a local shift in the balance towards excitation or towards inhibition. When averaged over large number of synapses, however, the balance of excitation and inhibition remains unchanged by moderate (20 μM or less) adenosine concentrations, allowing the maintenance of large scale cortical networks in the balanced state, despite changing concentrations of adenosine.

Discussion

The results of the present study demonstrate the following points. (1) Adenosine suppresses inhibitory synaptic transmission to layer 2/3 pyramids in rat visual cortex via activation of presynaptic A_1 receptors; (2) In half of cases (12/24) blockade of A_{2A} receptors led to a small but significant further increase of this suppression, and this action required operational A_1 receptors. This provides evidence for functional A_{2A}Rs at a portion of inhibitory terminals. (3) Adenosine may change the balance between excitation and inhibition in a concentration-dependent way: at low concentration (1 μM) it starts to suppress inhibition while having heterogeneous effects on excitation, but at higher concentrations (10 μM and higher) excitatory transmission is suppressed more strongly than inhibitory. Moreover, while the 'local' balance between excitatory and inhibitory inputs to an individual neuron may change in both directions, the overall balance averaged over all neurons is maintained under varying adenosine concentrations.

Suppression of cortical inhibitory transmission by adenosine

Prior studies revealed highly diverse effects of adenosine on inhibitory transmission in the cortex. Results from the hippocampus consistently show that adenosine had no effect on inhibitory transmission, although it did suppress excitatory transmission (Lambert & Teyler, 1991; Yoon & Rothman, 1991; Brundege & Dunwiddie, 1996). In the neocortex, the picture is more complex: adenosine had no effect on inhibitory transmission to layer 2/3 pyramids in prefrontal cortex of rats (Mathew *et al.* 2008), but did suppress inhibitory transmission in mice, to layer 5 pyramids in somatosensory cortex (Kruglikov & Rudy, 2008) and Cajal–Retzius neurons in immature visual cortex (Kirmse *et al.* 2008). Our results provide three lines of evidence for adenosine-mediated suppression of inhibitory synapses at layer 2/3 pyramidal neurons in rat visual cortex. Adenosine reduced the frequency of spontaneous and miniature IPSPs, decreased the amplitude of pharmacologically isolated evoked IPSPs and decreased inhibitory conductance in compound PSPs. Although sparse, these results allow us to exclude at least some general dichotomies. The adenosine sensitivity of inhibition in the neocortex is now demonstrated in both rat and mouse, and thus is not species specific, and it occurs in pyramidal neurons from both layer 2/3 and layer 5, and thus is not generally restricted to supragranular or infragranular layers. At the same time, the area specificity of adenosine's effects on inhibition remains to be elucidated: existing evidence shows adenosine-mediated suppression of inhibition in sensory areas (Kirmse *et al.* 2008; Kruglikov & Rudy, 2008; and our present results) but not in layer 2/3 neurons from prefrontal cortex (Mathew *et al.* 2008).

Our results show that adenosine-mediated suppression of inhibitory transmission to layer 2/3 pyramids is

presynaptic, because it is associated with changes in release indices – paired-pulse ratio (PPR), inversed coefficient of variation (CV^{-2}) and frequency of miniature events – and is mediated by A_1 receptors. These results are consistent with documented mechanisms of adenosine-suppression at other inhibitory synapses in the neocortex (Kirmse *et al.* 2008; Kruglikov & Rudy, 2008) as well as with the wealth of data for excitatory synapses in the neocortex (Murakoshi *et al.* 2001; Mathew *et al.* 2008; Kerr *et al.* 2013; Bannon *et al.* 2014) and hippocampus (Dunwiddie & Haas, 1985; Lambert & Teyler, 1991; Yoon & Rothman, 1991; Scanziani *et al.* 1992; Thompson *et al.* 1992; Wu & Saggau, 1994; Brundege & Dunwiddie, 1996).

