Adenosine A$_{2A}$ Receptor Modulation of Hippocampal CA3-CA1 Synapse Plasticity During Associative Learning in Behaving Mice

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Previous *in vitro* studies have characterized the electrophysiological and molecular signaling pathways of adenosine tonic modulation on long-lasting synaptic plasticity events, particularly for hippocampal long-term potentiation (LTP). However, it remains to be elucidated whether the long-term changes produced by endogenous adenosine in the efficiency of synapses are related to those required for learning and memory formation. Our goal was to understand how endogenous activation of adenosine excitatory A$_{2A}$ receptors modulates the associative learning evolution in conscious behaving mice. We have studied here the effects of the application of a highly selective A$_{2A}$ receptor antagonist, SCH58261, upon a well-known associative learning paradigm—classical eyeblink conditioning. We used a trace paradigm, with a tone as the conditioned stimulus (CS) and an electric shock presented to the supraorbital nerve as the unconditioned stimulus (US). A single electrical pulse was presented to the Schaffer collateral–commissural pathway to evoke field EPSPs (fEPSPs) in the pyramidal CA1 area during the CS–US interval. In vehicle-injected animals, there was a progressive increase in the percentage of conditioning responses (CRs) and in the slope of fEPSPs through conditioning sessions, an effect that was completely prevented (and lost) in SCH58261 (0.5 mg/kg, i.p.) -injected animals. Moreover, experimentally evoked LTP was impaired in SCH58261-injected mice. In conclusion, the endogenous activation of adenosine A$_{2A}$ receptors plays a pivotal effect on the associative learning process and its relevant hippocampal circuits, including activity-dependent changes at the CA3-CA1 synapse.
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INTRODUCTION

Learning is the mechanism by which the nervous system adapts its activities to environmental changes and constraints for the generation of appropriate behaviors. In spite of intense *in vitro* work that has helped to elucidate many electrophysiological and molecular mechanisms supporting the activity-dependent synaptic changes taking place at different cerebral sites (Bliss and Collingridge, 1993; Engert and Bonhoeffer, 1999; Kandel, 2001), it should be stressed that neuronal processes underlying learning must be studied under the best possible physiological conditions —namely, in alert-behaving animals (Delgado-Garcia and Gruart, 2006; Neves *et al*, 2008). In this regard, the eyelid motor system is an excellent model for the study of

associative learning, particularly using classical conditioning techniques (Woody, 1986; Thompson, 1988). A brain area critically involved in the acquisition of this type of associative learning is the hippocampus (Delgado-Garcia and Gruart, 2006; Gruart *et al*, 2006; Takatsuki *et al*, 2003; Tseng *et al*, 2004). The fact that mice can be trained with conditioning trace paradigms, and that the activity-dependent changes in hippocampal CA3-CA1 synapses are specifically involved in the acquisition and extinction of CRs (Gruart *et al*, 2006; Takatsuki *et al*, 2003) makes feasible the study of neural processes affecting cognitive functions in genetically (Dominguez-del-Toro *et al*, 2004; Gruart *et al*, 2007; Sahún *et al*, 2007) and/or pharmacologically (Gruart *et al*, 2006; Kopf *et al*, 1999) manipulated mice.

Adenosine is a prototypic neuromodulator present in the nervous system which tunes on-going synaptic transmission (Sebastião and Ribeiro, 2000) through the activation of high-affinity G-protein inhibitory (A$_1$) and excitatory (A$_{2A}$)—coupled receptors. It is known that endogenous extracellular adenosine (or its precursor, ATP) affects *in*

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vitro models of hippocampal LTP (de Mendonça and Ribeiro, 1994; de Mendonça and Ribeiro, 1997; Rex *et al*, 2005; Wang *et al*, 2004). It also modifies the animal's performance in learning and memory tests (Ohno and Watanabe, 1996; Suzuki *et al*, 1993; von Lubitz *et al*, 1993), particularly for conditioned eyeblinks (Winsky and Harvey, 1986), where there is impairment in the acquisition of CRs, due to the activation of adenosine A$_1$ receptors. The antagonism of A$_1$ receptors is widely proposed for the treatment of various memory disorders (Burnstock, 2007; Kopf *et al*, 1999), but little is known about the influence of A$_{2A}$ receptors upon learning processes—particularly those involving the participation of hippocampal circuits.

We studied here the changes in synaptic strength at the CA3-CA1 synapse during classical eyeblink conditioning. Animals were presented with a tone as CS and an electric shock to the supraorbital nerve as US. Eyelid CRs were determined from the electromyographic (EMG) activity of the orbicularis oculi muscle ipsilateral to the US presentation. Animals were implanted with chronic stimulating electrodes on Schaffer collaterals and with a recording electrode in the CA1 area. Field EPSPs evoked at the CA3-CA1 synapse during the CS–US interval was recorded across conditioning sessions and during experimentally evoked LTP. A selective A$_{2A}$ receptor antagonist (SCH58261) was injected in two different experimental situations to determine the role of A$_{2A}$ receptors in associative learning and CA3-CA1 changes in synaptic strength during both classical conditioning and LTP. Results indicated that the adenosine A$_{2A}$ receptor plays a crucial role in associative learning and the underlying CA3-CA1 synaptic plasticity.

MATERIALS AND METHODS

Animals

Experiments were carried out with C57BL/6 male adult mice (3–5 months old; 25–30 g) obtained from a commercial supplier (Iffa-Credo, Barcelona, Spain). Upon arrival, animals were housed in groups ($n = 10$ per cage), but were switched to individual cages after surgery. Mice were kept on a 12 h light–dark cycle with constant ambient temperature ($22 \pm 1^{\circ}$C) and humidity ($55 \pm 8\%$). Food and water were available *ad libitum*. Experimental procedures were performed in accordance with the Declaration of Helsinki, European Union guidelines (2003/65/CE), and recent Spanish regulations (BOE 252/34367-91, 2005) for the use of laboratory animals in chronic recordings. The local Ethical Committee for animal care and handling also approved all experimental protocols.

