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Research Report

Presynaptic adenosine A_1 receptors modulate excitatory synaptic transmission in the posterior piriform cortex in rats

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ABSTRACT

The effect of adenosine on the fEPSP was examined in the lateral olfactory tract (Ia input) and associative tract (Ib input) of the rat piriform cortex. The fEPSP evoked in the Ia input showed paired-pulse facilitation, while that in the Ib input showed paired-pulse depression, suggesting a lower resting release probability in the Ia input. This was supported by results showing that MK801 blocked the NMDA receptor-induced fEPSP more rapidly in the Ib input than the Ia input. Adenosine caused dose-dependent inhibition of the fEPSP in both inputs, the sensitivity being higher in the Ib input. This effect was mimicked by the A_1 receptor agonist, CHA, and antagonized by co-application of the A_1 receptor antagonist, DPCPX, showing that adenosine was acting at A_1 receptors. Application of DPCPX alone caused an increase in the fEPSP, the increase being larger in the Ia input. DPCPX also caused paired-pulse depression in both inputs, and the paired-pulse ratio measured in its presence was very similar in both inputs. These results suggest there is a lower endogenous concentration of adenosine in the Ib sublayer than the Ia sublayer, which might account for the native difference in the resting release probability of the two inputs. The adenosine-induced inhibition of the fEPSP in both inputs was associated with a significant reduction in the rate at which MK801 blocked NMDA receptor-mediated fEPSP activity, suggesting a presynaptic location of the A_1 receptors. Blocking of N-, P/Q-type calcium channels occluded the inhibition by adenosine, indicating that they are downstream effectors of presynaptic A_1 receptor activation.

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Abbreviations: aCSF, artificial cerebrospinal fluid; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; AP5, DL-2-amino-5-phosphonopentanoic acid; CHA, N^6-cyclohexyladenosine; CNS, central nervous system; DNQX, 6,7-dinitroquinoxaline-2,3-dione; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; DPMA, N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine; fEPSP, field excitatory postsynaptic potential; GABA, gamma-aminobutyric acid; NMDA, *N*-methyl-D-aspartic acid; PPS, paired-pulse stimulation; VDCC, voltage-dependent calcium channel

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1. Introduction

Adenosine plays an important role as a modulator of synaptic transmission in the central nervous system (CNS) (Ribeiro, 1995; de Mendonca and Ribeiro, 1997). It exerts its neuromodulating effect by acting at type 1 purinergic receptors, namely A_1 and A_2 receptors (Van Calker et al., 1979), in many CNS regions (Fields and Burnstock, 2006). Adenosine enhances excitatory synaptic transmission by activating A_2 receptors (Nishimura et al., 1990; Sebastiao and Ribeiro, 1996). In contrast, it potently inhibits glutamate release from excitatory nerve terminals by activating presynaptic A_1 receptors, thereby strongly depressing excitatory synaptic transmission presynaptically (Prince and Stevens, 1992; Moore et al., 2003). Adenosine also acts on A_1 receptors to depress the release of other neurotransmitters, for example, acetylcholine and γ-amino-butyric acid (GABA) (Harms et al., 1979; Holins and Stone, 1980). The mechanism underlying the depressant effect of A_1 receptor activation on transmitter release has been shown to involve a reduction in calcium influx upon arrival of the action potential at presynaptic terminals (Manita et al., 2004). In agreement with the above physiological and pharmacological observations, autoradiographic experiments using tritiated A_1 ligands have shown a high density of A_1 receptors in many CNS regions, including the cerebellum, hippocampus, cerebral cortex, piriform cortex, caudate putamen, and nucleus accumbens (Goodman and Synder, 1982; Moore et al., 2000; Ribeiro et al., 2002). Because of its potent depressant effect on synaptic transmission by its action on A_1 receptors, adenosine is considered as an endogenous modulator regulating synaptic plasticity (Moore et al., 2003) and as an agent protecting neurons from metabolic insult caused by ischemia (Saransaari and Oja, 2003; Sugino et al., 2001) or hypoxia (Arlinghaus and Lee, 1996; Fowler et al., 2003; Schmidt et al., 1996) and from the development of seizure (Birnstiel et al., 1992; Bruno et al., 2003; Schubert, 1992). It has also been shown to have sedative, anticonvulsant, anxiolytic, and locomotor depressant effects (Jacobson and Gao, 2006).

The piriform cortex makes up the major part of the olfactory cortex, and consists of anterior, lateral, and posterior parts (Nevill and Haberly, 2004). Activity-dependent synaptic plasticity, e.g. long-term potentiation and depression, can be easily induced in the piriform cortex, making it an excellent candidate for a cellular substrate underlying sensory odor-related information storage in the piriform cortex (Hasselmo and Barkai, 1995; Saar et al., 1999). In addition, the piriform cortex is one of the brain regions in which epileptiform activity can be easily induced in animal models of epilepsy (Demir et al., 1999a,b). It is therefore of interest to examine the effect of adenosine on excitatory synaptic transmission in the piriform cortex. The principal neurons of the piriform cortex are pyramidal cells that receive two principal inputs from the olfactory bulb and from other cortical areas, including other parts of the piriform cortex (Nevill and Haberly, 2004). The fibers of these inputs are well organized in the most superficial part of the piriform cortex, layer I, in which the apical dendrites of the pyramidal cells are located and receive contacts from synaptic inputs. The fibers from the olfactory bulb, forming the lateral olfactory tract and carrying odor sensory information, project directly to the piriform cortex without thalamic intermediation and are located in the outer half of layer I; these are referred to as the Ia input in this study. The intrinsic associative fibers from other cortical areas are located in the inner half of layer I (Hasselmo and Bower, 1990; Nevill and Haberly, 2004) and are referred to as the Ib input in this study. In addition to this physical separation, previous studies have established certain criteria for distinguishing physiologically between the Ia and Ib inputs. For instance, paired-pulses delivered to the Ia input result in paired-pulse facilitation of the fEPSP, in which the synaptic response to the second stimulating pulse is larger than that to the first pulse. On the other hand, paired-pulse stimulation (PPS) of the Ib input causes paired-pulse depression of the fEPSP (Bower and Haberly, 1986; Franks and Isaacson, 2005). Moreover, it has been shown that the EPSP(C) evoked by stimulation of the Ib input is sensitive to the $GABA_B$ receptor agonist, baclofen, but that evoked by stimulation of the Ia input is not (Franks and Isaacson, 2005; Tang and Hasselmo, 1994). In the present study, we used these two criteria to confirm that the evoked fEPSP were in the Ia or the Ib input in the posterior piriform cortex and examined the effect of adenosine. Our aims were to examine whether adenosine had any differential effect on the Ia and Ib inputs and determine the underlying mechanism, to identify the receptor subtype involved in the effect of adenosine and its location, and to explore the molecular target affected by adenosine receptor activation.

2. Results

With the exception of the experiments shown in Fig. 5, all recordings were made at room temperature (25 °C). With the recording and stimulating electrodes placed in the outer half of layer I (hereafter referred to as the Ia sublayer) of the posterior piriform cortex (Figs. 1A, B1), the fEPSP evoked by two consecutive stimulation pulses showed paired-pulse facilitation when the inter-pulse interval (IPI) was between 20 and 500 ms (Fig. 1C1 upper trace), the maximal facilitation of 140% being obtained when the IPI was between 50 and 100 ms (Fig. 1C2, black circles, $n=8$ slices). The evoked fEPSP was insensitive to bath application of 10 μM baclofen, a $GABA_B$ receptor agonist, or to 100 μM CGP35348, a $GABA_B$ receptor antagonist, applied after the baclofen ($n=4$ slices) (Fig. 1D1). In contrast, with the recording and stimulating electrodes placed in the inner half of layer I (hereafter referred to as the Ib sublayer) (Figs. 1A, B2), the fEPSP showed paired-pulse depression when the IPI was less than 500 ms (Fig. 1C1 lower trace), with the maximal depression of 46% being obtained when the IPI was between 20 and 75 ms (Fig. 1C2, white circles, $n=6$ slices). Moreover, the fEPSP evoked in this condition was markedly attenuated by bath application of baclofen (56±10% of baseline; $n=4$, $p<0.005$, $t=5$, $df=3$, paired t-test), and this effect was antagonized by subsequent bath application of CGP35348 (84±9% of baseline; $n=4$, $p<0.005$, $t=8$, $df=3$, paired t-test) (Fig. 1D2). These results are consistent with previous reports (Franks and Isaacson, 2005; Tang and Hasselmo, 1994), which used the same criteria to verify the EPSPs activity of the Ia or Ib input, and also show that we were able to precisely evoke fEPSPs activity in the Ia or Ib input.