Evidence for functional A_{2A} receptors at inhibitory synapses in the neocortex

The results of the present study demonstrate the existence of functional A_{2A} receptors at inhibitory synapses at layer 2/3 pyramids of the visual cortex. Application of a specific antagonist of A_{2A} receptors, SCH-58261, in the presence of 100 μM adenosine led to a further decrease in IPSP amplitude in half of all cases (12/24). However, this effect was not present if adenosine and SCH-58261 were applied in the presence of the A_1R antagonist DPCPX. We interpret these results as follows. Activation of A_{2A}Rs does not have a direct effect on synaptic transmission, but it diminishes the suppressive effect of A_1Rs on inhibitory transmission. A high concentration of adenosine activates both A_1Rs, which suppress the transmission, and A_{2A}Rs, which interact with A_1Rs, diminishing the suppression at a subset of inhibitory synapses. Blockade of A_{2A}Rs leads to disinhibition of A_1Rs, revealing the full suppressive effect of A_1R on synaptic transmission. Because A_1Rs modulate synaptic transmission via presynaptic mechanisms, the above scenario implies that A_{2A}Rs are also located presynaptically. These results and their interpretation are consistent with the hypothesis suggested by Sebastião & Ribeiro (2009) that A_{2A} receptors are less related to direct modulation of synaptic transmission, but rather interact with receptors for other modulators, and 'fine-tune' synaptic function by modulating the effect of modulators. Support for these ideas comes from experiments on synaptosomes showing that A_{2A}Rs modulate the affinity of A_1Rs to ligands (Lopes *et al.* 1999), and from the finding that A_1Rs and A_{2A}Rs can form heteromers in membranes of striatal neurons, with activation of A_{2A}Rs reducing affinity of A_1Rs through direct receptor–receptor interaction (Ciruela *et al.* 2006, 2011; Ferre *et al.* 2007). Finally, present results for inhibitory synapses are consistent with our recent finding of functional A_{2A}Rs at a portion of excitatory neocortical synapses (Bannon *et al.* 2014).

Specificity and heterogeneity of adenosine effects on cortical synaptic transmission

Published data show great variety in the magnitude of adenosine's effects on transmission at cortical synapses. Factors contributing to this variability might include differences between synapses in the expression of the adenosine receptors, the ratio of A_1 and A_{2A} receptors, their affinity with and efficiency in intracellular cascades triggered by receptor activation, as well as the balance between local pathways for adenosine production, release and utilization. Experimental results show that each of these factors may be specific to the region of the brain, type of neuron and synapse, but also may change with age.

A case for region-specific expression of adenosine receptors is provided by inhibitory transmission, which is not affected by adenosine in the hippocampus (Lambert & Teyler, 1991; Yoon & Rothman, 1991; Brundege & Dunwiddie, 1996) and layer 2/3 pyramids from prefrontal cortex (Mathew *et al.* 2008), but can be suppressed by adenosine in sensory areas of the neocortex (Kirmse *et al.* 2008; Kruglikov & Rudy, 2008; and our present results). Results from hippocampus and layer 2/3 pyramids from prefrontal cortex also provide a clear case for synapse-type specificity of expression of adenosine receptors only at excitatory synapses, which are suppressed by adenosine, but not at inhibitory synapses, which are insensitive to adenosine (Lambert & Teyler, 1991; Yoon & Rothman, 1991; Brundege & Dunwiddie, 1996; Mathew *et al.* 2008). Notably, an example from a brain area in which the effects of adenosine have been investigated in great detail, the basal forebrain, shows that even excitatory and inhibitory synapses at neighboring neurons may be either sensitive to adenosine or not (Yang *et al.* 2013). In the basal forebrain, adenosine reduced the frequency of spontaneous and miniature IPSCs in GABAergic neurons with large H-currents, but not in GABAergic neurons with small H-currents and not in cholinergic neurons. The frequency of excitatory events was reduced by adenosine in all these neurons (Yang *et al.* 2013).

