Synaptic Mechanisms of Adenosine A2A Receptor-Mediated Hyperexcitability in the Hippocampus

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ABSTRACT: Adenosine inhibits excitatory neurons widely in the brain through adenosine A1 receptor, but activation of adenosine A2A receptor (A2AR) has an opposite effect promoting discharge in neuronal networks. In the hippocampus A2AR expression level is low, and the receptor’s effect on identified neuronal circuits is unknown. Using optogenetic afferent stimulation and whole-cell recording from identified postsynaptic neurons we show that A2AR facilitates excitatory glutamatergic Schaffer collateral synapses to CA1 pyramidal cells, but not to GABAergic inhibitory interneurons. In addition, A2AR enhances GABAergic inhibitory transmission between CA1 area interneurons leading to disinhibition of pyramidal cells. Adenosine A2AR has no direct modulatory effect on GABAergic synapses to pyramidal cells. As a result adenosine A2AR activation alters the synaptic excitation–inhibition balance in the CA1 area resulting in increased pyramidal cell discharge to glutamatergic Schaffer collateral stimulation. In line with this, we show that A2AR promotes synchronous pyramidal cell firing in hyperexcitable conditions where extracellular potassium is elevated or following high-frequency electrical stimulation. Our results revealed selective synapse- and cell type specific adenosine A2AR effects in hippocampal CA1 area. The uncovered mechanisms help our understanding of A2AR’s facilitatory effect on cortical network activity. © 2014 The Authors Hippocampus Published by Wiley Periodicals, Inc.

KEY WORDS: antiepileptic; basket cell; cholecystokinin; disinhibition; parvalbumin; synchrony

INTRODUCTION

Adenosine is well known for its inhibitory effect on neocortical and hippocampal glutamatergic principal cells via the A1 receptor (A1R) (Dias et al., 2013). In addition, the high affinity adenosine A2A receptor (A2AR) is expressed in the brain, and although present at low levels in the neocortex and hippocampus (Schiffmann et al., 1991; Dixon et al., 1996) its activation in pathological conditions promotes epileptiform activity and facilitates excitotoxic neuronal death (Jones et al., 1998; Etherington and Frénguelli, 2004; Zeraati et al., 2006; El Yacoubi et al., 2009). However, evidence for A2AR-mediated facilitation of cortical excitatory neuron discharge is largely based on results in epilepsy and neuronal trauma models, and function of A2AR under physiological conditions in the cortex is less well known. Facilitatory effect of A2AR on excitatory neurons in healthy brain is well characterized in basal ganglia where it is involved in controlling arousal and motor responses (Rebola et al., 2005a; Ciruela et al., 2006; Shook and Jackson, 2011; Wei et al., 2011; Lazarus et al., 2012). Adenosine A2AR-mediated modulation of neuronal activity has also been reported in the hippocampus and neocortex where the receptor activation facilitates excitatory input from the CA3 area to CA1 enhancing glutamatergic synapses directly or by altering glutamate transport (Cunha et al., 1994; Rebola et al., 2005c; Dias et al., 2012; Matos et al., 2013). In physiological conditions adenosine A2ARs are involved in synaptic long-term plasticity in hippocampal glutamatergic mossy fibers (Rebola et al., 2008; Chamberlain et al., 2013), and a recent study demonstrated that deletion of A2AR selectively in the hippocampus compromises contextual memory formation (Wei et al., 2013).

The paucity of apparent adenosine A2AR expression in the hippocampus hints that the receptor may be localized to specific neuron subpopulations or subtypes of synapses (Schiffmann and Vanderhaeghen, 1991; Dixon et al., 1996). Although reported facilitatory effects on glutamatergic transmission between pyramidal cells could explain, at least partly, why A2AR activation promotes cortical pyramidal cell discharge (Jones et al., 1998; Zeraati et al., 2006; El
Yacoubi et al., 2008; El Yacoubi et al., 2009; Moschovos et al., 2012), it is unknown if modulation of GABAergic inhibitory interneurons contributes to A2AR-mediated effects on hippocamal function. Adenosine A2AR expression level increases in posttraumatic and epileptic neocortex and hippocampus (Dixon et al., 1996; Rebola et al., 2005b), and this may emphasize a role of the receptor in the activity modulation in pathological conditions. Knowledge of the action of A2AR on identified hippocampal synaptic circuits is crucial for understanding adenosine function in physiological conditions in the cortex and the therapeutic potential of high affinity adenosine receptors in pathological conditions such as epilepsy.

MATERIALS AND METHODS

Animals

Mice were anaesthetized with Na-pentobarbitone and decapitated in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986), and the European Community guidelines (86/609/EEC). Experiments were conducted on 4–8 week old heterozygous PV-Cre mice (The Jackson Laboratory B6;129P2-Pvalbtm1(cre)Arbr/J), BAC-CCK-Cre (Geibel et al., 2014) and CaMKII-Cre (B6.Cg-Tg(Camk2a-cre)T29-1Stl/J) and their wild type littermates. Homozygous PV-Cre mice were crossed with homozygous Ai9 mice (B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/J) to produce tdTomato fluorescence expression specifically in PV+ cells.