Surgery

Animals were deeply anesthetized with 4% chloral hydrate (0.1 ml/10 g, i.p.; Sigma-Aldrich, Madrid, Spain) and implanted with bipolar recording electrodes in the left orbicularis oculi muscle and with bipolar-stimulating electrodes on the ipsilateral supraorbitary branch of the trigeminal nerve, near its entrance into the orbit (Figure 1). Recording and stimulating electrodes were made of 50 µm, teflon-coated, annealed stainless-steel wire (A-M Systems, Carlsborg, WA). Electrode tips were bared of their isolating
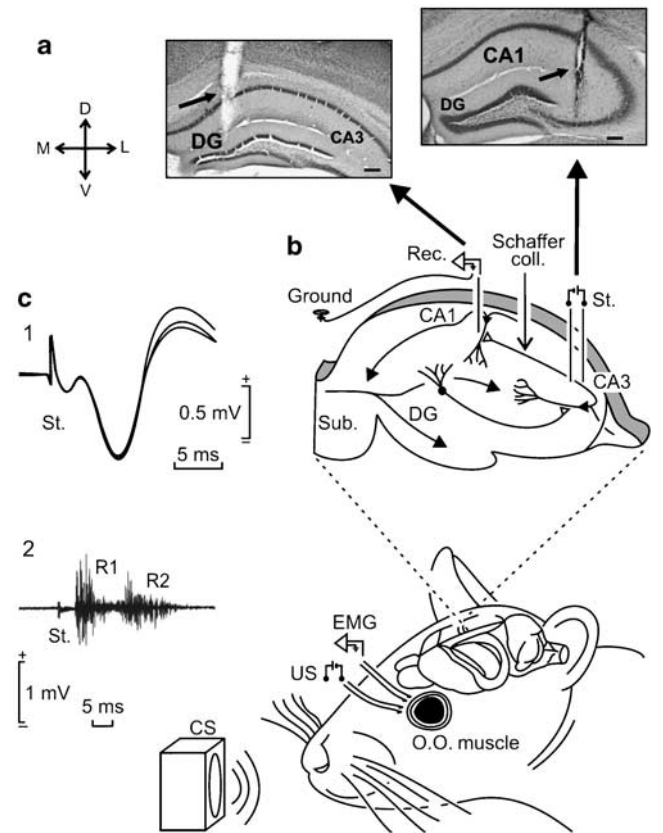
Figure 1 Experimental design. (a) Photomicrographs illustrating the location (arrows) of recording (left) and stimulating (right) sites in the dorsal hippocampus. Calibration bar is 200 µm. (b) EMG recording electrodes were implanted in the left orbicularis oculi (OO) muscle of the upper lid, whereas stimulating electrodes were implanted on the ipsilateral supraorbital nerve. The latter were used for US presentation. For classical eyelid conditioning, we used a tone (20 ms, 2.4 kHz, 85 dB) as a CS. The loudspeaker was located 30 cm from the animal's head. Stimulating electrodes (St) were implanted on the Schaffer collaterals of the right hippocampus, whereas a recording electrode (Rec) was aimed toward the pyramidal CA1 area. (c) The three superimposed records at the top (1) illustrate the fEPSP recorded in the stratum radiatum of the CA1 area following electrical stimulation of Schaffer collaterals. Superimposed records ($n = 3$) at the bottom (2) correspond to the blink reflex evoked in the OO muscle by the electrical stimulation of the trigeminal nerve. Note the two short (R1) and long (R2) latency components characterizing the blink reflex in mammals. Abbreviations: D, L, M, V, dorsal, lateral, medial, ventral; DG, dentate gyrus.

cover for 0.5 mm and bent as a hook to allow a stable insertion in the lid. During the same surgical step, animals were also implanted with bipolar-stimulating electrodes in the right Schaffer collateral pathway of the dorsal hippocampus (2 mm lateral and 1.5 mm posterior to Bregma, and 1–1.5 mm from the brain surface; Paxinos and Franklin, 2001) and with a recording electrode aimed at the right CA1 stratum radiatum (1.2 mm lateral and 2.2 mm posterior to Bregma, and 1–1.5 from the brain surface). Hippocampal electrodes were made of 50 µm, Teflon-coated tungsten wire (Advent Research Materials, Eynsham, UK). The final location of the recording electrode in the CA1 area was determined following the field potential depth profile evoked by paired (40 ms interval) pulses presented to Schaffer collaterals (Figure 1). A bare silver wire was affixed

to the skull as ground. The eight wires were soldered to two four-pin sockets (RS Amidata, Madrid, Spain), which were then fixed to the skull with dental cement. Further details of this chronic preparation can be found elsewhere (Gruart et al, 2006; Madroñal et al, 2007). Experiments were started $\geqslant 1$ week after surgery.

Recording and Stimulation Procedures

Recording sessions were performed with three animals at a time. Animals were placed in separate plastic chambers ($5 \times 5 \times 10$ cm) located inside a larger Faraday box ($30 \times 30 \times 20$ cm). The EMG activity of the orbicularis oculi muscle was recorded with Grass P511 differential amplifiers (Grass-Telefactor, West Warwick, RI) within a bandwidth of 0.1 Hz to 10 kHz. Field EPSP recordings were also carried out with Grass P511 differential amplifiers through a high-impedance probe ($2 \times 10^{12}\,\Omega$, 10 pF).