The results of the present study show, in agreement with published data, that even at synapses of the same type investigated under the same experimental conditions, saturating concentrations of adenosine may suppress transmission to a very different degree. This holds both for excitatory and for inhibitory synapses (e.g. Fig. 9). We interpret these results as evidence for a differential, synapse-specific, level of expression of adenosine receptors. By the same logic, the highly variable magnitude of the additional suppression of synaptic transmission by the blockade of A_{2A} receptors in the presence of a high adenosine concentration (Fig. 5 for inhibitory synapses, and Fig. 9 in Bannon *et al.* 2014 for excitatory synapses), suggests a variable ratio of A_1/A_{2A} receptor expression. The possibility of area-specific and cell-type

specific expression of adenosine receptors in cortical neurons is further supported by reports on postsynaptic effects of adenosine. In somatosensory and prefrontal cortex, adenosine application led to hyperpolarization and a decrease in input resistance in layer 5 pyramids and in some layer 3 pyramids, but not in layer 2 pyramids and not in inhibitory neurons (van Aerde *et al.* 2013). In the visual cortex, adenosine changed the membrane potential and input resistance of layer 2/3 pyramids (Bannon *et al.* 2014; and present results), but no such effect was reported in layer 5 pyramids (Murakoshi *et al.* 2001).

Finally, activity and maturation of the pathways for adenosine production, release and utilization, which determine the activity-dependent dynamics of adenosine concentration at a synapse, might be an important factor for determining the ultimate effect of adenosine on synaptic transmission. A recent study shows that in rat somatosensory cortex, excitatory synapses between layer 5 pyramids are subject to a tonic adenosine-mediated suppression, because application of the A_1R antagonist 8-CPT, which relieves synapses from the suppression, leads to an increase in the EPSP amplitude (Kerr *et al.* 2013). Moreover, the magnitude of the 8-CPT effect increases between postnatal days 17 and 32, indicating that the tonic adenosine-mediated suppression increases with age. Consistent with this interpretation, we have observed that in layer 2/3 pyramids from visual cortex, the A_1R antagonist DPCPX leads to a stronger increase in the frequency of miniature EPSPs in slices from P27–28 than in slices from P19–22 rats (N.B., P.Z., M.V. unpublished observations). The interplay between activity-dependent release of adenosine and availability of local mechanisms for its utilization may be an important factor determining the heterogeneity of local dynamics of adenosine concentration and its effects on synaptic transmission *in vivo*.

One clear conclusion from the broad diversity of adenosine effects considered above is that adenosine's effects on synaptic transmission cannot be generalized across brain structures or cell types, and more data on characterization of adenosine actions at specific connections are necessary for revealing possible organizing principles of regulation of cortical function by adenosine.

Modulation of the balance between excitation and inhibition by adenosine

Cortical networks operate in a balanced regime (Wehr & Zador, 2003; Okun & Lampl, 2008; Ozeki *et al.* 2009; Dorrn *et al.* 2010; Sun *et al.* 2010). Thus, for network operation, not just the magnitude of a change at excitatory or inhibitory synapses is important, but a change of the balance between excitation and inhibition.

Our results expose two interesting aspects of the modulation of excitation–inhibition balance by adenosine. The first is the concentration dependence of adenosine effects on excitation–inhibition balance. At low concentration adenosine preferentially suppresses evoked IPSPs, but has heterogeneous effect on EPSPs, so that on average excitation does not change. Because increases in adenosine concentration are activity related, a functional role for lessening inhibition around the locus of weak activation might help maintain this 'low profile' activity, e.g. for detection of threshold signals. At high concentrations of adenosine the suppression of EPSPs is stronger than suppression of IPSPs. A high concentration of adenosine is a correlate of strong activation, and stronger suppression of excitation by adenosine may be one of the mechanisms preventing excessive activity in the system. Thus, the concentration dependence of adenosine's effects on the excitation–inhibition balance endorses the adenosine-feedback with homeostatic function: protecting weak activity from an excessive inhibition, but also restricting excitation during excessively strong activation.