Slice Preparations

The brain was removed and placed in 4°C solution (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 0.5 CaCl2, 7 MgCl2, 1.0 NaH2PO4, 25 NaHCO3, 25 glucose (pH 7.4). For experiments in submerge chamber (Figs. 1–6) coronal slices (250 μm) were cut from both hemispheres using a vibratome (Microm HM650V, Carl Zeiss). For Figure 7 experiments in interface chamber slices were 400 μm thick. In either configurations slices were kept submerged in 32°C cutting solution for 20 min, then stored in interface chamber at 20–25°C for at least 60 min in recording solution (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl2, 1.3 MgSO4, 1.25 NaH2PO4, 25 NaHCO3, and 11 glucose (pH 7.4). For data in Figure 2, slices were stored and experiments performed in the continuous presence of KN-62 (3 μM) and MCPG (200 μM) to prevent long-term plasticity with repetitive glutamatergic fiber burst stimulation (Perez et al., 2001; Lamsa et al., 2007; Campanac et al., 2013). A surgical cut was made between CA1 and CA3 areas. Slices in a submerged recording chamber (Luigs and Neu mann) mounted on the stage of BX51WI microscope (Olympus), were visualized using a 20X immersion objective (2–4 zoom) with epifluorescence for YFP and tdTomato and with DIC-IR optics in combination with a CCD camera (Till-Photonics). Slices were superfused with recording solution at 5 mL/min and oxygenated with 95% O2 /5% CO2.

Electrophysiology

Whole cell and field potential recording electrodes (5–9 MΩ) were pulled (P-97, Sutter Instrument Co.) from borosilicate glass capillaries (GC150F-10, Harvard Apparatus). Intracellular solution for experiments in Figure 2 was (in mM): 145 Cs-Methansulphonate, 20 HEPES, 10 CsOH, 0.2 CsOH-EGTA, 2 ATP-Mg, 0.3 GTP-Na (295 mOsm, pH 7.2); in Figure 3 (in mM), 145 K-glucuronate, 10 KOH, 0.2 KOH-EGTA were used instead; in figs 4, Cs-Methanosulphonate was replaced with CsCl. QX-314 (5 mM) and Neurobiotin (0.2–0.5%, Vector Laboratories) were included in all intracellular filling solutions. Field potential electrodes were filled with saline. Ratio of baseline fEPSP slope values and popspike amplitudes evoked with different intensities were fitted with regression line in each experiment baseline. The fEPSP slope–popspike relation was considered linear when regression fitting index was > 0.8 (0.89 ± 0.03, n = 11, mean ± s.e.m, Sigma Plot). fEPSP slope values recorded following wash-in of CGS21680 were fitted in the baseline condition regression line. Then, measured popspike amplitude in CGS21680 and popspike estimate given to same fEPSP value in baseline linear slope–popspike relation were compared. This gave Δ popsipe/fEPSP used in Figure 1E. Because lowest intensity often failed to elicit stable popspike in baseline, intensities from 75 μS till 150 μS stimulus duration were used to determine linear relation of fEPSP slope and popspike amplitude in baseline conditions with regression line. The fEPSP values in the presence of agonist, which were potentiated out of the baseline fEPSP slope range, were excluded in analyses because no linear relation between fEPSP slope and popspike could be confirmed.

Data in Figures 1–6 were recorded with a Multiclamp 700B amplifier (Molecular Devices), low-pass filtered (4 kHz), digitized (10 kHz), and acquired by Clampex software (Molecular Devices). Field potential recordings in interface chamber (data for Fig. 7) were performed with an AC preamplifier and AC/DC amplifiers Neurolog NL104 and NL106 (0.3 Hz high-pass filtering) (Digitimer Ltd.). The signal was digitized by a Power 1401 plus (Cambridge Electronic Design). Additionally, a Humbug 50/60 Hz (Digitimer Ltd.) was used to remove noise locked to the electrical mains supply. Data were stored for offline analysis using Signal5 software (Cambridge Electronic Design) at 10 kHz acquisition rate. In Figure 7 experiments a single-pulse electrical stimulus was delivered (every 20 s), and elicited fEPSPs (100 ms from stimulation) were excluded from spontaneous activity analysis.

Access resistance (< 20 MΩ) was not compensated. Whole-cell recordings with >25% change were rejected. Liquid-junction potential was not corrected. Single, paired-pulse and HFS electrical stimuli (50–250 μA) were applied with concentric bipolar electrodes (CBAPC75PL1, FHC) connected to stimulus isolator boxes and triggered via computer. In Figure