For input/output curves (Figure 2), animals were stimulated at the Schaffer collaterals with paired pulses (40 ms interval) at increasing intensities (0.04–0.3 mA). The effects of paired pulses at different inter-stimulus intervals (10, 20, 40, 100, 200, and 500 ms) were also checked (Figure 2). For this, we used stimulus intensities corre-

sponding to 40% of the amount necessary for evoking a saturating response (Gureviciene et al, 2004). For both input/output curves and paired-pulse facilitation, pairs of stimuli were repeated $\geqslant 5$ times with time intervals $\geqslant 30$ s, to avoid interferences with slower short-term potentiation (augmentation) or depression processes (Zucker and Regehr, 2002). Moreover, to avoid any cumulative effects, intensities and/or intervals were presented at random.

Classical Conditioning Procedures

For classical eyeblink conditioning, a tone (20 ms, 2.4 kHz, 85 dB) was presented to the animals as a CS. The US consisted of a 500 µs, 3 × threshold, square, cathodal pulse applied to the supraorbital nerve presented 500 ms after the end of the CS (Figure 3). A total of two habituation, 10 conditioning, and five extinction sessions were performed for each animal. A conditioning session consisted of 60 CS–US presentations, and lasted ∼ 30 min. CS–US presentations were separated at random by 30 ± 5 s. For a proper analysis of the CR, the CS was presented alone in 10% of the cases. For habituation and extinction sessions, the CS was presented alone, also for 60 times per session, at intervals of 30 ± 5 s. As criteria, we considered a 'CR' the presence,
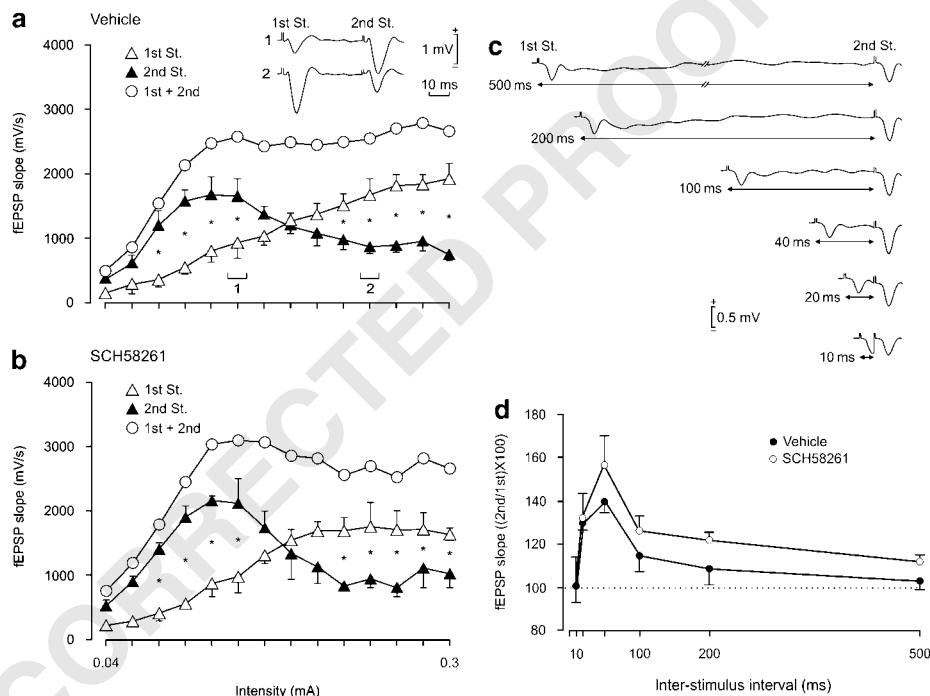
Figure 2 Input/output curves and paired-pulse stimulation evoked at the CA3-CA1 synapse in controls and in SCH58261-injected mice. (a) Relationships between the intensity (in mA) of pairs of stimuli (40 ms inter-stimulus interval) presented to Schaffer collaterals and the slope of the fEPSPs evoked in the CA1 layer, corresponding to the first (white triangles) and second (black triangles) stimuli. Values for the first + second fEPSPs (white circles) are also indicated. Data collected from five control animals. At the top are illustrated representative averaged ($n = 3$) records of fEPSPs recorded in the CA1 area following paired-pulse stimulation of the ipsilateral Schaffer collaterals at two different (1, 0.14 mA, and 2, 0.24 mA) intensities. Illustrated data correspond to mean ± SEM. Asterisks (*) indicate significant ($P < 0.001$) differences between fEPSPs evoked by the first and second stimuli ($F_{(13,52)} = 35.314$). (b) Same as in (a) but illustrating data collected from SCH58261-injected animals ($n = 5$). *, $P < 0.001$, [$F_{(13,52)} = 43.962$]. (c) Representative control records (averaged three times) of fEPSPs evoked by paired-pulse stimulation at six different (500, 200, 100, 40, 20, and 10 ms) time intervals, and using and intensities (mA) ∼40% of the asymptotic value, as illustrated in Figure 2. (d) Averaged paired-pulse ratio [(second pulse/first pulse) × 100] of fEPSPs evoked by paired-pulse stimulation at the indicated inter-stimulus intervals in both control and SCH58261-injected animals ($n = 5$ for each group). Illustrated data correspond to mean ± SEM. Note a significant facilitation [$F_{(5,20)} = 3.916$; $P \leqslant 0.01$] of fEPSPs evoked by the second pulse with respect to the first at 40 ms inter-pulse intervals for both control and SCH58261-injected animals. No significant differences ($P \geqslant 0.702$) were observed for data collected from the two groups of animals.
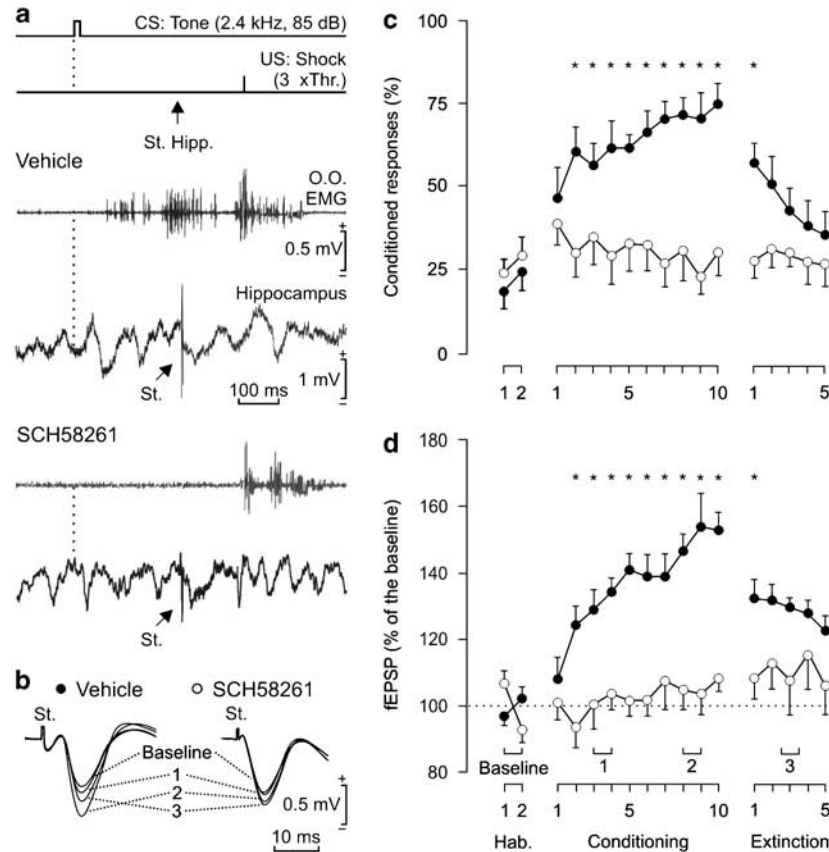
Figure 3 Learning curves and evolution of the synaptic field potential for control and SCH58261-injected groups. (a) A schematic representation of the conditioning paradigm, illustrating CS and US, and the moment at which a single pulse (100 μs, square, biphasic) was presented to Schaffer collaterals (St Hipp.). Below are illustrated two examples of EMG records from the orbicularis oculi (OO) muscle, as well as extracellular records of hippocampal activity collected from representative animals of the control (vehicle) and experimental (SCH58261) groups. Illustrated data were obtained from the ninth conditioning session of each animal. Note the fEPSP evoked by the single pulse presented to Schaffer collaterals. (b) Representative records (averaged three times) of fEPSPs collected at different times across the classical conditioning test, from a control and an SCH58261-injected animal. (c) Evolution in the percentage of CRs during the successive sessions for control (black circles) and SCH58261-injected (white circles) groups. Differences between control and SCH58261-injected groups were statistically significant from the second to the tenth conditioning sessions and for the first extinction session [$F_{(16,144)} = 5.604$; $P < 0.001$]. (d) Evolution of fEPSP slopes for control (black circles) and SCH58261-injected (white circles) groups, expressed as the % of change with respect to mean values collected during the two habituation sessions (baseline). The sample records illustrated at (b) were collected at the indicated times (baseline, 1, 2, and 3). Mean values are followed by ± SEM. Differences between control and SCH58261-injected groups were statistically significant from the second to the tenth conditioning sessions and for the first extinction session [$F_{(16,144)} = 3.154$; $P \leqslant 0.01$].