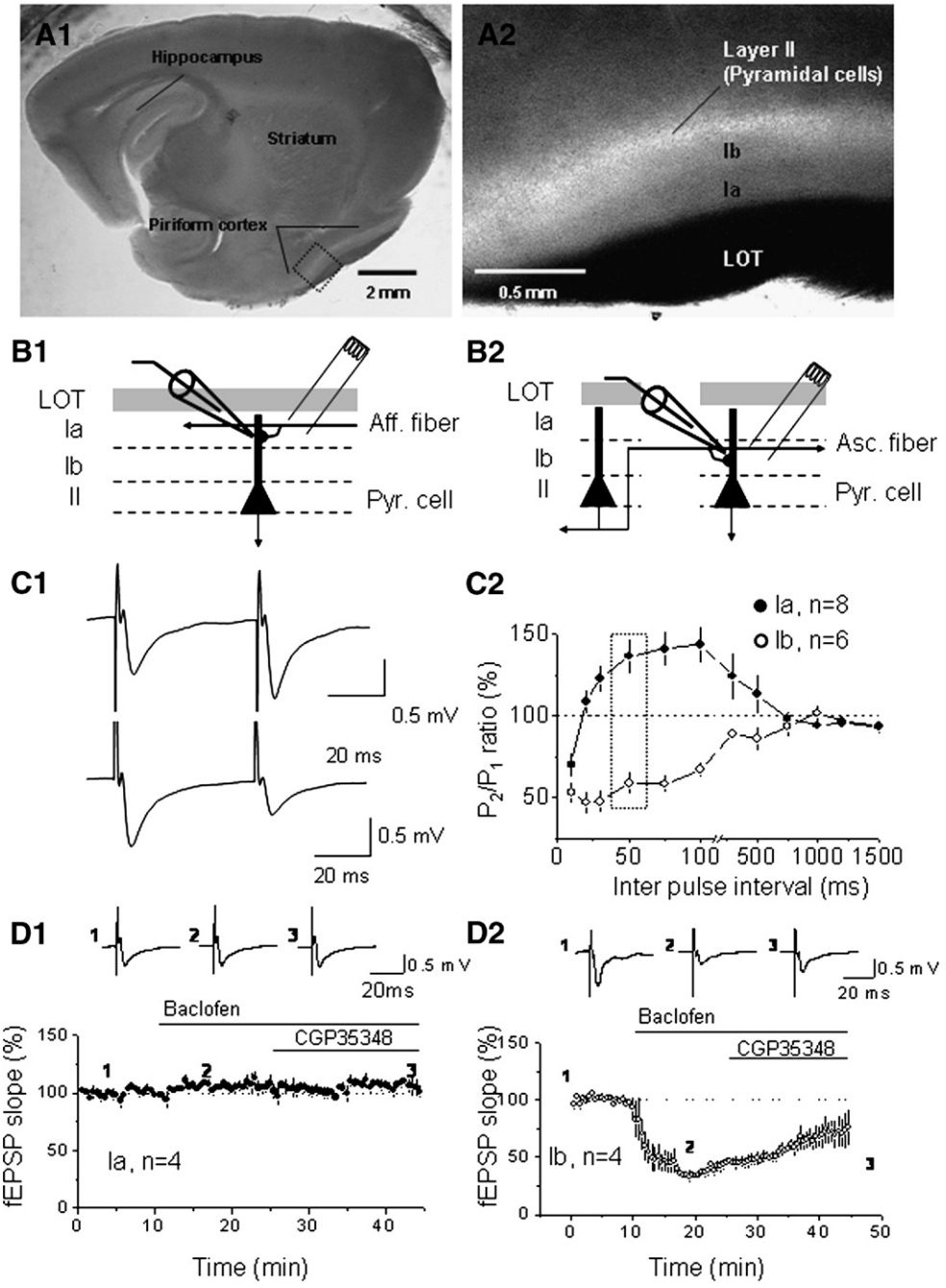

Fig. 1 – Evoking of fEPSP activity in the Ia and Ib inputs in the piriform cortex. A1 shows a photograph of a sagittal slice of rat cerebrum containing the posterior piriform cortex as indicated by the dotted square, shown in higher magnification in A2. Note that the lateral olfactory tract (LOT), Ia, Ib, and superficial pyramidal cell layers can be easily identified because of the darker appearance of the Ia sublayer. B1 and B2 are schematic plots showing the neuronal circuits and electrode arrangement for evoking and recording fEPSPs in, respectively, the Ia (B1) or Ib (B2) input in the piriform cortex. The fEPSP activity evoked in the Ia sublayer showed paired-pulse facilitation (C1, upper trace) and was insensitive to bath application of 50 μM baclofen (D1). The fEPSP activity evoked in the Ib sublayer showed paired-pulse depression (C1, lower trace), and was attenuated by baclofen application, this effect being subsequently antagonized by CGP35348 (D2). Also note that the responses of the Ia and Ib inputs to PPS with different IPIs are also markedly different (C2). The dotted rectangle in C2 indicates the IPI used for Fig. 4.

We then tested the effect of bath application of adenosine on the synaptic activity of the two inputs. In response to 10 μM adenosine, the fEPSPs in the Ia (Fig. 2A) and Ib (Fig. 2B) inputs were markedly attenuated to 65±8% (n=5 slices; p<0.01, t=5.5, df=4, paired t-test) and 59±4% (n=5 slices; p<0.01, t=15, df=4, paired t-test) of the baseline values, respectively, without a significant change in presynaptic volley (see the superimposed traces in Fig. 2). These results suggest that adenosine

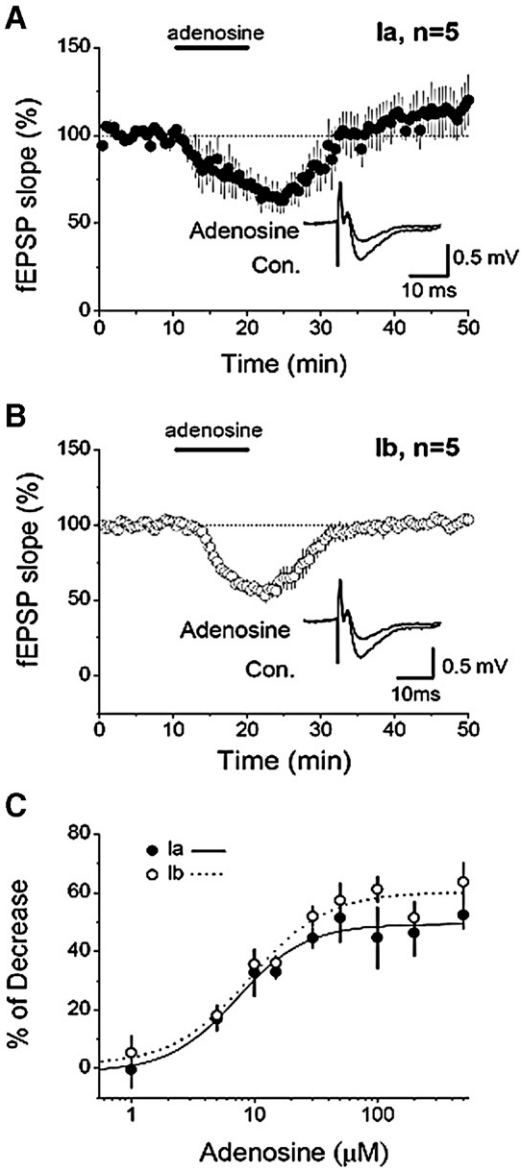

Fig. 2 – Inhibitory effect of adenosine on the fEPSPs evoked in the Ia and Ib sublayers. Bath application of 10 μM adenosine significantly attenuated the fEPSPs in the Ia input (A) and Ib input (B). C shows dose-dependent inhibition by adenosine of the fEPSPs in both inputs.