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FIGURE 1. Activation of adenosine A2A receptor facilitates glutamatergic transmission in hippocampal Schaffer collaterals and amplifies CA1 pyramidal cell input-output function. A-C: A selective agonist CGS21680 (30 nM) increases fEPSP slope and population spike amplitude evoked by stimulation of Schaffer collaterals. A: Schematic shows experimental design. Paired-pulse (50 ms interval) electrical stimulation (S) was delivered in the CA1 area. The CA3 area was removed by surgical cut to avoid recurrent excitation. Averaged field potential traces (10) evoked with mid-strength stimulation (100 μs pulse duration) in baseline (bl, black) and following application of CGS21680 (30 nM) (CGS, red). (a) shows prespike volley amplitude (between horizontal dotted lines), (b) fEPSP slope was measured between dotted vertical lines, and (c) popspike amplitude between horizontal lines. Stimulation artifact (S) is truncated. B: Increase of fEPSP slope by CGS21680 (30 nM). fEPSPs were elicited in every experiment with five stimulation intensities gradually increasing stimulus pulse duration from 50 to 150 μs. Open boxes show median (with 25% and 75% quartiles) of baseline-normalized fEPSP slope in 8 experiments following wash-in of CGS21680. Solid boxes show CGS21680 wash-in results in presence of the A2AR antagonist SCH58261 (100 nM) (n = 3). Significant difference between open and solid boxes (*P<0.05, Mann-Whitney test). D, E: CGS21680 increases popspike amplitude - fEPSP slope ratio. D: Relation of popspike amplitude and fEPSP slope in one experiment in baseline (black trace and symbols) and following wash-in of CGS21680 (red). fEPSPs were evoked with various intensities using stimulation pulse duration from 75 to 125 μs. Inset: Averaged (10) field potential responses in baseline (black) and following wash-in of CGS21680 (red). Popspikes appearing in the fEPSP following wash-in of CGS21680 are indicated by arrows. (Data in the plot show first popspike amplitude when more than one popspike is elicited in CGS21680.) E: Effect of CGS21680 on popspike amplitude - fEPSP slope relation in all experiments. In baseline conditions popspike – fEPSP slope relation was determined in each experiment (see Materials and Methods). Plot shows a relation of popspike amplitude associated with similar size fEPSP slope in CGS21680 and baseline. This is indicated as Δ popspike/fEPSP slope. Open boxes represent median of means of individual experiments (circles). fEPSPs upon 2nd stimulation of paired-pulse generated significantly higher popspikes than similar magnitude fEPSPs in baseline (*P<0.05, Mann-Whitney test). For 1st stimulation pulse response, there was no significant difference between baseline and CGS21680. Solid boxes correspond to control experiments where CGS21680 was applied in the presence of A2AR blocker SCH58261 (30 nM). Antagonist blocks the agonist-induced increase in Δ popspike/fEPSP slope (*P<0.05, Mann-Whitney test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
stimulation with S2 electrode was suspended after baseline during SCH58261 wash-in and resumed after 10 min. Data were analyzed offline using Clampfit 10.2 software (Molecular Devices) or Spike2 software (Cambridge Electronic Design). Recorded signals were low-pass filtered on-line at 6 kHz and off-line in Figure 7 experiments as reported in results using Spike2 software. Drugs were diluted (1:1,000) in ddH2O, DMSO or ethanol, and applied via superfusion.

Statistics

All data presented were tested for normal distribution (Shapiro-Wilk test, Sigma Plot), and when passed t-test or single way ANOVA and Tukey’s post hoc test was used to confirm
significance, and data were shown as mean ± sem. Otherwise Mann-Whitney was used instead and data shown as median and quartiles.

Stereotaxic Injections

An adeno-associated virus serotype 2 or 5 construct (AAV2/5:ChR2-eYFP) was stereotaxically injected into dorsal hippocampus of heterozygous PV-Cre, CCK-Cre, and CaMKII-Cre mice (CA1-CA3 area) via 33-gauge needle attached to a MicroSyringe (Hamilton). Craniotomy was made for mice anesthetized with 2–4% isoflurane. In each hemisphere, 800 nL of virus suspension was delivered at 80 nL/min by a MicroSyringe Pump Controller (World Precision Instruments). Following suturing of the wound, mice were allowed to recover for 14–21 days after injections.

Optogenetics

ChR2 was activated by a fixed-spot 20 or 80 μm diameter laser light spot (pulse 3 ms, max. 100 mW, Rapp OptoElectronics) via the microscope objective (diameter measured under objective). All experiments with 20 Hz 5-pulse stimulation were performed in the presence of blockers for high-frequency stimulation-elicited long-term plasticity. Paired-pulse ratios are presented as 2nd versus 1st IPSC amplitude. Compound IPSC and EPSC charge was measured in 500 ms window from current onset.

Cell Visualization, Anatomical Analysis, and Immunohistochemistry

Processes and analyses are described in Oren et al. (2009). Briefly, slices were fixed overnight at 4°C, washed in 0.1 M
phosphate buffer (PB), embedded in 20% gelatine, and sectioned (60–70 μm) with a vibratome (Leica Microsystems) in 0.1 M PB. Then, washed in 50 mM Tris-buffered saline (TBS, pH 7.4) with 0.3% Triton X-100 (TBS-Tx), incubated overnight with streptavidin conjugated to either AlexaFluor-488 or Cy3, washed in 50 mM TBS-Tx, mounted in Vectashield.
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(A Vector Laboratories) and examined with an epifluorescent microscope (DM5000 B, Leica Microsystems) using appropriate filter sets (L5 or Y3) and a CCD camera (ORCA-ER, Hamamatsu). Pyramidal cells were identified by mushroom spines on dendrites, basket cells and oriens-lacunosum molecular (O-LM) cells by their axon arborisation inside stratum pyramidale or lacunosum moleculare, respectively (Oren et al., 2009; Nissen et al., 2010). Digital micrographs were constructed from z-stack images recorded with epifluorescence microscope, collapsed, and analyzed with Image-J software (Somogyi et al., 2012).

Free-floating sections were washed in 50 mM TBS-Tx, blocked in 20% normal horse serum (NHS, Vector Laboratories) in TBS-Tx, and incubated in primary antibodies at 4°C for 48 h. Fluorochrome-conjugated secondary antibodies were applied overnight at 4°C. After another wash-in TBS-Tx, sections were mounted in Vectashield under coverslips. Immunoreactivity was evaluated at 40X magnification with 2X zoom using confocal laser-scanning microscopy (LSM710, Carl Zeiss) with Zen2008 software. Details of primary and secondary antibodies are reported in Nissen et al. (2010).