during the CS–US interval, of EMG activity in the orbicularis oculi muscle lasting > 10 ms and initiated > 50 ms after CS onset. In addition, the integrated EMG activity recorded during the CS–US interval had to be at least 2.5 times greater than the average activity recorded immediately before CS presentation (Gruart *et al*, 2006; Porras-García *et al*, 2005).

Synaptic field potentials in the CA1 area were evoked during habituation, conditioning, and extinction sessions by a single 100 μs, square, biphasic (negative–positive) pulse applied to Schaffer collaterals 300 ms after CS presentation (Figure 3). For each animal, the stimulus intensity was set at 40% of the intensity necessary for evoking a maximum fEPSP response as determined from data collected from input/output curves (see Figure 2). An additional criterion for selecting the stimulus intensity during conditioning sessions was that a second stimulus, presented 40 ms after a conditioning pulse, evoked a larger (> 20%) synaptic field potential (Bliss and Gardner-Medwin, 1973).

LTP Induction

For evoking LTP, each animal was presented with a high-frequency stimulation (HFS) session consisting of five 200 Hz, 100 ms trains of pulses at a rate of 1/s. These trains of pulses were presented 6 times in total, at intervals of 1 min. The 100 μs, square, biphasic pulses used to evoke LTP were applied at an intensity of ∼ 40% of the amount necessary for evoking a saturating response. Baseline values were collected for 15 min at a rate of 3/min using the same stimulus intensity. After the HFS session, field EPSPs (fEPSPs) were evoked again at the same rate (3/min) and intensity (40% of asymptotic value, see above) for 30 min. An additional recording session was repeated 24 h after HFS for 15 min (Figure 4).