affects synaptic transmission, but not the excitability of presynaptic neurons. The effect of adenosine on the fEPSPs in both inputs was dose-dependent, the IC_{50} being 7 μM for the Ia input and 9 μM for the Ib input (Fig. 2C). At all concentrations of adenosine tested, no significant difference was noted between the extent by which the fEPSP of the two inputs was attenuated by adenosine, although the maximal effect, obtained from the curve fitting shown in Fig. 2C, was greater for the Ib input (59%) than for the Ia input (49%). The effect of adenosine could be mimicked by 10 μM CHA, an A_1 receptor agonist (Figs. 3A and B; see also Fowler et al., 1999; Heinbockel and Pape, 1999), but not by 100 nM DPMA, an A_2 receptor agonist (Ia: 113 ± 7%; $n = 6$, $p = 0.053$, $t = -2$, $df = 5$, paired t-test; Ib: 110 ± 8%; $n = 6$, $p = 0.3$, $t = -1.2$, $df = 5$, paired t-test) (Figs. 3C and D; see also Diogenes et al., 2004; Wirkner et al., 2004; Zhang and Schmidt, 1998), showing that adenosine was acting at A_1, and not A_2, receptors. Interestingly, CHA caused significantly greater attenuation of the fEPSP in the Ib input (42 ± 7% of baseline; $n = 6$ slices, $p < 0.001$, $t = 9.35$, $df = 5$, paired t-test) than the Ia input (60 ± 7% of baseline; $n = 6$ slices, $p < 0.001$, $t = 7$, $df = 5$, paired t-test; $n = 6$ slices) (comparison of the two inputs: $p < 0.05$, $F = 6$, ANOVA test). This was supported by results showing that 10 μM adenosine had no effect on the fEPSP in either input when the A_1 receptor was blocked by 1 μM DPCPX, an A_1 receptor antagonist (Figs. 3E and F; see also Hallworth et al., 2002; Wong et al., 2006). In addition, DPCPX alone increased the fEPSPs to 184 ± 18% ($n = 6$ slices, $p < 0.001$, $t = -8.5$, $df = 5$, paired t-test) or 152 ± 13% ($n = 5$ slices, $p < 0.001$, $t = -1.4$, $df = 4$, paired t-test) of baseline levels in the Ia or Ib pathway, respectively (Figs. 3E and F), the difference between the two inputs being significant ($p < 0.05$, $F = 5.8$, ANOVA test). These results suggest that endogenous adenosine is being continuously released and depressing the synaptic activity of both inputs. When the effect of DPCPX was included in the dose–response curve for adenosine and the approach of Prince and Stevens (1992) used to estimate the endogenous adenosine concentration, it was found to be 30 μM for the Ia input and 12 μM for the Ib input. The higher endogenous concentration of adenosine in the Ia subfield than the Ib subfield is consistent with the result that the fEPSP in the Ia input was less sensitive to applied adenosine than that in the Ib input.

We next used three different approaches to examine whether the A_1 receptor was located at a presynaptic or postsynaptic site.

We first used PPS with an IPI of 50 ms, a well known and sensitive method for examining this question (Zucker, 1973). An IPI of 50 ms was chosen because it produced a reliable and maximum paired-pulse facilitation or paired-pulse depression in the Ia or Ib input, respectively (see Fig. 1C). Bath application of 10 μM adenosine increased the PPS ratio above control levels in both the Ia input (Fig. 4A, left trace) and Ib input (Fig. 4C, left trace). In contrast, when the A_1 receptor was blocked by DPCPX, a decrease in the PPS ratio was seen in both the Ia input (Fig. 4A, right trace) and Ib input (Fig. 4C, right trace). Fig. 4B shows the summarized results for the Ia input for 4 and 8 slices tested for adenosine and DPCPX, respectively. The PPS ratio recorded before and after adenosine application, or before and after DPCPX application slices was 129 ± 6% vs. 155 ± 13% ($n = 4$ slices, $p < 0.05$, $t = -3.4$; $df = 3$, paired t-test) or 133 ± 8% vs. 75 ± 10% ($n = 8$ slices, $p < 0.01$, $t = 5$, $df = 7$, paired t-test), respectively, showing significant effect of adenosine and DPCPX on PPS ratio in Ia input. Fig. 4D shows the results for 4 and 6 slices tested for, respectively, adenosine and DPCPX for the Ib input. The PPS ratio recorded before and after adenosine application or before and after DPCPX application was 74 ± 5% vs. 113 ± 13% or 80 ± 5% vs. 60 ± 10%, respectively, and, again, the differences compared to the controls were both significant (paired t-test for control vs. adenosine or DPCPX; adenosine: $p < 0.01$, $t = -7.5$, $df = 3$; DPCPX: $p < 0.005$, $t = 3.9$, $df = 5$). Interestingly, when DPCPX was bath applied to block A_1 receptors, no significant difference in the PPS ratio was seen between the two inputs (PPS ratio: Ia = 75 ± 10%, Ib = 60 ± 5%; $p = 0.25$, $F = 1.48$, ANOVA test). Taken together, these results favor a presynaptic location for the adenosine A_1 receptor.

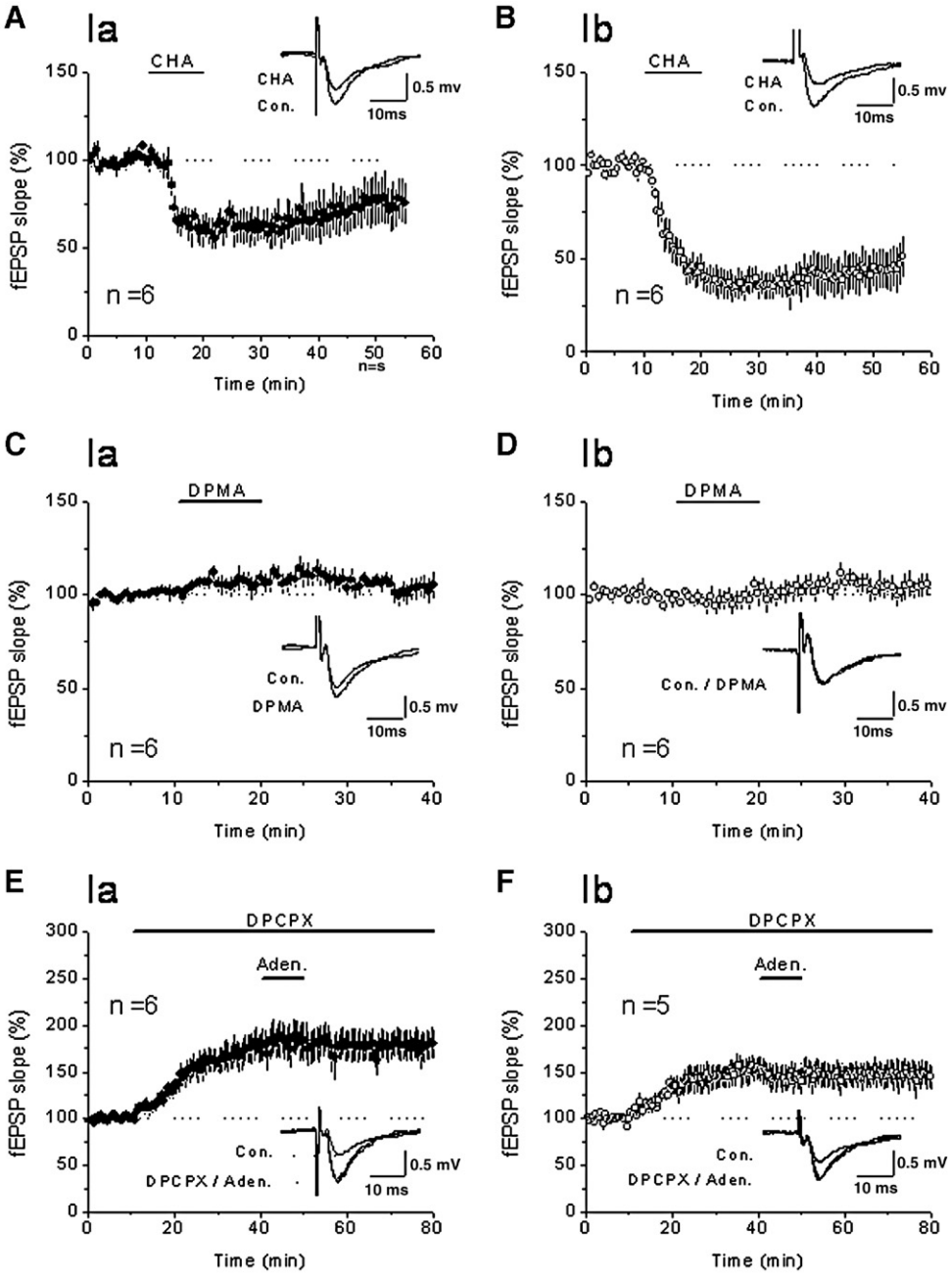

Fig. 3 – The effect of adenosine is due to activation of the purinergic A_1 receptor. Bath application of 10 μM CHA significantly attenuated the fEPSPs in the Ia input (A) and Ib input (B). In contrast, bath application of 100 nM DPMA resulted in no significant change in the fEPSPs in the Ia (C) or Ib (D) input. Application of 1 μM DPCPX significantly increased the fEPSPs in the Ia (E) and Ib (F) input. Note that, during application of DPCPX, application of 10 μM adenosine did not have any further effect on the fEPSP in either input.