**RESULTS**

**Adenosine A2AR Facilitates Glutamatergic Schaffer Collateral Synapses and Amplifies CA1 Pyramidal Cell Input–Output Transformation**

We studied the effect of A2AR activation on hippocampal Schaffer collateral synapses in the CA1 area using paired-pulse microelectrode stimulation (50 ms interval, delivered every 15 s) and field potential recording in mouse hippocampal slices. Wash-in of selective A2AR agonist CGS21680 (30 nM) after a baseline (at least 10 min) enhanced stimulus-evoked fEPSP slope and increased population spike (popspike) amplitude (P < 0.05), but did not alter prespike volley (Mann-Whitney test) (Figs. 1A–C). Stimulus-evoked fEPSP and popspike details are shown in Figures 1B,C. Baseline-normalized prespike volley in CGS21680 was 1.02 ± 0.02 for 1st stimulation pulse and 1.01 ± 0.03 for 2nd (n = 11) (Sebastiao and Ribeiro, 1992). Facilitatory effects of CGS21680 on fEPSP slope and popspike amplitude were fully blocked in CCK+ cells are not modulated by CGS21680 (30 nM) either in postsynaptic interneurons (B) nor in pyramidal cells (C) (mean ± sem, t-test). All recordings were in the presence of NBQX (25 μM) and DL-APV (100 μM). **Insets:** schematic shows experimental design. Averaged IPSCs (10) from sample recordings. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**FIGURE 5.** Facilitation of efferent GABAergic synapses by CGS21680 is specific to PV+ cells. The IPSCs elicited from CCK+ interneurons are not modulated by the A2AR agonist. A: Optogenetic stimulation of axons from CCK-expressing GABAergic cells. Confocal images of AAV-transduced Cre-dependent eYFP-ChR2 (above) in proCCK+ neurons (below at Cy5). Fluorophore-positive somata are pointed with arrows (fixed slice). IPSCs evoked from CCK+ cells are not modulated by CGS21680 (30 nM) either in postsynaptic interneurons (B) nor in pyramidal cells (C) (mean ± sem, t-test). All recordings were in the presence of NBQX (25 μM) and DL-APV (100 μM). **Insets:** schematic shows experimental design. Averaged IPSCs (10) from sample recordings. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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experiments with continuous presence of the A2AR antagonist SCH58261 (100 nM) (Mann-Whitney test) (Figs. 1B,C). Effect of CGS21680 on field potential responses was studied in each experiment with five stimulation intensities. In all experiments stimulation intensity was adjusted to generate a popspike with mid-range intensity in baseline conditions (popspike amplitude $0.25 \pm 0.06$ mV for 1st pulse, and $0.59 \pm 0.17$ mV for 2nd pulse, $n = 11$, mean $\pm$ sem. This corresponded to fEPSP slope of $0.32 \pm 0.06$ mV/ms and $0.57 \pm 0.11$ mV/ms, respectively).

We discovered that following wash-in of CGS21680 (30 nM), popspike amplitude–fEPSP slope ratio also changed. In the presence of CGS21680, fEPSPs were associated with higher amplitude popspikes than during baseline (Fig. 1D). We used linear regression to fit fEPSP slope and popspike amplitude values (evoked with various stimulus intensities) in baseline conditions in each experiment (see Material and Methods). Following wash-in of CGS21680 (30 nM), fEPSPs upon 2nd stimulation of the paired-pulse generated significantly higher amplitude popspikes than similar magnitude fEPSPs during baseline ($P < 0.05$, Mann-Whitney test) (Fig. 1E). Popspike amplitude–fEPSP slope relation details are shown in Figures 1D and E. The results show that A2AR facilitates glutamatergic synapses in the hippocampus, and in addition increases CA1 pyramidal cells’ output in response to Schaffer collateral excitation.

Adenosine A2AR Increases Glutamatergic Excitation and Suppresses GABAergic Feed-Forward Inhibition to CA1 Pyramidal Cells

Next, we investigated how A2AR activation modulates monosynaptic excitatory and disynaptic inhibitory currents in the CA1 hippocampal pyramidal cells. We selectively stimulated Schaffer collaterals delivering 473 nm laser light-pulses (3 ms, 5 pulses at 50-ms interval, delivered every 30 s) to CA1 stratum radiatum in slices expressing channelrhodopsin 2 (ChR2) in glutamatergic neurons (Fig. 2A). Slices were prepared from hippocampi of heterozygous CaMKII-Cre ($^{+}$/Cre) mice transduced with AAV2/5-Chr2-eYFP to express Chr2 in a Cre-dependent manner in CA1-CA3 pyramidal cells (see Materials and Methods). Postsynaptic cells were voltage-
FIGURE 7.

(A) A<sub>2A</sub>R antagonist + SCH58261 (100 nM)

(B) Unfiltered, BP 1-100 Hz, HP 60 Hz

(C) Count (2 min bin) vs. time (min)

(D) Baseline in CGS21680 (30 nM)

(E) Unfiltered, BP 1-100 Hz, HP 60 Hz

(F) Count (2 min bin) vs. time (min)