Drugs

We used here the adenosine A$_{2A}$ receptor antagonist SCH58261 {7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-
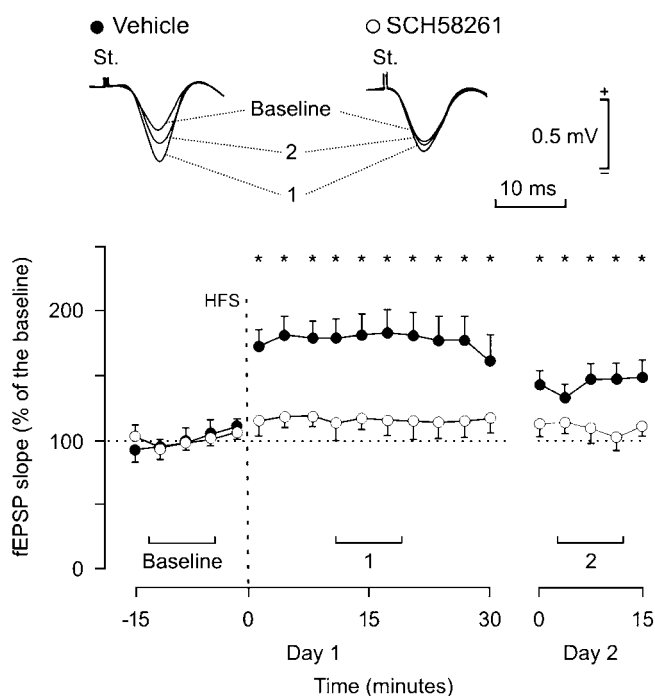
Figure 4 Evolution of fEPSPs evoked in the pyramidal CA1 area by single-pulse stimulation of Schaffer collaterals before and after HFS. For LTP induction, each animal was subjected to an HFS session (dashed line) consisting of five 200 Hz, 100 ms trains of pulses at a rate of 1/s. This protocol was presented six times, at intervals of 1 min. The 100 µs, square, biphasic pulses used to evoke LTP were applied at the same intensity as that used for the single pulse presented before (ie, for baseline records) and following HFS presentation. LTP evolution was followed for 30 min immediately after HFS (day 1) and 24 h later (day 2). Recordings were collected from 10 animals for both control (black circles) and SCH58261-injected (white circles) groups. Illustrated data correspond to mean ± SEM. Representative control records (averaged three times) of fEPSPs evoked before and after the HFS session are illustrated at the top for the two groups of animals. Field EPSPs were collected at the indicated time periods (baseline, 1, and 2). Asterisks (*) indicate significant differences [F$_{(19,171)}$ = 8.243; P < 0.001] between LTP evoked in controls vs SCH58261-injected mice.

[4,3-e]-1,2,4 triazolo[1,5-] pyrimidine}, obtained from Tocris Cookson (Ballwin, MO, USA). SCH58261 was dissolved in saline with 10% dimethylsulfoxide and administered i.p. at a dose of 0.5 mg/kg. At this dose, SCH58261 reaches the brain rapidly (El Yacoubi et al, 2001), has no effect on cardiovascular hemodynamics and retains A$_{2A}$ selective receptor antagonist properties in vivo (Monopoli et al, 1998). The SCH58261 dose used here (0.5 mg/kg) was selected from a range of doses (0.5–2 mg/kg) checked in a set of preliminary HFS experiments. Control animals received the same volume of the vehicle solution. For experiments illustrated in Figures 2 and 4 (input–output curves, paired-pulse test, and HFS-induced LTP), the drug was injected 30 min before the corresponding test. For classical conditioning (Figure 3), animals were injected 30 min before each conditioning session (ie, 10 times).

Data Collection and Analysis

EMG and extracellular hippocampal activity, and 1-volt rectangular pulses corresponding to CS and US presenta-

tions, were stored digitally on a computer through an analog/digital converter (1401 Plus; CED, Cambridge, England), at a sampling frequency of 11–22 kHz and with an amplitude resolution of 12 bits. Data were analyzed offline for quantification of CRs and fEPSP slopes with the help of commercial (Spike 2 and SIGAVG from CEDM) and home-made (Porras-García et al, 2005; Gruart et al, 2006) representation programs. The slope of evoked fEPSPs was represented as the first derivative (volts per second) of fEPSP recordings (volts). For this, five successive fEPSPs were averaged, and the mean value of the slope during the rise time (ie, the period of the slope between the initial 10% and the final 10% of the fEPSP) was determined. Computed results were processed for statistical analysis using the Sigma Stat for Windows package. Unless otherwise indicated, data are represented as the mean ± SEM. Acquired data were analyzed using a two-way ANOVA test, with session as repeated measure.

RESULTS

EMG and CA3-CA1 Synaptic Field Potentials Recorded in Alert-Behaving Mice

As illustrated in Figure 1, the experimental design used in this work enables the simultaneous recording of conditioned eyeblinks and of fEPSPs evoked at hippocampal CA3-CA1 synapses (Gruart et al, 2006; Madroñal et al, 2007). Stimulating and recording electrodes implanted in the upper lid did not disturb its normal kinematics, and allowed the generation of spontaneous and electrically evoked eyelid responses (Figure 1c2). Conditioned eyeblinks were easily distinguished in EMG recordings and were quantified following criteria described previously (Porras-García et al, 2005; Gruart et al, 2006). In simultaneity with the acquisition of CRs, we recorded the evolution of CA3–CA1 field synaptic potentials. The electrical stimulation of Schaffer collaterals presented 300 ms after the CS evoked a definite fEPSP in the CA1 area (Figure 1C2). According to recorded fEPSP profiles (see examples in Figures 2–4) most recording electrodes were located near CA1 apical dendrites (ie, in the stratum radiatum). Only on occasions, recorded waves presented a positive profile, corresponding to a more dorsal location of the electrode (Schwartzkroin, 1986). The stimulus applied to Schaffer collaterals disrupted the ongoing θ rhythm recorded in the CA1 area, but the rhythm reappeared in phase 100–500 ms later. Field EPSPs recorded with this procedure do not present any spontaneous tendency toward a sustained increase or decrease (de Jonge and Racine, 1985; Madroñal et al, 2007).