In the second approach, if the effect of adenosine were presynaptic, adenosine would be expected to have the same effect on the fEPSPs caused by the two principal ionotropic glutamate receptors, the AMPA receptor (AMPA-R) and the NMDA receptor (NMDA-R). To test this, we compared the extent by which 10 μM adenosine attenuated the AMPA-R and NMDA-R-mediated fEPSPs. The effect of adenosine on the AMPA-R-mediated fEPSP was first determined, then the effect on the NMDA-R mediated fEPSP was measured in the same slice by blocking the AMPA-R fEPSP and switching from normal artificial cerebrospinal fluid (aCSF) to low magnesium aCSF, as described in Experimental procedures (Fig. 5A). In the Ib input, adenosine attenuated the AMPA-R- and NMDA-R-mediated fEPSPs by the same extent (AMPA-R 50 ± 5% of baseline, NMDA-R 51 ± 7% of baseline; $n = 6$ slices, $p = 0.92$, $t = -0.1$, $df = 5$, paired t-test) (Fig. 5B), while, in the Ia input, the degree of attenuation of the AMPA-R-fEPSP was much less than that of the NMDA-R-mediated fEPSP (AMPA-R 51 ± 4% of baseline; NMDA-R 31 ± 7% of baseline; $n = 6$, $p < 0.01$, $t = 4.35$, $df = 5$, paired t-test) (Fig. 5C). We then asked whether this

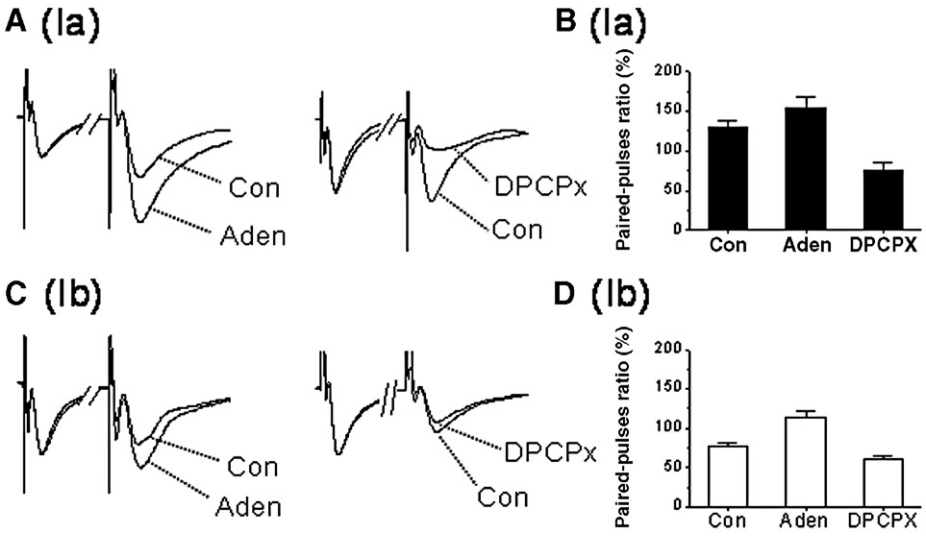

Fig. 4 – Effect of adenosine on the PP ratios of the fEPSPs in the Ia and Ib inputs. Superimposition of the fEPSP responses in the Ia (A) or Ib (C) input in response to PPS before (Con) and after application of 10 μM adenosine (left traces) or DPCPX (right traces), the second response being expressed as a percentage of the first for each condition. Note the responses to the second pulse in the control and adenosine- or DPCPX-treated samples. The summarized results for each treatment are shown in B and D. Controls of adenosine and DPCPX are pooled together.

discrepancy could be attributed to the spilling over of glutamate, which then acted at NMDA-Rs located at neighboring synapses in the Ia sublayer (Asztely et al., 1997; Kullmann and Asztely, 1998). To test this, we repeated the experiments using a recording temperature of 35 °C, and found that, at this physiological temperature, the attenuation in the Ia input by adenosine was the same for both fEPSPs (AMPA-R 64 ± 4% of baseline, NMDA-R 58 ± 4% of baseline; $n = 6$ slices, $p = 0.32$, $t = 1$, $df = 5$) (Fig. 5D). Moreover, on addition of the glutamate transporter blocker, D,L-TBOA (50 μM), the difference between the degree of attenuation of the AMPA-R-fEPSP and NMDA-R-fEPSP was again observed (AMPA-R 70 ± 5% of baseline, NMDA-R 48 ± 7% of baseline; $n = 7$ slices, $p < 0.01$, $t = -3.8$, $df = 6$, paired t-test) (Fig. 5E), further supporting the argument. Taken together, the above results are consistent with a presynaptic role of the A_1 receptor in modulating excitatory synaptic transmission in the piriform cortex.

The third method used to address this question was to examine the rate at which MK801, a blocker of activated NMDA-Rs, blocked the NMDA-R-mediated fEPSP (Rosenmund et al., 1993; Hessler et al., 1993). We first tested whether this method was sensitive enough to detect the difference in release probability between the Ia and Ib inputs. An example of the time-course of an experiment performed at synapses in the Ia sublayer is shown in Fig. 6A. In the control condition, the decay time constant (τ) of the MK801 blocking rate for the Ia input was 18 ± 2 ($n = 9$ slices, white triangles and the fitted line in Fig. 6A2), which was larger than that for the Ib pathway ($\tau = 13 \pm 1$, $n = 11$ slices; black inverted triangles and fitted line in Fig. 6A2) (comparison of the two inputs: $p < 0.05$, $F = 6.2$, ANOVA test). This suggested a lower resting release probability in the Ia input than in the Ib input (Figs. 6A2 and D), which is consistent with the fact that the Ia input showed paired-pulse facilitation and the Ib input paired-pulse depression (Figs. 1B and C). To examine the effect of adenosine on the MK801 blocking rate, 10 μM adenosine was applied. After the fEPSP had stabilized, the stimulating intensity was increased to recruit more NMDA-R-mediated fEPSPs, so that the peak amplitude was similar to that before adenosine application (Fig. 6B), then the MK801 blocking rate was measured as described for the control condition. As shown in Figs. 6C–E, activation of A_1 receptors by adenosine significantly decreased the MK801 blocking rate in both the Ia ($\tau = 36 \pm 7$, $n = 8$ slices, $p < 0.05$, $F = 6.3$, ANOVA test) and Ib ($\tau = 40 \pm 6$, $n = 9$ slices, $p < 0.01$, $F = 13.4$, ANOVA test) inputs. We also used a low concentration (1 μM) of AP5 to partially reduce the NMDA-R-mediated fEPSP by a similar amount to that produced by adenosine and measured the MK801 blocking rate as in the adenosine experiments. In both inputs, no significant difference was noted in the MK801 blocking rate after the postsynaptic blocking of NMDA-R by AP5 (τ for Ia = 17 ± 2, and Ib = 14 ± 1) (Figs. 6C–E). These results all favor a presynaptic effect of adenosine.