(G) Normalized data (exp. 1, 2, 3, 4)
clamped sequentially at −70 mV and at a reversal potential of EPSCs (11 ± 1 mV, n = 7 cells) to record glutamatergic EPSCs and disynaptic GABAergic IPSCs, respectively (Fig. 2B). Wash-in of A2AR agonist CGS21680 (30 nM) potentiated glutamatergic EPSCs and simultaneously suppressed disynaptic GABAergic IPSCs in CA1 pyramidal cells (Figs. 2B,C,E). Charge of baseline-normalized EPSCs increased to 1.25 ± 0.08 (P < 0.05, n = 7 cells, t-test), and disynaptic IPSCs decreased to 0.77 ± 0.07 (P < 0.05, n = 7 cells, t-test). Baseline EPSC and IPSC were 25.3 ± 4.7 pC and 55.2 ± 12.6 pC, respectively. When we repeated experiments in the presence of the A2AR antagonist SCH58261 (100 nM) applied at least 30 min prior to agonist wash-in, A2AR agonist effect was fully blocked and neither EPSCs nor IPSCs were altered (Figs. 2D–F). Baseline-normalized EPSCs and IPSCs were 0.98 ± 0.02 and 0.94 ± 0.03, respectively (n = 6, t-test). During baseline, mean ± sem of EPSCs was 48.0 ± 8.5 pC and IPSCs was 70.1 ± 7.6 pC.

Because pyramidal cells in the CA1 area can express low levels of CCK and GABAergic IPSCs, we could mask synaptic EPSCs in these experiments (Geibel et al., 2014). We therefore washed in glutamate receptor blockers NBQX (25 μM) and DL-APV (100 μM) at the end to measure ChR2 contribution to light-stimulation-evoked excitatory currents. In all tested cells glutamatergic current was predominant (78 ± 8% of total charge, n = 7 cells) showing that the facilitatory effect of A2AR agonist on excitatory currents is caused by increased glutamatergic EPSCs.

The results show that A2AR activation modulates Schaffer collateral-driven synaptic input from CA3 area to CA1 pyramidal cells in two ways; facilitating monosynaptic glutamatergic excitation and suppressing network-driven disynaptic GABAergic inhibition simultaneously. These changes can at least partially explain our above findings on A2AR-mediated facilitation of Schaffer collateral fEPSP (see Figs. 1A,B) and popspike upon Schaffer collateral paired pulse stimulation (see Figs. 1C, and the observed facilitation in CA1 pyramidal cells input–output transformation (see Figs. 1D,E).

### Adenosine A2AR Facilitates Glutamatergic Schaffer Collateral Synapses Selectively to Pyramidal Cells

We repeated Schaffer collateral electrical stimulation experiments (see Fig. 1) and recorded intracellularly from either postsynaptic CA1 pyramidal cells or interneurons. Bath-applied adenosine A2AR agonist CGS21680 (30 nM) facilitated glutamatergic EPSC amplitude to 1.30 ± 0.04 from baseline (10–15 min following application, P < 0.001, n = 9, t-test) in synapses onto identified CA1 pyramidal cells (see Material and Methods). Wash-in of A2AR antagonist SCH58261 (100 nM) after baseline failed to change EPSCs, and baseline-normalized EPSC amplitude in SCH58261 was 0.94 ± 0.04 (n = 6, t-test) indicating that A2ARs are not activated by endogenous adenosine under the experimental conditions (Figs. 3A,B). Next, we studied EPSCs in two major interneuron populations involved in feed-forward inhibition in area CA1; GABAergic cells expressing either parvalbumin (PV+) or cholecystokinin (CCK+) with axonal cannabinoid receptor Type 1 (CB1R) (Katona et al., 1999; Glickfeld and Scanziani, 2006; Nissen et al., 2010; Armstrong and Soltész, 2012). EPSCs in PV+ and CCK+ interneurons were not altered by A2AR agonist (t-test), and baseline-normalized EPSC amplitudes in CGS21680 (30 nM) were 1.05 ± 0.05 (n = 8) and 1.04 ± 0.02 (n = 7) accordingly (Figs. 3C–F). Thus, activation of A2AR facilitates excitatory Schaffer collateral synapses in target-specific manner.

Mean ± sem of EPSCs during baseline was 79.6 ± 8.1 pA in pyramidal cells (n = 9) and 77.8 ± 15.8 pA in the interneurons (n = 15). GABA receptors were blocked with PiTX (100 μM) and CGP55845 (1 μM), and cells were filled with neurobiotin for post hoc anatomical and immunohistochemical studies (Figs. 3A,E).

![FIGURE 7. Modulation of spontaneous epileptiform pyramidal cell discharge by A2AR antagonist and agonist in hyperexcitable conditions with elevated extracellular potassium. A–C: Adenosine A2AR blocker SCH58261 (100 nM) suppresses spontaneous epileptiform discharges in hippocampal slices exposed to elevated (8–9 mM) extracellular potassium. Spontaneous interictal-like synchronous bursting activity was recorded with field potential electrode in CA3 area. A: A sample trace from one experiment showing inhibition of spontaneous epileptiform burst activity by SCH58261 (unfiltered trace). Timing for wash-in of A2AR antagonist SCH58261 (100 nM) is indicated by horizontal bar. Histogram below shows occurrence of spontaneous epileptiform bursts in 2 min bins. For burst occurrence analysis data were band-pass filtered (1–100 Hz) to avoid detection of occasional single unitary extracellular spikes. B: Epileptiform population bursts are characterized by 1–100 Hz band-pass filtered (BP 1–100 Hz) field potential deflection associated with extracellular spikes (high-pass filtered at 60 Hz, HP 60 Hz). An unfiltered epoch shown on top with filtering below as indicated. C: Plot shows suppression in occurrence of spontaneous epileptiform events by SCH58261 in the three of three experiments.](http://example.com/figure7.png)
Adenosine A2AR Enhances GABAergic Inhibition in the CA1 Area Selectively Between Interneurons