Input/Output Relationships at the CA3-CA1 Synapse in Controls and in SCH58261-Injected Mice

The facilitation evoked by the presentation of a pair of pulses is a typical presynaptic short-term plastic property of some excitatory synapses of the hippocampus, including the CA3-CA1 synapses, and it has been correlated with neurotransmitter release (Thomson, 2000; Zucker and Regehr, 2002). In a first series of experiments, we studied in control and SCH58261-injected mice the changes in

fEPSP slopes evoked in the pyramidal CA1 area by paired-pulse (40 ms interval) stimulation of the ipsilateral Schaffer collaterals (see Madroñal *et al*, 2008 for details). The slope of fEPSPs (in mV/s) evoked in the CA1 area by the first stimulus in controls (vehicle, $n = 5$ animals, Figure 2a) increased steadily with current strength (range 0.04–0.3 mA) until reaching asymptotic values for intensities > 0.24 mA (white triangles, Figure 2a; Student's *t*-test, $P \geqslant 0.079$). In contrast, fEPSPs evoked by the second stimulus (black triangles, Figure 2a) increased initially in parallel with fEPSPs evoked by the first pulse (but with significantly larger values) until stimuli > 0.18 mA, from which the fEPSP slopes evoked by the second stimulus were significantly smaller than those evoked by the first $[F_{(13,52)} = 35.314; P < 0.001]$. The addition of fEPSP slopes evoked by the first and second stimuli (first + second, white circles, Figure 2a) presented a sigmoid-like shape. Similar results have been described recently for the CA3–CA1 synapse in behaving mice (Madroñal *et al*, 2008).

SCH58261-injected mice ($n = 5$ animals) presented input/output curves non-significantly different ($P \geqslant 0.315$) from those obtained from vehicle-injected animals (Figure 2b). In addition, the paired-pulse facilitation evoked with low intensities (< 0.16 mA) was changed into paired-pulse depression for larger paired-pulse intensities (> 0.2 mA). Results collected for fEPSPs evoked by the first and second stimuli in SCH58261-injected animals were also significantly different at both low (< 0.16 mA) and high (> 0.2 mA) stimulus intensities $[F_{(13,52)} = 43.962; P < 0.001]$. These results also indicate that SCH58261 injections will not modify baseline slope of fEPSPs during classical conditioning and LTP experiments (see below).

We checked the effects of paired-pulse stimulation at a fixed intensity (~40% of the amount needed for evoking a maximum fEPSP response) of the CA3–CA1 synapse at different time intervals in controls and in SCH58261-injected mice (Figure 2c and d). Again, no significant differences were observed between data collected from the two groups of animals $[F_{(5,59)} = 0.598; P \geqslant 0.702]$. Nevertheless, both groups of animals presented significant paired-pulse facilitation of fEPSPs evoked by the second pulse with respect to the first at 40 ms inter-pulse intervals $[F_{(5,20)} = 3.916; P \leqslant 0.01]$.

In conclusion, and as indicated in a recent report (Madroñal *et al*, 2008), paired-pulse facilitation evoked with low stimulus intensities can be changed into paired-pulse depression by stronger pairs of stimuli, suggesting a homeostatic role to either facilitate or damp afferent volleys arriving at pyramidal CA1 cells following a conditioning input (Turrigiano and Nelson, 2004). Apparently, this interesting property of short-term plasticity at CA3–CA1 synapses is not modified by the pharmacological manipulation of adenosine A_{2A} receptors.

Classical Conditioning of Eyelid Responses in Controls and in SCH58261-Injected Mice

In each experimental group (vehicle and SCH58261-injected), nine successful animals were classically conditioned using a trace (CS, tone; US, shock) paradigm. The time interval between the end of the CS and the beginning of the US was 500 ms. As shown in Figure 3a and c, the percentage of CRs increased steadily across conditioning sessions for the vehicle-injected animals, with a profile similar to that in previous descriptions in mice, using similar trace conditioning procedures (Takatsuki *et al*, 2003; Dominguez-del-Toro *et al*, 2004; Gruart *et al*, 2006). These animals presented a mean of $46.5 \pm 7.9\%$ responses during the first conditioning session, and reached asymptotic values from the 7th session onward ($> 70\%$ of CRs).

The mean percentage of CRs collected for control animals was significantly larger from the second conditioning session onward compared with values reached by the SCH58261-injected mice, as well as for the first extinction session $[F_{(16\ 144)} = 5.604; P < 0.001]$. In SCH58261-injected mice, we were unable to observe a normal conditioning profile like that of the control group. In fact, mice injected daily with SCH58261 presented a profile of CRs that decreased progressively across conditioning sessions, and by the 9th session reached values ($22.7 \pm 4.2\%$) well below ($P < 0.05$) those observed for the first conditioning session ($39.7 \pm 5.6\%$ of CRs). Thus, the daily injection of SCH58261 presented a cumulative (negative) effect on the associative learning capabilities of injected animals.