We next asked whether the A_1 receptor-mediated attenuation of the fEPSP was caused by modulation of presynaptic calcium influx by comparing the effect of adenosine on the fEPSP in control and low extracellular calcium conditions. The effect of adenosine was first tested in normal aCSF. After complete wash out of adenosine, the normal aCSF was replaced with aCSF containing 0.65 mM calcium and the effect of adenosine again tested. Lowering the extracellular calcium concentration decreased basal synaptic transmission to 60 ± 10% and 52 ± 7% of baseline in the Ia and Ib inputs, respectively. Subsequent application of 10 μM adenosine further reduced fEPSP activity in both inputs. Expressing the data as a percentage of the baseline activity under the same conditions (control or low calcium), adenosine was found to attenuate fEPSP activity to 54 ± 4% ($n = 10$ slices) and 87 ± 6% ($n = 7$ slices) of baseline levels in normal and low Ca^{2+} aCSF, respectively, in the Ia input and to 48 ± 4% ($n = 9$ slices) and 100 ± 7% ($n = 7$ slices) in the Ib input. A

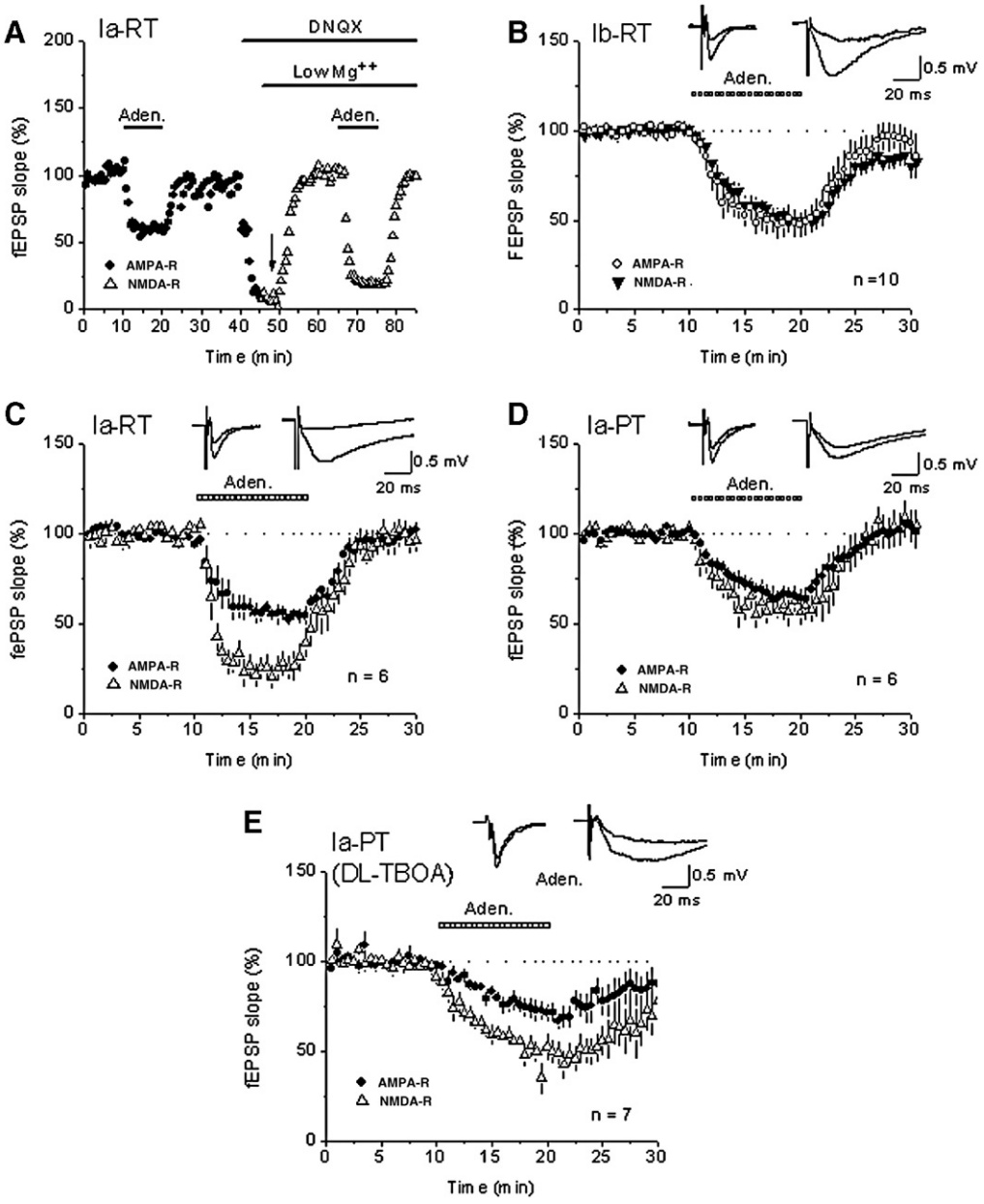

Fig. 5 – Adenosine is equally effective in attenuating the AMPA-R- and NMDA-R-mediated fEPSPs in both the Ia and Ib inputs. A shows the time-course of a single experiment to illustrate how the effects of adenosine on the AMPA-R- and NMDA-R-mediated fEPSPs were measured in the same slice. The AMPA-R-mediated fEPSP is shown as black circles and the NMDA-R-mediated fEPSP as white triangles. B shows the summarized results from 10 slices tested for Ib input at room temperature (RT). Note that adenosine was equally effective at attenuating AMPA-R and NMDA-R activity. C shows the results for the Ia input at room temperature. Note adenosine had less attenuating activity on AMPA-R activity than NMDA-R activity. D shows that, at physiological temperature (PT), adenosine caused equal attenuation of AMPA-R and NMDA-R activity. E shows that, at physiological temperature and with glutamate uptake blocked by D,L-TBOA, adenosine again attenuated AMPA-R activity less than NMDA-R activity in the Ia input.

significant difference between the results in normal and low Ca^{2+} conditions was found in both inputs (ANOVA test, $p < 0.001$; Ia: $F = 26$, Ib: $F = 73$).

We also tested the effect of directly blocking voltage-dependent calcium channels (VDCCs). Of the VDCCs, the P/Q- and N-types are known to be located at the presynaptic terminal and to be involved in the control of transmitter release. Bath application of ϖ-conotoxin GVIA, a peptide neurotoxin selective for the N-type VDCC, or of ϖ-conotoxin MVIIC, a peptide neurotoxin acting on the P/Q- and N-type VDCCs, dramatically attenuated the fEPSP in both the Ia and Ib inputs (Figs. 7A, B), with ϖ-conotoxin MVIIC being much more effective. The fEPSP was attenuated by ϖ-conotoxin GVIA and ϖ-conotoxin MVIIC to, respectively, 35 ± 7% ($n = 4$ slices; $p < 0.001$, $t = 12.6$, $df = 3$, paired t-test) and 12 ± 3% ($n = 3$ slices; $p < 0.001$, $t = 19$, $df = 2$, paired t-test) of baseline in the Ia input and to 47 ± 7% ($n = 7$ slices; $p < 0.001$,