The results above do not explain why feed-forward IPSCs were strongly suppressed by A2AR activation in experiments shown in Figure 2. To explore this, we investigated whether GABAergic synapses from interneurons to pyramidal cells are modulated by A2AR agonist, or if GABAergic synapses between interneurons are altered. We utilized Cre-dependent ChR2 expression to optogenetically activate GABAergic synapses from either PV- or CCK-expressing CA1 interneurons. Slices were prepared from heterozygous PV-Cre (Fig. 4) and BAC-CCK-Cre<sup>Neo<sup>fl/fl</sup></sup> mice (Fig. 5) transduced with AAV:ChR2-eYFP (see Materials and Methods) (Geibel et al., 2014). We first stimulated ChR2-expressing PV+ GABAergic interneuron axons with paired-pulse laser light pulses (3 ms, 50 ms interval) in the CA1 area, and found that wash-in of the agonist CGS21680 (30 nM) increased IPSC amplitude in postsynaptic interneurons to 1.35 ± 0.04 of baseline (P < 0.001, n = 12, t-test) (Figs. 4A,B). The facilitation was significant in 11 of 12 anatomically verified interneurons, and was fully blocked when studied in the presence of the A2AR antagonist SCH58261 (100 nM) (n = 5, t-test) (Fig. 4B). However, CGS21680 (30 nM) failed to directly modulate GABAergic synapses from PV+ cells to postsynaptic pyramidal cells (t-test) (Fig. 4C). Baseline-normalized IPSC amplitude in postsynaptic pyramidal cells was 0.93 ± 0.04 in the presence of CGS21680 (30 nM) (n = 12).

The IPSC facilitation by CGS21680 (30 nM) in interneurons was associated with a decrease in the paired-pulse ratio to 0.67 ± 0.08 from baseline (P < 0.001, n = 10, t-test), suggesting presynaptic modulation of transmission by A2AR in GABAergic fibers (Fig. 4D). In addition, facilitation of IPSC by CGS21680 was blocked in the presence of a PKA inhibitor H-89 dihydrochloride hydrate (1 μM) (baseline-normalized IPSC amplitude was to 1.02 ± 0.01, n = 5) (Fig. 4E). In PKA-inhibitor studies, IPSCs were elicited with afferent electrical stimulation in the presence of glutamate receptor blockers (NBQX, 25 μM and DL-APV, 100 μM) and in control experiments IPSC increased to 1.14 ± 0.03 from baseline by CGS21680 (30 nM) (P < 0.01, 15 min wash-in, n = 6, t-test). Wash-in of A2AR antagonist SCH58261 after baseline (100 nM) failed to change IPSCs (amplitude 0.99 ± 0.11 of baseline, n = 6, t-test).

The results on IPSCs in postsynaptic pyramidal cells and interneurons show that A2AR-mediated modulation of inhibitory synapses from PV+ GABAergic fibers depends on the postsynaptic cell type. Postsynaptic neurons were filled with neurobiotin during recording for post hoc analysis of the cells (see Materials and Methods). This confirmed that A2AR-mediated facilitation of IPSCs occurs in various postsynaptic interneuron types including oriens-lacunosum moleculare (OLM) cells (n = 2), and basket cells with negative (n = 2) or positive (n = 6) axonal immunochemistry for CB1R (Fig. 4F) (Glickfeld and Scanziani, 2006; Lawrence et al., 2006; Klaus-berger and Somogyi, 2008). Two interneurons, of which one showed IPSC facilitation by A2AR, remained unidentified (Fig. 4F).

Conversely, IPSCs elicited from CCK+ GABAergic fibers (Fig. 5A) were not modulated by A2AR. Exposure to CGS21680 (30 nM) failed to alter IPSCs either in postsynaptic interneurons (n = 8) or pyramidal cells (n = 5) (t-test, baseline IPSCs = 94.0 ± 25.2 pA and 52.7 ± 9.9 pA, respectively) (Figs. 5B,C). Inhibitory PSCs were elicited by paired-pulse optical stimulation in slices from BAC-CCK-Cre<sup>Neo<sup>fl/fl</sup></sup> mice transfected with AAV:ChR2-eYFP. Ionotropic glutamate receptors were blocked with NBQX (25 μM) and DL-APV (100 μM), because in addition to GABAergic neurons also CCK-containing glutamatergic fibers in the CA1 area may express Cre (Geibel et al., 2014). We also confirmed that optogenetically evoked IPSCs in the slices were elicited from CCK+ interneuron axons demonstrating suppression of the IPSCs by CBR1 agonist WIN55,212-2 (5 μM) to 0.62 ± 0.03 of baseline (P < 0.001, n = 6, t-test) with a characteristic increase in paired-pulse ratio (to 1.49 ± 0.18 from baseline, P < 0.05, n = 5, t-test) (Katona et al., 1999; Glickfeld and Scanziani, 2006; Nissen et al., 2010).