Evolution of CA3–CA1 fEPSP Across Classical Conditioning of Eyelid Responses Controls and in SCH58261-Injected Mice

As shown in Figure 3b and d, fEPSPs evoked in control animals by the electrical stimulation of Schaffer collaterals increased progressively in slope (taking the slope of fEPSPs collected during the two habituation sessions as 100%, see baseline values in Figure 3d) across conditioning, to ~140% during the 4th conditioning session and to $> 150\%$ for the ninth and tenth conditioning sessions ($P \leqslant 0.05$; black circles, Figure 3d). In contrast, animals injected daily with SCH58261 (white circles, Figure 3d) presented significantly lower values for fEPSP slopes than those of controls from the second to the tenth conditioning sessions and for the first extinction session $[F_{(16\ 144)} = 5.604; P < 0.001]$. Indeed, fEPSPs evoked in SCH58261-injected mice across conditioning were similar to (and sometimes even lower than) baseline records.

As expected (Gruart *et al*, 2006; Sahún *et al*, 2007), during the five extinction sessions, fEPSP slopes in the control situation decreased progressively (black circles, Figure 3d), but did not reach an expected value of ~80% of baseline fEPSP responses (see Gruart *et al*, 2006). In contrast, the slope of fEPSPs recorded from SCH58261-injected mice remained close to baseline values (white circles, Figure 3d).

Comparison of LTP Evoked in Alert-Behaving Controls and SCH58261-Injected Mice

For the LTP study, and to obtain a baseline for evoked fEPSPs, animals ($n = 10$ for each group) were stimulated for 15 min at Schaffer collaterals (Figure 4). The stimulus consisted of a single 100 μs, square, biphasic pulse presented 3 times/min. Pulse intensity (0.05–0.3 mA) was set at ~40% of the amount necessary to evoke a maximum fEPSP response (Gruart *et al*, 2006, Sahún *et al*, 2007). For LTP induction, each animal was subjected to an HFS session consisting of five trains (200 Hz, 100 ms) of pulses at a rate

of 1/s. These trains of stimuli were presented six times in total, at intervals of 1 min. To avoid evoking a population spike and/or unwanted EEG seizures, the stimulus intensity for HFS was set at the same amount as that used for generating the baseline record. After HFS, the same single stimulus used to generate baseline records was presented at the initial rate (3/min) for another 30 min (Figure 4). With this protocol, the control group presented a significant LTP lasting at least 24 h [$F_{(19,171)} = 8.101$; $P < 0.001$]. In contrast, SCH58261-injected mice presented a minimum (120%), non-significant ($\geqslant 0.27$) increase in the slope of fEPSPs evoked by the test pulses following the HFS session.

In summary, although SCH58261-injected animals presented a normal performance for input/output curves and paired-pulse modulation (Figure 2), they had evident limitations for associative learning and for activity-dependent synaptic plasticity (Figure 3), a result confirmed by the significant deficit in HFS-evoked LTP (Figure 4).

DISCUSSION

The present results, obtained from alert-behaving mice, provide evidence for a direct and endogenous role of adenosine operating through the excitatory A$_{2A}$ receptor subtype in the physiological potentiation of fEPSPs evoked at hippocampal CA3-CA1 synapses during the acquisition of an associative learning task. These results were further confirmed by the absence of HFS-evoked LTP in SCH58261-injected animals.

We found that mice, injected daily with SCH58261, in a concentration that has been shown to prevent beta-amyloid (25–35)-induced cognitive deficits in mice (Dall'Igna et al, 2007), were unable to be conditioned with a simple associative learning task, such as the classical conditioning of eyelid responses. SCH58261-injected animals presented a decrease in the percentage of eyelid responses across conditioning and extinction sessions, most probably due to blockade of adenosine A$_{2A}$ receptors. Indeed, this compound has about 50-fold selectivity for rat A$_{2A}$ receptors (Ki $\cong 2$ nM) over rat adenosine A1 receptors (Zocchi et al, 1996) and has even higher A$_{2A}$ selectivity when adenosine A$_{2B}$ (580-fold) or rat adenosine A3 (1561-fold) receptors are concerned (Yang et al, 2007). SCH58261, at doses similar or even higher than that used in the present work, retains A$_{2A}$ receptor antagonist properties in vivo as it does not mimic centrally mediated actions of selective adenosine A1 receptor antagonists (Connole et al, 2004; Kelsey et al, 2009). Moreover, selective antagonism of A1 receptors does not affect centrally mediated actions of SCH58261 (El Yacoubi et al, 2000). Finally, SCH58261 (i.p.) was shown to antagonize the action of a selective A2A receptor agonist but not that of a selective A1 receptor agonist (Monopoli et al, 1998).

Interestingly, impaired associative learning was also observed whereas recording hippocampal fEPSPs, in a synaptic relay (ie, the CA3-CA1 synapse) that is critically involved in the acquisition of associative learning in alert-behaving mice (Gruart et al, 2006; Madroñal et al, 2007) and rats (Whitlock et al, 2006). In the present experiments, with SCH58261-injected mice we observed a non-significant increase in fEPSP slopes across conditioning sessions,

which was probably related to the observed decrease in the percentage of CRs.

Although the effects of a systemic injection of SCH58261 reported here could be a secondary result of changes occurring elsewhere in the brain, the fact that we concentrate on fEPSP evoked monosynaptically in pyramidal CA1 cells by the electrical stimulation of the ipsilateral Shaffer collaterals strongly suggest that these effects are taking place at the least in the CA3-CA1 synapse. Obviously, this assumption does not discard other actions of SCH58261 taking place at selected brain sites in which A$_{2A}$ receptors are also present.