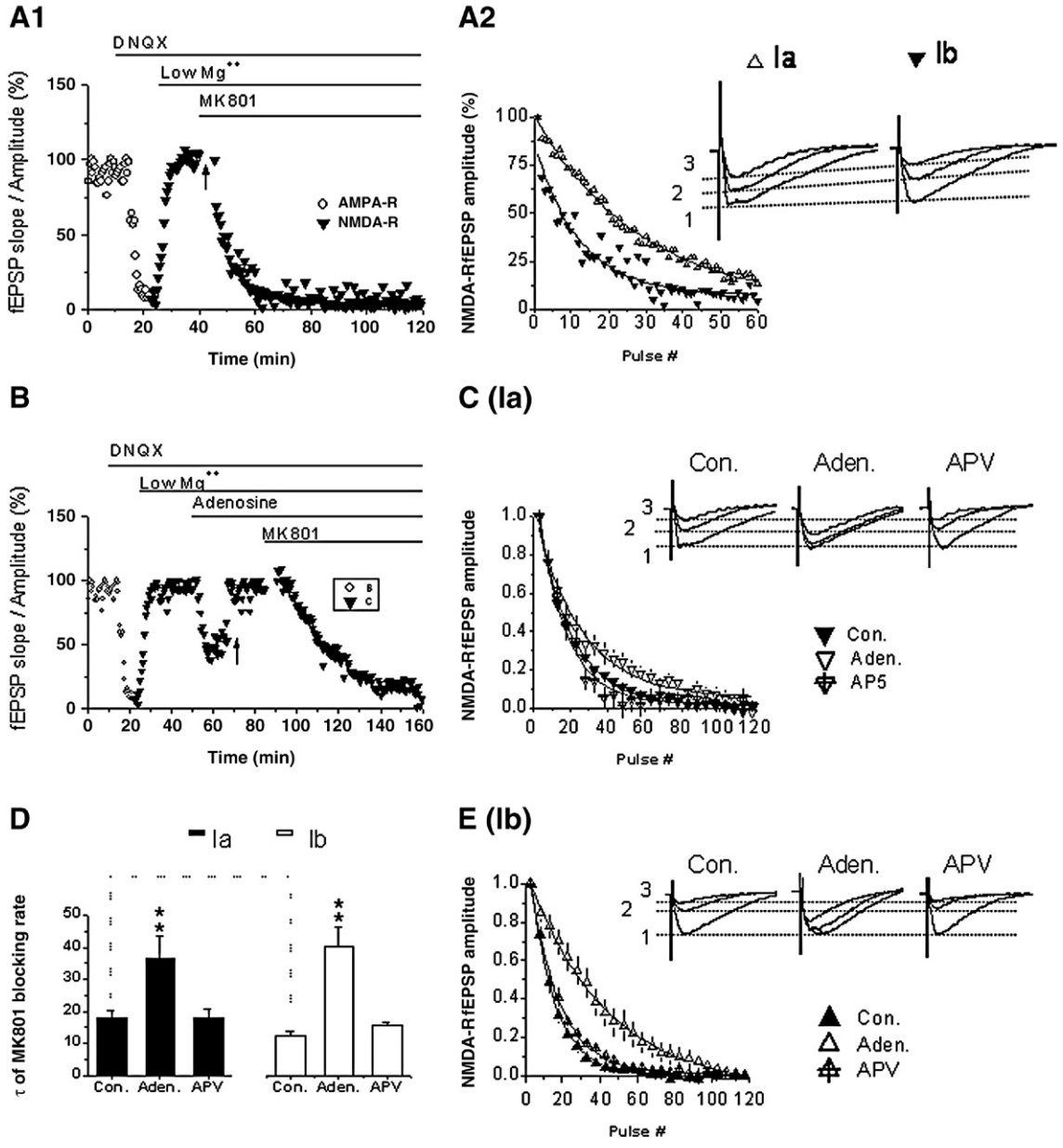

Fig. 6 – Adenosine reduces the MK801 blocking rate of NMDA-R-mediated fEPSP activity in both the Ia and Ib inputs. A1 shows the time-course of a single experiment on the Ib input to illustrate how the time-constant of the MK801 blocking rate of the NMDA-R-mediated fEPSP was measured in control conditions. A2 shows data from two experiments, one testing the Ia input (white triangles) and the other the Ib input (inverted black triangles; data from the experiment shown in A1). Note that the time constant was larger in the Ia input than in the Ib input. The insets show the averaged response for pulses #1 to #5, #6 to #10, and #11 to #15, as indicated by the Arabic numbers. Again, note the more rapid decay of the NMDA-R-mediated fEPSP in the Ib input than in the Ia input. B shows the time-course of a single experiment on the Ia input to illustrate how the effect of adenosine on the MK801 blocking rate was measured. Note that, before MK801 application, 10 μM adenosine was applied for 30 min and the stimulation intensity increased (see arrow), so that the fEPSP activity was comparable to that before adenosine application. C and E show the summarized results for the NMDA-R-mediated fEPSP plotted against pulse number in controls (black triangles) or in the presence of adenosine (white triangles) or AP5 (crossed triangles) for the Ia (C) or Ib (E) input. The insets show the averaged response for pulses #1 to #5, #6 to #10, and #11 to #15 for each condition. Note that adenosine, but not AP5, significantly decreased the MK801 blocking rate of the NMDA-R-mediated fEPSP. D shows chart plots summarizing the τ values extracted under different experimental conditions in the Ia (black columns) or Ib (white columns) input.

$t = 7.2$, $df = 6$, paired t-test) and 20 ± 4% ($n = 3$ slices; $p < 0.005$, $t = 18$, $df = 2$, paired t-test) of baseline in the Ib input. A significant difference was found between the effect of the two toxins in both inputs (ANOVA test: Ia: $p < 0.05$, $F = 8$; Ib: $p < 0.05$, $F = 6.2$). The difference between the effects of the two toxins yielded the pure effect of the P/Q-type VDCC, which was less effective than the N-

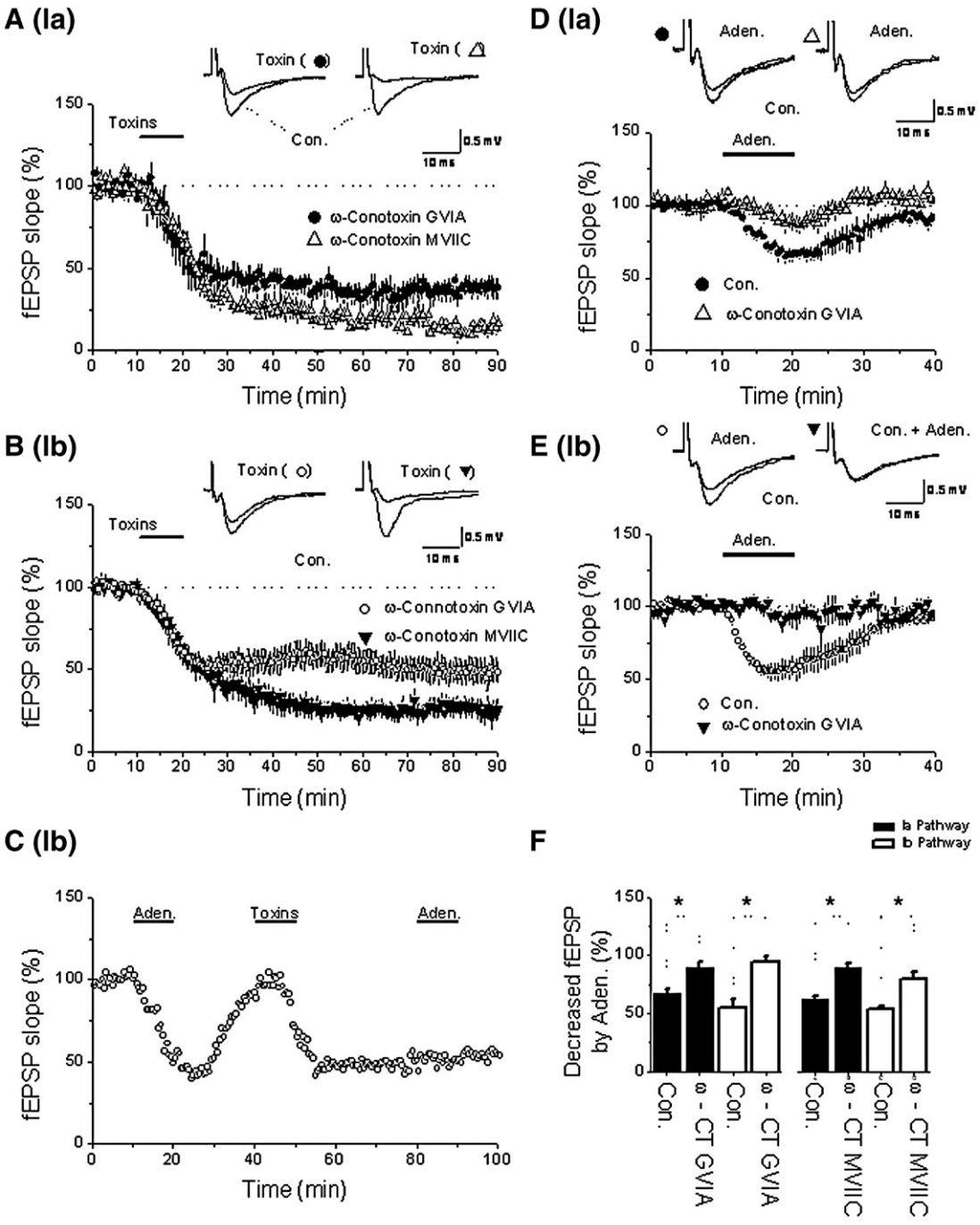

Fig. 7 – Blocking of VDCCs occludes the inhibitory effect of adenosine on fEPSP activity in both the Ia and Ib inputs. A and B show the inhibitory effect of 1 μM ϖ-conotoxin GVIA (black circles) or 1 μM ϖ-conotoxin MVIIC (white triangles) on the fEPSP in the Ia (A) or Ib (B) input. C shows the time-course of a single experiment (Ib input) to illustrate how the effect of the toxins on the adenosine-induced attenuation of the fEPSP was measured in a slice. The effect of 10 μM adenosine was first measured, then the adenosine was completely washed out, the toxin applied, and the effect of adenosine again tested. D and E show summarized results for the effect of ϖ-conotoxin GVIA for the Ia (D) or Ib (E) input. F shows chart plots of the summarized results for the different experimental conditions.