Endogenous Adenosine Promotes Synchronous Pyramidal Cell Discharge Via A2ARs in Hippocampal Slices

We next studied whether endogenous adenosine released by high-frequency electrical stimulation is sufficient to modulate hippocampal pyramidal cell discharge through adenosine A2AR (Chamberlain et al., 2013). We utilized experimental design used above in Figure 1 to electrically stimulate Schaffer collaterals with paired pulses (50 ms interval), while recording field potential in the CA1 area. In addition, we applied high-frequency stimulation (HFS, 50 Hz 100 pulse) with a second stimulation electrode positioned in the vicinity of recording electrode aiming to elicit local release of adenosine (Fig. 6A) (Chamberlain et al., 2013). Schaffer collaterals were stimulated every 5 s and HFS delivered with second electrode every 2 min. To uncover adenosine A2AR-mediated modulation the experiments were performed in continuous presence of blockers for CB1R (AM-251 2 μM), GABAB receptor (CGP555485, 1 μM), adenosine A1R (DPCPX, 200 nM) as well as with DL-APV (100 μM). We analyzed same fEPSP parameters as in Figure 1 and found that HFS was followed by significant increase of pop-spike amplitude in Schaffer collateral-mediated field potential response. Popspike was elicited by 2nd stimulation pulse of the paired-pulse and they were significantly increased from baseline up to 40 s following the HFS. Importantly, the facilitation was blocked after wash-in of SCH58261 (100 nM) (P < 0.001, ANOVA, Tukey’s HSD test, Fig. 6B). Although HFS transiently also modulated fEPSP slope in the experiments, application of the A2AR blocker failed to cause any change in the effect on slope. Neither did HFS or SCH58261 affect prespike volley (ANOVA, Tukey’s HSD test, data not
Finally, we investigated whether A2AR activation by endogenous adenosine modulates spontaneous epileptiform discharge of hippocampal pyramidal cells in hyperexcitable conditions. Spontaneous inter-ictal like pyramidal cell population bursts were generated exposing slices to elevated (8–9 mM) extracellular potassium ([K\textsubscript{o}]) in perfusion solution (Korn et al., 1987; Sagratella et al., 1987). Field potential was recorded in the CA3 area in an interface chamber. Following stable baseline (at least 10 min), either A2AR blocker SCH58261 (100 nM) or agonist CGS21680 (30 nM) was washed in. Epileptiform activity was quantified analyzing the occurrence of spontaneous inter-ictal like events characterized by a low frequency content field potential deflection associated with a barrage of extracellular spikes. Recordings were band-pass (1–100 Hz) filtered offline to uncover low-frequency deflections and analyze event occurrence (Figs. 7A,B). Amplitude threshold was set to 0.25 mV, and event detection was visually verified. Parallel high-pass filtering (>60 Hz) of recordings uncovered extracellular spikes associated with the events. Occurrence of inter-ictal like events in baseline conditions was 32.7 ± 11.7 events/min, ranging from 6.3 to 97.4 events/min (n = 7). The adenosine A2AR blocker SCH58261 strongly inhibited the occurrence spontaneous population bursts to 36 ± 9% (P < 0.01, n = 3, t-test) of baseline in 20–30 min following drug application. The activity-suppressing effect of antagonist persisted and in 40–50 min from drug application the burst occurrence dropped to 16 ± 5% of baseline level (P < 0.001, n = 3, t-test) (Fig. 7C). Adenosine A2AR agonist CGS21680 (100 nM) increased spontaneous epileptiform burst occurrence (Figs. 7D–G) from baseline to 140 ± 16% (P < 0.05, n = 4, t-test) in 20–30 min following drug application. Increase of burst occurrence was significant in three of four experiments, but varied in magnitude (Figs. 7F,G). Samples of band-pass and high-pass-filtered events are illustrated in Figures 7B,E. Modulation of spontaneous activity with A2AR drugs suggests the receptors are tonically activated in slices with elevated [K\textsubscript{o}], possibly because of increased ambient adenosine levels (Marichich and Nasello, 1973; Etherington and Frenguelli, 2004; Dias et al., 2013).

DISCUSSION

Adenosine has a well-established role as an endogenous neuronal inhibitor in the brain. Adenosine's suppressive effect on excitatory glutamatergic transmission via A1R is well characterized, but its effect via other adenosine receptor types is not as well known (Dunwiddie and Masino, 2001; Sebastiao and Ribeiro, 2009). In the hippocampus and neocortex the high-affinity A2AR is expressed in low quantities (Dixon et al., 1996), but elevated levels of extracellular adenosine activate these receptors to facilitate neuronal discharge (Etherington and Frenguelli, 2004; Zeraati et al., 2006; El Yacoubi et al., 2008; El Yacoubi et al., 2009). It has been proposed that excitatory effects of adenosine in the cortex may mainly occur in pathological conditions, because A2AR expression levels increase in those circumstances in parallel with desensitization and down-regulation of A1R (Rebola et al., 2005b; D’Alimonte et al., 2009; Hamil et al., 2012; Moschovos et al., 2012). In addition evidence for A2AR-mediated modulation of activity in the hippocampus in physiological conditions is emerging (Cunha and Ribeiro, 2000; Rebola et al., 2005a; Rebola et al., 2008; Dias et al., 2012; Chamberlain et al., 2013; Dias et al., 2013; Wei et al., 2013), but A2AR effect on identified neuronal circuits in this area is still poorly understood.