Role of Adenosine in Associative Learning and Activity-Dependent Synaptic Plasticity

It is widely accepted that adenosine, acting through activation of high-affinity inhibitory (A$_1$) and excitatory (A$_{2A}$) receptors, modulates synaptic plasticity events, particularly in in vitro models of LTP and long-term depression (LTD)/depotentiation (see de Mendonça and Ribeiro, 1997). As adenosine is an endogenous purine with an important role in the regulation of neuronal excitability and low-frequency synaptic transmission, the hypothesis was advanced that adenosine could also modulate phenomena of use-dependent changes in synaptic strength. In this regard, it has been reported that endogenous adenosine, through activation of A$_2$ receptors, could facilitate HFS-induced LTP in the pyramidal CA1 area (Kessey and Mogul, 1997, 1998; Sekino et al, 1991). Indeed, activation of A$_{2A}$ receptors with highly selective agonists facilitates LTP in vitro (de Mendonça and Ribeiro, 1994). As the adenosinergic system is able to modulate long-term changes in the efficiency of synapses that are considered the neurophysiological basis for learning and memory (Bliss and Collingridge, 1993), it would be expected that these compounds could also modify the performance of experimental animals in learning and memory tests. Accordingly, it was found that adenosine, through the activation of the widespread adenosine A$_1$ receptor, could impair the acquisition of the classical conditioning of nictitating membrane responses (Winsky and Harvey, 1986), or even impair the retention in a passive avoidance task (Normile and Barraco, 1991). In spite of the fact that most of the effects produced by endogenous adenosine are through the activation of the widespread inhibitory A$_1$ receptor, there are some grounds for proposing that the actions of these inhibitory receptors could be supplemented by an autocrine-like role of facilitatory A$_{2A}$ receptors restricted to activated synapses (reviewed in Ferré et al, 2005). However, little is known yet about the influence of endogenous activation of synaptically localized A$_{2A}$ receptors in the modulation of learning and memory tasks (see Kopf et al, 1999).

In the present experiments, we have demonstrated for the first time that A$_{2A}$ receptors are critically involved in the acquisition of classically conditioned eyelid responses in behaving mice. Moreover, the deficit in associative learning observed following the pharmacological blockade of A$_{2A}$ receptors, with a highly specific antagonist, is possibly related with an impairment of hippocampal synapses to undergo the expected potentiation (Gruart et al, 2006;

Whitlock *et al*, 2006), as revealed by the absence of change in the slopes of fEPSPs across conditioning sessions. In fact, there is evidence that the increase in the synaptic strength of relevant hippocampal circuits involved in associative learning, and the acquisition of classically conditioned eyelid responses, require the activation of hippocampal NMDA receptors. The genetic alteration (Kishimoto *et al*, 2001, 2006) or the *in vivo* pharmacological blockade of NMDA receptors (Gruart *et al*, 2006; Maren *et al*, 1992; Sakamoto *et al*, 2005) prevents both the acquisition of a classically conditioned eyelid response and the enhancement of the synaptic transmission between the CA3-CA1 synapse using a trace paradigm similar to the one used here. Although systemic injections of SCH58261 could obviously block other neuronal centers expressing the A$_{2A}$ receptor (see for example, Mingote *et al*, 2008), results collected here clearly involve the CA3-CA1 synapse as demonstrated by the significant decrease in both LTP and physiological synaptic potentiation during associative learning.

Adenosine A$_{2A}$ receptors localized postsynaptically at synapses between mossy fibers and CA3 pyramidal cells are essential for a form of LTP of NMDA currents (Rebola *et al*, 2008). It has also been shown that adenosine A$_{2A}$ receptors co-locate with mGluR receptors and act synergistically to potentiate NMDA effects at the hippocampal CA3-CA1 synapse (Tebano *et al*, 2005). It is therefore feasible to speculate that the mechanism by which there is an impairment of the acquisition of conditioned responses by blockade of A$_{2A}$ receptors could be a reduced activation of NMDA receptors. Another possibility is the interaction of adenosine A$_{2A}$ receptors with TrkB receptors, as adenosine A$_{2A}$ receptors, through a cyclic AMP-mediated process (Boulanger and Poo, 1999; Diógenes *et al*, 2004) or through transactivation of TrkB receptors (Lee and Chao, 2001), trigger BDNF actions at synapses. In this regard, it has been shown recently that TrkB receptors located in the hippocampal circuit are involved in associative learning and in learning-induced changes in synaptic strength (Gruart *et al*, 2007). Adenosine A$_{2A}$ receptors seem to be required for normal BDNF levels (Tebano *et al*, 2008), for BDNF-induced potentiation of synaptic transmission (Diógenes *et al*, 2007; Tebano *et al*, 2008), for BDNF influence upon alpha7-nicotinic acetylcholine receptors (Fernandes *et al*, 2008), and—most relevant in the context of the present work—for BDNF-induced facilitation of LTP (Fontinha *et al*, 2008) in the CA1 area of hippocampal slices. Indeed, upon blockade of A$_{2A}$ receptors, BDNF was no longer able to facilitate theta-burst-induced CA1 LTP in hippocampal slices (Fontinha *et al*, 2008). Therefore, it is probable that a reduced influence of BDNF upon synaptic plasticity phenomena, due to the lack of activation of A$_{2A}$ receptors by endogenous adenosine, might explain the now-reported associative learning disability of the animals with A$_{2A}$ receptors blocked. Further studies, clearly outside the scope of the present work, are required to directly evaluate this hypothesis.

The present results allow determining the role of endogenous activation of adenosine A$_{2A}$ receptors during hippocampal LTP in behaving animals, shedding light on the mechanisms involved in the induction, expression, and extinction of LTP processes. In this regard, we have convincingly demonstrated that HFS-induced LTP in alert-behaving mice is disrupted in the presence of a potent and selective antagonist for A$_{2A}$ receptors, demonstrating a crucial role of these receptors in associative learning. Therefore, adenosine A$_{2A}$ receptors in the hippocampus, though less abundant than adenosine A$_1$ receptors (Ribeiro *et al*, 2003), emerge as essential for hippocampal-dependent cognitive functions.

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