type channel in both inputs. These results showed that N- and P/Q-type VDCCs were involved in the control of transmitter release in both the Ia and Ib inputs. Reducing calcium influx by blocking VDCCs with the peptide toxins also reduced the A_1 receptor-mediated attenuation of the fEPSP in both inputs (Figs. 7C–F). In the Ia input, the fEPSP was attenuated to 67 ± 4% of baseline by adenosine in normal aCSF, but only to 89 ± 5% of baseline in ϖ-conotoxin GVIA-containing aCSF (Fig. 7D; $p < 0.01$, $n = 7$ slices,

$p < 0.01$, $t = -4.7$, $df = 6$, paired t-test) and to 59 ± 6% of baseline in normal CSF and 89 ± 4% of baseline in ϖ-conotoxin MVIIC-containing aCSF (Fig. 7F; $n = 7$ slices, $p < 0.01$, $t = -4.8$; $df = 6$, paired t-test). The corresponding values for the Ib input in control aCSF and ϖ-conotoxin GVIA-containing aCSF were 55 ± 7% and 94 ± 5% (Fig. 7E; $n = 7$ slices, $p < 0.01$, $t = -4.2$, $df = 6$, paired t-test) and 53 ± 3% and 80 ± 5% for control aCSF and ϖ-conotoxin MVIIC toxin-containing aCSF (Fig. 7F; $n = 7$ slices, $p < 0.01$, $t = -3.6$, $df = 6$, paired t-test). These results are consistent with the VDCCs being downstream effectors of A_1 receptor activation.

3. Discussion

In the present study, we demonstrated that the excitatory synaptic inputs from the lateral olfactory tract (Ia input) and from the intrinsic fibers of other parts of the piriform cortex (Ib input) onto pyramidal neurons in the posterior piriform cortex were subject to presynaptic inhibition by adenosine acting at A_1 receptors, the Ib input being more sensitive than the Ia input. This difference in sensitivity to adenosine might be due to the difference in the concentration of endogenous adenosine in the Ia and Ib sublayers, which might account for the difference in the resting release probability seen in the two inputs. The reduction in the release probability caused by adenosine was shown to be due to activation of A_1 receptors, which blocked N- and P/Q-types VDCCs and reduced calcium influx into presynaptic terminals.

Moore et al. (2003) have suggested several possibilities that might explain why adenosine modulates the function of hippocampal mossy fiber synapses on CA3 pyramidal neurons more than that of other synapses: (1) a different class of adenosine receptor, (2) a higher density of A_1 receptors, (3) a higher affinity of A_1 receptors, (4) better coupling of A_1 receptors to VDCCs, or (5) a higher endogenous adenosine concentration. The differential sensitivity to adenosine of the Ia and Ib inputs might also be due to the above mechanisms. A different class of adenosine receptor is unlikely to be the explanation in the present study, as we did not find any significant role for A_2 receptors in modulating fEPSP in the Ia or Ib input. Our results cannot determine whether there are different affinity A_1 receptors, a different density of A_1 receptors, or different coupling of A_1 receptors to VDCCs at Ia and Ib inputs. However, our results do support the possibility that different endogenous adenosine concentrations might account for the different adenosine sensitivities of the two excitatory inputs onto pyramidal neurons in the posterior piriform cortex. Using a similar approach to that of Prince and Stevens (1992), we estimated the endogenous adenosine concentrations in the Ia and Ib sublayers to be about 30 μM and 12 μM, respectively. Although the difference in the effect of bath-applied adenosine on the fEPSP in the Ia and Ib inputs was not significant and the IC_{50} for adenosine inhibition was similar in the Ia and Ib inputs, the effects of DPCPX (an A_1 receptor antagonist) and CHA (an A_1 receptor agonist) on the fEPSP activity were significantly different in the Ia and Ib inputs. This discrepancy may be attributed to the fact that a large number of A_1 receptors are under tonic activation by endogenous adenosine in both pathways, so additional bath-applied adenosine does not result in a significant difference between the two inputs, whereas higher affinity antagonists or agonists (DPCPX or CHA, respectively) have a greater effect.

The most prominent feature of the ultrastructural geometry of synapses between the Ia input and pyramidal neurons at the electron microscopic level is that they are wrapped by glial cell processes, a feature not seen at synapses between Ib fibers and pyramidal cells (Nevill and Haberly, 2004; Tseng and Haberly, 1989). Consistent with this, we have found that the density of glial fibrillary acidic protein-immunoreactive cells and their processes is significantly higher in the Ia sublayer than the Ib sublayer (Yang et al., unpublished observation). This prominent wrapping of glial processes around neuronal dendrites, fibers, and synapses could lead to a complicated geometry of the local environment, allowing easier accumulation of transmitter within the extracellular space in the local Ia sublayer, resulting in the activation of extrasynaptic receptors (Kullmann et al., 1999). Supporting evidence for the possible existence of a difference in geometry at the ultrastructural level in the Ia and Ib sublayers comes from the results showing a temperature- or glutamate uptake-dependent difference in the amount of adenosine-mediated attenuation of the AMPA-R-mediated and NMDA-R-mediated fEPSPs in the Ia input. In the Ia sublayer, the complicated geometry of the neuronal processes retards the diffusion of glutamate and allows it to accumulate in the local extracellular space, which allows activation of NMDA-Rs located at neighboring, non-activated synapses (see also Asztely et al., 1997; Kullmann and Asztely, 1998). Thus, the NMDA-R signal/AMPA-R signal ratio for every presynaptic stimulation is higher in the Ia input than in the Ib input, which may account for the observation that, in the Ia input, adenosine caused greater attenuation of the NMDA-R-mediated fEPSP than the AMPA-R-mediated fEPSP. Increasing the recording temperature from room temperature to 35 °C allows operation of the glutamate transporter, which is reported not to function at room temperature (25 °C), our normal recording temperature, and prevents the activation of NMDA-R at non-activated neighboring synapse by glutamate spill-over from activated synapses (Asztely et al., 1997; Kullmann and Asztely, 1998) and returns the NMDA-R/AMPA-R signal ratio to a normal value, as obtained in the Ib input. The NMDA-R-mediated fEPSP therefore becomes as sensitive as the AMPA-R-mediated fEPSP to adenosine.

Glial cells, e.g. astrocytes, are involved in purine metabolism (Fields and Burnstock, 2006), so adenosine may be continuously released in a tonic or phasic fashion from glial elements wrapped around Ia and Ib inputs and act as a volume transmitter between the Ia and Ib synaptic sites. Because the density of glial processes is significantly higher in the Ia sublayer than the Ib sublayer (Yang et al., unpublished observation), the estimated difference in adenosine concentration between the Ia and Ib sublayers could be due to differences in tonic vs. phasic release or on the distance to the adenosine release site. Moreover, as released adenosine might accumulate in the local extracellular space more easily in the Ia sublayer than in the Ib sublayer, as discussed above, these facts provide a possible explanation for the higher endogenous concentration of adenosine in the Ia sublayer.

The inhibitory effect of adenosine on the fEPSP evoked in the piriform cortex was mimicked by the A_1 receptor agonist,

CHA (Grover and Yan, 1999; Hallworth et al., 2002), and was antagonized by the A_1 receptor antagonist, DPCPX (Alexander et al., 2004; Wong et al., 2006), but was not affected by the A_2 receptor agonist, DPMA (Diogenes et al., 2004; Wirkner et al., 2004). These results show that adenosine acts at A_1, but not A_2, receptors. To verify the location of the A_1 receptor, the effects of adenosine on the PPS ratio (Zucker, 1973), the AMPA-R-mediated fEPSP/NMDA-R-mediated fEPSP ratio, and the MK801 blocking rate of the NMDA R-mediated fEPSP (Hessler et al., 1993; Min et al., 1998a,b; Rosenmund et al., 1993) were tested. The third approach used, the rate of blocking by MK801, may have potential weaknesses; for example, the requirement for low Mg^{2+} aCSF to activate the NMDA receptor-mediated field potential, as this alters the ratio of divalent cations, which might also alter membrane excitability. However, the other two approaches do not have such problems and the results of all three approaches suggested that the A_1 receptor is predominantly located on the presynaptic terminals of both the Ia and Ib pathways. The A_1 receptor is a G_i protein-coupled receptor and belongs to the family of purinergic receptors for adenosine (Burnstock et al., 1978; Fields and Burnstock, 2006). Activation of A_1 receptors activates G_i protein, which, in turn, decreases the concentration of cyclic AMP in the presynaptic cytoplasm, which reduces the release probability and inhibits synaptic transmission presynaptically (Zhong et al., 2004; Zhong and Zucker, 2005). The reduction in the release probability caused by activation of presynaptic A_1 receptors could be achieved by reducing calcium influx through blockage of VDCCs (Zhong et al., 2004; Zhong and Zucker, 2005) or by inhibition of enzymes involved in exocytosis (Chavez-Noriega and Stevens, 1994; Chen and Regehr, 1997). The present results showed that the effect of adenosine was occluded by lowering the extracellular calcium concentration or by blockade of P/Q- and/or N-type VDCCs, suggesting that these presynaptic VDCCs are downstream effectors of A_1 receptor activation. The modified function of VDCCs accounts for the inhibitory effect of A_1 receptors on synaptic transmission in the piriform cortex. In addition, our study is the first to report that both P/Q- and N-type calcium channels are involved in excitatory synaptic transmission in both sensory afferent and associative inputs in the piriform cortex.