We identified here two sites of synaptic modulation by which A2AR acts to shift the balance between synaptic excitation and inhibition in mouse hippocampus to facilitate principal cell discharge. Adenosine A2AR activation directly enhances excitatory glutamatergic Schaffer collateral synapses to CA1 pyramidal cells, and simultaneously suppresses feed-forward GABAergic inhibition to the same neurons. This at least partially explains the facilitatory effects of A2AR agonist on Schaffer collateral field potential responses in the CA1 area with increased fEPSP slope and popspike amplitude (Sebastiao and Ribeiro, 1992) (also shown here in Figs. 1–6). Our results also demonstrate that adenosine A2AR is unlikely to modulate glutamatergic Schaffer collateral axon excitability, for example through axonal receptors (Kullmann et al., 2005), because the agonist did not have effect on extracellular prespike volley. Together our findings provide a simple mechanistic explanation how A2AR activity increases excitability in the hippocampal CA3-CA1 circuitry modulating identified excitatory and inhibitory synapses. Although modulatory effects of A2AR are not restricted to synapses, but in addition can include alterations in intrinsic properties of neurons (Rebola et al., 2011) as well as glial glutamate transport (Matos et al., 2013), the synaptic modulatory action can at least partly explain proconvulsive effect of A2AR reported previously (Jones et al., 1998; Zeraati et al., 2006; El Yacoubi et al., 2008; El Yacoubi et al., 2009) and also demonstrated here.

Facilitation of epileptiform activity through low A2AR expression level in the hippocampus (Dixon et al., 1996) can be explained by synergistic action of the synaptic modulatory actions shown here. Increased Schaffer collateral excitation of pyramidal cells, but not feed-forward interneurons increases CA1 pyramidal firing to glutamatergic input from the CA3 area (Pouille and Scanziani, 2001; Lamsa et al., 2005; Xu et al., 2006; Pavlov et al., 2011; Lovett-Barron et al., 2012). We studied two major subpopulations of CA1 area GABAergic interneurons, either expressing PV or CCK, which both contribute to CA3-CA1 feed-forward inhibition controlling CA1 area pyramidal cell firing and their input-output transformation (Cobb et al., 1995; Buhl et al., 1996; Glickfeld and Scanziani, 2006; Klausberger and Somogyi, 2008; Lovett-Barron et al., 2012). Inhibitory transmission through these interneurons to CA1 pyramidal cells was not enhanced by A2AR. Instead A2AR activation suppressed feed-forward GABAergic inhibition in pyramidal cells through a mechanism, which is likely to
include disinhibition. Facilitation of inhibitory synapses between CA1 interneurons has been demonstrated to effectively suppress network activity-driven GABAergic inhibition in the CA1 area pyramidal cells (Chamberland and Topolnik, 2012; Lovett-Barron et al., 2012). This promotes synaptically-driven pyramidal cell discharge and increases their input-output transformation (Toth et al., 1997; Letzkus et al., 2011; Lovett-Barron et al., 2012; Xu et al., 2013). We report that $A_2\alpha$R-mediated facilitation of IPSCs was present in various postsynaptic CA1 area interneuron types, including O-LM cells specialized to inhibit distal dendrites of pyramidal cells, and basket cells that directly control pyramidal cell action potential firing via perisomatic inhibitory synapses (Zhang and McBain, 1995; Glickfeld and Scanziani, 2006; Klausberger and Somogyi, 2008). Through modulation of the GABAergic circuits $A_2\alpha$Rs can control co-ordinated rhythmic neuronal activities in the hippocampus (Cobb et al., 1995; Klausberger et al., 2005; Wulff et al., 2009). Interestingly, the $A_2\alpha$R-mediated facilitation of GABAergic efferents was specific to PV-expressing interneurons, and was not detected in CCK+ GABAergic interneuron fibers (Armstrong and Soltesz, 2012).

Importantly, we showed that $A_2\alpha$R-mediated facilitation of CA1 pyramidal cell activity also occurs through endogenous adenosine. High-frequency electrical stimulation experiment demonstrated that CA1 area pyramidal cell input–output transformation to Schaffer collateral stimulation is similarly facilitated via endogenous and agonist-induced $A_2\alpha$R activity. Although high-frequency stimulation-evoked $A_2\alpha$R activation failed to significantly change synaptic Schaffer collateral responses in the experiments, this can be explained by higher sensitivity of the network-driven input–output function than a monosynaptic pathway to synaptic modulations (Lovett-Barron et al., 2012).

Our results on spontaneous activity modulation by $A_2\alpha$R antagonist and agonist in hyperexcitable conditions confirm the previously reported findings that $A_2\alpha$R controls spontaneous epileptiform pyramidal cell discharge in the hippocampus (Sebastiao and Ribeiro, 2009). In addition, the results indicate that in slices with elevated extracellular potassium adenosine $A_2\alpha$Rs are tonically active promoting synchronous discharge in the hippocampus. This was evidenced by robust effect with $A_2\alpha$R antagonist suppressing the spontaneous interictal like events in the CA3 area. Variability and occasionally a lack of $A_2\alpha$R agonist effect to promote synchronous discharge in these conditions could also be explained by vigorous tonic $A_2\alpha$R activity in baseline conditions (Dias et al., 2013). Given that ambient adenosine levels elevate in epileptic tissue and $A_2\alpha$R expression increases whereas $A_1$R levels go down, $A_2\alpha$R blockers might provide an effective supplementary treatment in specific forms of epilepsy (Sebastiao and Ribeiro, 2009; Gomes et al., 2011). Adenosine’s therapeutic effect via $A_1$R might benefit from inhibition of $A_2\alpha$Rs. A seizure promoting role of $A_2\alpha$R in humans has recently been highlighted (Shinohara et al., 2013), and adenosine $A_2\alpha$R antagonists have already entered clinical trials and are safe to use with relatively mild side effects (Lopes et al., 2011; Shook and Jackson, 2011; Muller, 2013). Our findings here identify specific synaptic targets for $A_2$AR-modulation. This helps to understand how these receptors are involved in generation of aberrant hippocampal activity and can point out specific therapeutic targets in cortical microcircuits.

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