The second feature, exposed by the analysis of compound responses, is that adenosine may change the balance in a subset of excitatory and inhibitory synapses in both directions, either towards excitation or towards inhibition. Notably, despite these local changes, the overall balance of excitation and inhibition a cell receives does not change. In experiments with compound responses, although adenosine did change the balance in individual experiments, the average over all cells and inputs did not change significantly. This conclusion is supported by results of analysis of miniature events. The frequency of both miniature IPSPs and miniature EPSPs was changed to the same extent (to ∼80% of control) by adenosine application. Thus, adenosine may change the excitation–inhibition balance in a selected set of synapses, without compromising the overall balanced state of a network. The set of synapses subject to selective modulation may be defined by activity or by structural features, such as glial islands or individual glial cells which control the adenosine concentration over local regions including 300–600 dendrites (Fellin, 2009; Halassa *et al.* 2009). By selective up- or down-regulation of local excitation–inhibition balance, adenosine has the potential to modulate the spread of activity in neuronal networks.

Conclusions and outlook: how adenosine regulates synaptic transmission and excitation–inhibition balance in the neocortex

The following conclusions on adenosine's effects on synaptic transmission in the cortex can be drawn from the

results of the present study and the wealth of published work.

The most pronounced and common effect of adenosine on neocortical synapses is suppression of transmitter release mediated by presynaptic A_1 receptors. This suppression has been invariably found at all excitatory synapses in the cortex studied so far, indicating that presynaptic A_1 receptors are expressed at all excitatory presynaptic terminals in the cortex. In contrast, only a fraction of inhibitory connections are affected by adenosine, and thus presumably adenosine receptors are expressed in only a fraction of inhibitory presynaptic terminals. Elucidating guiding principles of this selective expression and its functional role remains a challenge for future studies.

At both excitatory and A_1R-expressing inhibitory synapses, the suppressive effect of A_1 receptors can be partially counteracted by A_{2A}Rs. The effects of A_{2A}Rs were small relative to the magnitude of A1-mediated suppression, but were clearly present in half of all cases (12/24). Our results show that an effect of A_{2A}R blockade was observed only when A_1Rs were functional, but not when A_1Rs were blocked. This suggests that at both excitatory and inhibitory synapses, A_{2A}Rs do not have their own effect on transmission, but can modulate the A_1R-mediated suppression. This interpretation is consistent with the hypothesis by Sebastião & Ribeiro (2009) on the role of A_{2A}Rs as modulators of the effects of other neuromodulators. Interestingly, we found evidence for A_{2A}Rs in only a fraction of excitatory and inhibitory synapses. Understanding which factors govern the expression of A_{2A} receptors at some, but not at some other synapses is another challenge for future studies.

A salient feature of adenosine's effects is high variability of the magnitude of suppression in different experiments, even in a homogeneous population of synapses. This holds both for the magnitude of A_1R-mediated suppression, and, when present, the effect of A_{2A}R–A_1R interaction. The most straightforward interpretation of this heterogeneity is the variable level of expression of adenosine receptors at presynaptic fibres. Understanding the rules and functional role of this heterogeneous expression, especially at inhibitory synapses remains an open challenge.

The heterogeneity of the adenosine sensitivity of cortical synapses sets the stage for possible differential local modulation of the excitatory–inhibitory balance in cortical circuits by adenosine. In combination with the concentration dependence of adenosine effects on the excitation–inhibition balance, this provides adenosine with an ability to combine activity-dependent fine-tuning of synaptic transmission with a homeostatic function, keeping activity in cortical networks within the operating range.

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Additional information

Competing interests

The authors declare no competing interests.

Author contributions

All authors contributed to the conception and design of the experiments, the collection, analysis and interpretation of data and drafting the article or revising it critically for important intellectual content. All authors have approved the final version of the manuscript.

Funding

This work was supported by the grant R01MH087631 from the NIH and Startup funds from the University of Connecticut to M.V. M.V. was partially supported by Humboldt Research Award from the Alexander von Humboldt-Stiftung.

Acknowledgements

We are grateful to Dr Stanislav Volgushev for advice on statistical analyis.