Consistent with previous results (Franks and Isaacson, 2005; Tang and Hasselmo, 1994), we found that, while the fEPSP of the Ib input was very sensitive to bath-applied baclofen, this was not the case for the Ia input. These results suggest that, in the piriform cortex, presynaptic $GABA_B$ receptors are present on the terminals of associative fibers, but not on sensory afferent fibers. Another physiological feature distinguishing these two inputs is that the Ia input showed PPS facilitation, whereas the Ib input showed PPS depression (see also Bower and Haberly, 1986; Franks and Isaacson, 2005). This difference might stem from the fact that the Ia input has a lower resting release probability than the Ib input. This was supported by our results showing that the MK801 blocking rate of the NMDA-R-mediated fEPSP was significantly lower in the Ia input than in the Ib input (see also Min et al., 1998a; Hessler et al., 1993; Rosenmund et al., 1993, for other synapses). The difference in release probability might simply reflect the nature of the two inputs or it might be due to the difference in the endogenous concentration of adenosine in the Ia and Ib sublayers. Since the present data showed that, in both inputs, no significant difference in the PPS ratio was detected when A_1 receptors were either completely blocked by DPCPX or saturated by adenosine, it is very likely that the difference in concentration could, at least in part, account for the difference in the release probability of the two inputs. Interestingly, extracellular adenosine in the stratum lucidam of the hippocampal CA3 area causes tonic activation of presynaptic A_1 receptors, which results in a low release probability of mossy fiber synapses (Moore et al., 2003) and enzymatic removal of extracellular adenosine or pharmacological blocking of A_1 receptor function results in a dramatic increase in synaptic transmission at mossy fiber synapses and largely occludes frequency-dependent facilitation, a form of short-term synaptic plasticity that is one of the unique features of mossy fiber synapses and is mainly dependent on the resting release probability.

In conclusion, adenosine is widely distributed in almost every type of tissue, as it is a product of purine metabolism. In the CNS, it also functions as a neuromodulator and is produced in a number of purine metabolic pathways and subsequently released into the extracellular space from neurons and surrounding glia cells. In the piriform cortex, it has a dramatic inhibitory effect on synaptic transmission, as reported in many other CNS synapses, via activation of presynaptic A_1 receptors. Together with the differences in geometry and other properties, including the different distribution of enzymes involved in synthesis and turnover of adenosine, adenosine differentially regulates synaptic transmission in two principal inputs, i.e. the sensory afferent and intrinsic association fibers, on to pyramidal neurons in the piriform cortex.

4. Experimental procedures

The use of animals in this study was in accordance with the guidelines of the Ethical Committee for Animal Research of the National Taiwan University. Male Sprague-Dawley rats, aged 25–35 days, were decapitated and the brains rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing (mM): NaCl 119, KCl 2.5, $NaHCO_3$ 26.2, NaH_2PO_4 1, $MgSO_4$ 1.3, $CaCl_2$ 2.5, glucose 11, pH adjusted to 7.4 by gassing with 5% CO_2/95% O_2. Sagittal forebrain slices (450 μm thick) were cut using a vibrating tissue slicer (Campden Instruments, Loughborough, UK) and slices containing the piriform cortex area (Fig. 1A) collected and placed in an interface-type holding chamber at room temperature (25 °C). For extracellular field potential recording, slices were transferred to an immersion-type recording chamber and perfused at a rate of 2 ml/min with aCSF containing 0.1 mM picrotoxin at 25 °C, with the exception of some experiments in Fig. 5, in which the recording temperature was raised to 35 °C. To record the fEPSP activity of the afferent sensory input (the Ia input), a glass pipette filled with 3 M NaCl and a bipolar stainless steel electrode (FHC, Bowdoinham, ME 04008, USA) were positioned in the outer half of layer I (Figs. 1A2, B1), while, to record the fEPSP in the intrinsic fibers (the Ib input) connecting different parts of the piriform cortex, the two electrodes were placed in the inner half of layer I (Figs. 1A2 and C1). All recordings were made in the posterior piriform cortex

(Fig. 1A1). The quality of the recording of field potential activity was only accepted when the amplitude of the fEPSP was at least twice that of presynaptic volley. To measure the NMDA-R-mediated fEPSP, 10 μM DNQX was applied to abolish the AMPA-R-mediated fEPSP and the NMDA-R-mediated fEPSP revealed by replacing the normal aCSF with low Mg^{2+} aCSF (0.2 mM Mg^{2+}). All signals were filtered at 2 kHz using a low pass Bessel filter provided by the amplifier (Axopatch-1D, Axon Instruments, Foster City, CA 94404, USA), and digitized at 5 kHz using a CED micro 1401 interface running Signal software (Cambridge Electronic Design, Cambridge, UK).

A stable period of at least 10 min of baseline fEPSP activity was recorded before drugs were bath-applied in aCSF. The initial slope of the fEPSP was measured for data analysis. Synaptic responses were normalized to the average values measured during the baseline period. The average slope of the fEPSPs recorded during drug application was used for statistical comparison. In some experiments, PPS with an IPI of 50 ms was delivered to evoke synaptic activity and the PPS ratio calculated as:

$$\text{Slope - II} \times 100\%/\text{Slope - I}, \quad (1)$$

where Slope I and Slope II are the slopes of the synaptic activity in response to the first and second stimulating pulses, respectively. To estimate the endogenous adenosine concentration, the effect of DPCPX was included in the dose–response curve for adenosine, and the equation:

$$f(c) = 1/[1 + (c_0 + c)/K] \quad (2)$$

of Prince and Stevens (1992) was used to fit the data. In the above equation, $f(c)$ is the decrease in the synaptic response caused by a given adenosine concentration, c, in the bath medium expressed as a percentage of the baseline level and normalized to the maximum value (the increase in the synaptic response caused by DPCPX); c_0 is the endogenous adenosine concentration; and K is the dissociation constant for adenosine and the A_1 receptor. The data were fitted by the least square method using Origin 7.5 software (OriginLab, Northampton, MA 01060, USA).

To measure the MK801 blocking rate of NMDA-R-mediated fEPSP activity in both the Ia and Ib inputs, the NMDA-R-mediated fEPSP was first isolated as described above. After a stable baseline period, 50 μM MK801 was added and stimulation interrupted for 5 min to allow full penetration of MK801 into the slices. Stimulation was then resumed and the decay in the NMDA-R-mediated fEPSP recorded (Fig. 6A1). The amplitude of the NMDA-R-mediated fEPSP was measured and plotted against the pulse number (Fig. 6A2). The first response after resumption of stimulation was numbered #1 and subsequent responses expressed as a percentage of this value. A single exponential decay was fitted to the data (see continuous lines in Fig. 6A2) using the least square method and Origin 7.5 software (OriginLab, Northampton, MA 01060, USA), and the time constant was extracted for statistical comparison. To evaluate the effect of adenosine on the MK801 blocking rate, the experimental procedure described above was used, but, before MK801 application, 10 μM adenosine was applied for 30 min and the stimulation intensity increased (see arrow), so that the fEPSP activity was comparable to that before adenosine application (Fig. 6B). In some experiments, adenosine was replaced by 1 μM AP5 as a control.

All data are presented as the mean ± standard error of the mean and were compared statistically using either the paired t-test or one-way ANOVA test. The criterion for significance was $p<0.05$. All chemicals used for aCSF preparation were purchased from Merck (Frankfurt, German). Adenosine, baclofen, CHA, DPMA, MK801, and picrotoxin were from Sigma (St. Louis, MO 63178, USA), while DPCPX, AP5, DNQX, CGP 35348, ϖ-conotoxin GVIA/MVIIC, and D,L-TBOA were from Tocris–Cookson (Bristol, UK).

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